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Pour l'obtention du grade de
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École doctorale : Sciences pour l'environnement - Gay Lussac (La Rochelle)
Secteur de recherche : Biologie de l?environnement, des populations, écologie

Présentée par :
Hanxiang Wu

Amino acid and glucose conjugates of a phenylpyrrole compound: synthesis, systemicity and biological properties

Directeur(s) de Thèse :
Rémi Lemoine, Jean-Louis Bonnemain, Jean-François Chollet

Soutenue le 07 avril 2017 devant le jury

Jury :

Président	**Sylvie Dinant**	Directeur de recherche, INRA de Versailles
Rapporteur	**Sylvie Dinant**	Directeur de recherche, INRA de Versailles
Rapporteur	**Michel Couderchet**	Professeur des Universités, Université de Reims
Membre	**Rémi Lemoine**	Directeur de recherche CNRS, Université de Poitiers
Membre	**Jean-Louis Bonnemain**	Professeur émérite, Université de Poitiers
Membre	**Jean-François Chollet**	Chargé de recherche CNRS, Université de Poitiers
Membre	**François Laurent**	Chargé de recherche, INRA de Toulouse

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AMINO ACID AND GLUCOSE CONJUGATES OF A PHENYLPYRROLE COMPOUND: SYNTHESIS, SYSTEMICITY AND BIOLOGICAL PROPERTIES

Directeur de Thèse : Rémi Lemoine

Co-directeurs : Jean-François Chollet & Jean-Louis Bonnemain

soutenue le **7 avril 2017** devant la Commission d'Examen

JURY :

Michel COUDERCHET, Professeur, Université de Reims	*Rapporteur*
Sylvie DINANT, Directeur de Recherche INRA, INRA Versailles	*Rapporteur*
François LAURENT, Chargé de Recherche INRA (HDR), INRA Toulouse	*Examinateur*
Rémi LEMOINE, Directeur de Recherche CNRS, Université de Poitiers	*Examinateur*
Jean-Louis BONNEMAIN, Professeur Émérite, Université de Poitiers	*Examinateur*
Jean-François CHOLLET, Chargé de Recherche CNRS, Université de Poitiers	*Examinateur*

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Your endless love makes me have the courage to face any difficulty.

Hanxiang WU

University of Poitiers, France

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Abstract

To reduce pesticide use without affecting the quantity and quality of agricultural products is one of the great challenges of the 21st century. New agrochemicals with optimal efficacy, environmental safety and user friendliness are needed for sustainable agriculture. Prodrug strategy provides a new possible way for achieving this goal by vectorization of agrochemicals, which may lead to site-targeted tanslocation and distribution. By applying prodrug concept into agrochemical design, the purpose of this thesis was to study profungicides which can concentrate in plant phloem tissues for controlling vascular or root diseases. The non-systemic fungicide fenpiclonil from the phenylpyrrole family was selected as model compound. According to carrier-mediated prodrug strategy, the resulting profungicide molecules were modified by introducing amino acid and glucose promoity respectively.

The first part of this thesis was to select an ideal promoiety for plant nutrient transport systems. The phloem mobility of L-glutamic acid and D-glucose fenpiclonil conjugate was compared in *Ricinus communis* seedlings. In systemicity tests, the concentration of L-amino acid fenpiclonil conjugate in phloem sap was about 20 times higher than that of D-glucose fenpiclonil conjugate. The results suggested that the amino acid promoiety was clearly more favorable to phloem mobility than that of glucose. Further investigation revealed that the systemicity of L-glutamic acid conjugate was pH dependent and almost completely inhibited by the protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP). Considering the concentration of L-glutamic acid conjugate in phloem sap that was about five times higher than its counterpart of the D series, we concluded that the phloem transport of the L-glutamic acid conjugate is governed by a stereospecific amino acid carrier system energized by the proton motive force.

In the second part, we investigated and exploited the limitations of our vectorisation strategy of D-glucose fenpiclonil conjugate (D-GFC) that exhibited an extremely low phloem systemicity. The effect of D-GFC at 0.5 mM concentration was studied

using *R. communis* seedlings, leaf discs of broad bean and a yeast mutant complemented with AtSUC2, coding for sucrose transporter involved in sucrose phloem loading in *Arabidopsis thaliana*. The results showed that D-GFC strongly inhibited the uptake and phloem transport of [^{14}C]-sucrose like *P*-chloromercuribenzenesulfonic acid (PCMBS) used at the same concentration. But unlike PCMBS which binds to the sulfhydryl groups of numerous membrane proteins, no inhibitory effect of D-GFC on [^{3}H]3-O-methyl-D-glucose and [^{3}H]-glutamine, as well as on PM-H^+-ATPase activity, was observed in the *Ricinus* model. Further results showed that D-GFC also inhibited the plant sucrose transporter involved in phloem loading in Fabaceae and Brassicaceae. Hence, D-GFC could be used as a new tool in phloemology to study the transport routes and compartmentation of endogenous sugars.

Finally, the studies centered on structural optimization by selecting the spacer arms of amino acid conjugates. The phloem mobility of three new spacer-linked amino acid conjugates was tested to investigate the effect of spacer arm structure on recognition and transport by carrier system using the *R. communis* seedlings. The conjugate which contains a triazole ring with the shortest chain length showed the best phloem systemicity among the four conjugates, indicating that reducing the length of the chain between the triazole ring and the L-α-amino acid function led to an improvement of the phloem systemicity. By contrast, removing the triazole ring did not improve systemicity although physicochemical properties were more favourable to diffusion through the plasma membrane. In addition, the amino acid conjugate exhibited clearly a broader ability to phloem systemicity than fenpiclonil acidic derivatives within the pH value range of the foliar apoplast (pHs from 5.0 to 6.5).

Keywords: Propesticide, Fungicide, Phenylpyrrole, *Ricinus communis*, *Vicia faba*, AtSUC2, Phloem transport, Systemicity, D-Glucose conjugate, L-Amino acid conjugate

Résumé

Réduire l'utilisation des pesticides sans affecter la quantité et la qualité des productions agricoles est l'un des grands défis du XXIe siècle. De nouveaux produits phytopharmaceutiques avec une efficacité optimale, une sécurité environnementale et dépourvus d'effets nocifs pour les utilisateurs, sont nécessaires pour une agriculture durable. La stratégie de prodrogue offre une nouvelle voie possible pour atteindre cet objectif en vectorisant les produits agrochimiques, ce qui peut conduire à un transport et à une distribution ciblés chez la plante. En utilisant le concept de prodrogue dans la conception de composés phytosanitaires, nos travaux ont pour objectif d'étudier de nouveaux profongicides potentiels qui peuvent se concentrer dans la sève phloémienne en vue de lutter contre les maladies vasculaires et racinaires. Le fenpiclonil, fongicide de contact de la famille des phénylpyrroles, a été choisi comme composé modèle. Selon une stratégie de prodrogues faisant appel à un transport actif via des protéines transmembranaires manipulant normalement les nutriments issus de la photosynthèse, des conjugués ont été synthétisés en introduisant respectivement un α-aminoacide ou un sucre dans sa structure.

La première partie de ce travail a consisté à sélectionner le meilleur nutriment capable de leurrer les systèmes de transport des plantes. Nous avons comparé la mobilité phloémienne, chez le ricin, de conjugués associant le fenpiclonil avec l'acide glutamique ou le glucose par le biai d'un espaceur. En utilisant le modèle Ricin comme test de systémie, nous avons montré que le conjugué fenpiclonil-L-acide glutamique est 20 fois plus concentré dans la sève phloémienne que le conjugué fenpiclonil-D-glucose. Ces résultats suggèrent donc que les acides aminés sont de meilleurs groupements vecteurs que le glucose, assurant ainsi une meilleure mobilité des conjugués dans la plante. D'autres études ont révélé que la systémie du conjugué avec l'acide L-glutamique est dépendante du pH et presque complètement inhibée par le carbonylcyanure m-chlorophénylhydrazone (CCCP). En considérant le fait que la concentration du conjugué avec l'acide L-glutamique dans la sève phloémienne est

environ cinq fois plus élevée que son homologue de la série D, nous pouvons conclure que le transport phloémien du conjugué avec l'aminoacide de la série L est régi par un système de transport d'acide aminé stéréospécifique activé par la force proton motrice.

Dans la deuxième partie de notre travail, nous sommes intéressé aux limites de notre stratégie de vectorisation révélées par la très faible systémie du conjugué fenpiclonil-D-glucose (D-GFC). Les expériences ont été conduites en utilisant des plantules de ricin, des disques folaires de fève et un mutant de levure exprimant At-SUC2. Nos résultats ont montré que ce conjugué inhibe aussi fortement que le PCMBS (utilisé à la même concentation) l'absorption et le transport phloémien du saccharose-[^{14}C], mais qu'il n'a pas d'effet inhibiteur sur le transport du 3-O-méthyl-D-glucose-[3H] ou de la glutamine-[3H] chez le ricin, ni même sur l'activité de l'ATPase-H+ de la membrane plasmique, contrairement au PCMBS qui se lie aux groupements sulfhydryl de nombreuses protéines membranaires. Des résultats complémentaires ont montré que D-GFC inhibe aussi le transporteur de saccharose impliqué dans le chargement phloémien chez les Fabaceae et Brassicaceae. Il constitue un nouvel outil en phloémologie pour étudier les voies de transport et la compartimentation des sucres endogènes.

Enfin, la mobilité phloémienne de trois nouveaux conjugués du fenpiclonil avec des acides aminés a été évaluée pour étudier l'influence de la structure de l'espaceur sur la reconnaissance et la manipulation par un système de transport actif chez le ricin. Parmi les quatre conjugués testés, celui qui contient un cycle 1,2,3-triazole avec la chaîne la plus courte a montré la meilleure mobilité phloémienne, indiquant que la réduction de la longueur de la chaîne entre le cycle triazole et la fonction L-α-aminoacide a permis d'optimiser la sytémie. En revanche, la suppression du cycle 1,2,3-triazole n'améliore pas la systémie, bien que les propriétés physico-chimiques du produit soient plus favorables à la diffusion au travers de la membrane plasmique. Enfin, nous avons montré que les conjugués avec les acides aminés présentent clairement une plus grande aptitude à la mobilité phloémienne que les dé-

rivés acides de fenpiclonil dans la plage des valeurs de pH de l'apoplasme foliaire (pH de 5,0 à 6,5).

Mots clés : Propesticide, Fongicide, Phenylpyrrole, Ricinus communis, Vicia faba, AtSUC2, Transport phloémien, Systémie, conjugué avec le D-glucose, conjugué avec un L-aminoacide

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List of abbreviations

BBB *Blood–brain barrier*

CCCP *Carbonyl cyanide m-chlorophenylhydrazone*

CF *Concentration factor*

D-GFC *D-Glucose fenpiclonil conjugate*

FRB *Free rotatable bonds*

Gln *Glutamine*

HBA *Number of hydrogen bond acceptors*

HBD *Number of hydrogen bond donors*

HEPES *4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid*

HPLC *High-pressure liquid chromatography*

IPM *Integrated pest management*

K_a *Acid dissociation constant of HA*

Log K_{ow} *the octanol/water partition coefficient*

Log P t*he octanol/water partition coefficient of the neutral molecule*

MES *2-(N-Morpholino)ethanesulfonic acid*

MW *Molecular weight*

PCMBS *Parachloromercuribenzenesulfonic acid*

pk_a *Log*(1/*K*a)

PM *Plasma membrane*

PSA *Polar surface area*

Suc *Sucrose*

TFA *Trifluoroacetic acid*

3-*O*-MeG *3-O-Methylglucose*

Chapter 1. Introduction and literature review

1.1 Challenges and opportunities for pesticide

The development of science and technology has greatly promoted agricultural civilization since 1950s, but the world food problem remains one of the greatest challenges confronting humankind, particularly in developing countries. According to a latest United Nations report, *World Population Prospects: The 2012 Revision*[1], it is estimated that the world population will reach more than 9.6 billion in 2050. This means that global food demand will be doubled until 2050 to feed a steadily growing population[2,3]. Meanwhile, Agriculture faces increasing pressures from environmental deterioration, climate change, land restrictions, loss of biodiversity and crop pests[4-6].

It is to be noted that plant health problem is a major cause of crop losses[7]. The spread of pests and pathogens have always been threatening the global crop production, especially for single crop species agroecosystems. Every year, estimates of 30–40 % crop losses due to plant health problems are common in the scientific literature[7-9]. Global crop pests include more than 10,000 species of insects, 30,000 species of weeds, 100,000 diseases (caused by fungi, viruses, bacteria and other microorganisms) and 3,000 species of nematodes[10]. Compared current distributions of 1901 pests and pathogens and historical observation, Bebber and co-workers warned that if current trends continue, many important agricultural nations could be saturated with pests within the next few decades[11]. Hence, the effective control of pests and pathogens is essential to global food security.

Crop chemical protection has been applied to agriculture to secure the sustainable production of food and fiber for more than 200 years. The increased use of pesticides since the early 1960s was a major factor for the huge productivity increases achieved[9]. Without pesticides, about 70% of crop yields could have been lost to pests[8]. Over the past 30 years, integrated pest management (IPM) programmes have

been accepted and implemented worldwide[12,13]. IPM combines various management strategies and measures to grow healthy crops and reduce the use of pesticides, including cultural, biological and chemical measures[14,15]. Although the IPM programmes substantially reduced pesticide use, chemical control still cannot be replaced and will continue to play an essential role in crop protection in the foreseeable future.

Despite pesticides have produced great benefits yet, the incorrect use of pesticides also poses significant threats to human health and environment[16,17]. With the growing public concern, there has been a clear trend of reducing pesticide use worldwide. The European Commission Directive 2009/128/EC on the sustainable use of pesticides established a framework to achieve a sustainable use of pesticides for reducing the risks and impacts of pesticide on human health and environment, meanwhile promoting the use of IPM and low pesticide-input management including non-chemical alternatives[18]. France, which is the fourth largest market following the USA, Brazil, and Japan, has implemented an action plan called "Écophyto" in 2008[19]. The entire sector, firms, wholesalers, cooperatives, agricultural advisers and farmers, is included in the Écophyto II, which is expected to reduce the 50 % nationwide use of pesticides by 2025[20].

Reducing pesticide use while at the same time maintaining the quantity and quality of agricultural products creates both challenges and opportunites for agrochemical development. New innovations are needed to discover more selective and efficient chemicals. However, a serious problem is only a very small part of pesticides (less than 0.1%) actually reaches their target pests after application, and off-target portion becomes environmental pollutant[21]. To meet these demands on reducing pesticide doses and improving the efficiency, targeted delivery of pesticides will be a new trend. Advances in targeted drug delivery strategies have been phenomenal throughout the past few decades[22]. It is interesting to explore the possible applications on pesticide targeting using the same approaches, such as ion-trap strategy and carrier-based ap-

proaches. The structure and function of plants also provides various potential pesticide delivery systems.

1.2 Transporter-Mediated Prodrug for Delivery

1.2.1 Prodrug strategy

Agrochemical and drug discovery share the common goal of innovation. To keep the dose at the lowest possible level and enhance bioavailability are prime properties to new active ingredients. As commonly defined, bioavailability implies the extent and rate at which a drug becomes available in the systemic circulation[23]. This term also can be used in agrochemical research to describe a pesticide that exerts its biological action in certain plant organs or cells at a distance from the site of application. Both agrochemicals and drugs require targeted delivery to ensure high localization at the site of action, while limit toxicity in non-target organs or organisms.

There are various barriers which can hinder drug from reaching its site of action, including poor aqueous solubility, inappropriate lipophilicity, insufficient chemical stability and lack of site specificity. Furthermore, the physiological structures and functions of the body are capable to protect human tissues from pathogens and toxins, but such functions can produce numerous hurdles to drug delivery as well. The permeability of biological membranes is a key determinant of drug bioavailability[24,25]. After oral administration, the pharmacological activity may be restricted by the inability to be absorbed from the gastrointestinal tract[26,27]. The blood–brain barrier (BBB), formed by brain vessel endothelial cells linking together by tight junctions, is a diffusion barrier[28,29]. The BBB is the most important factor limiting the drug permeability to the central nervous system[28,30].

The prodrug concept (Fig. 1-1), as first introduced by Adrian Albert in 1958[31], can be used to improve the physicochemical properties or bioproperties of the parent drug molecule in such a way as to enhance its deliverability[32]. Prodrug strategies have been used to solve the problems in oral absorption, organ distribution, metabo-

lism and excretion. Over the last two decades, it is estimated that the percentage of drugs classified as prodrugs has significantly increased to about 10% worldwide[33].

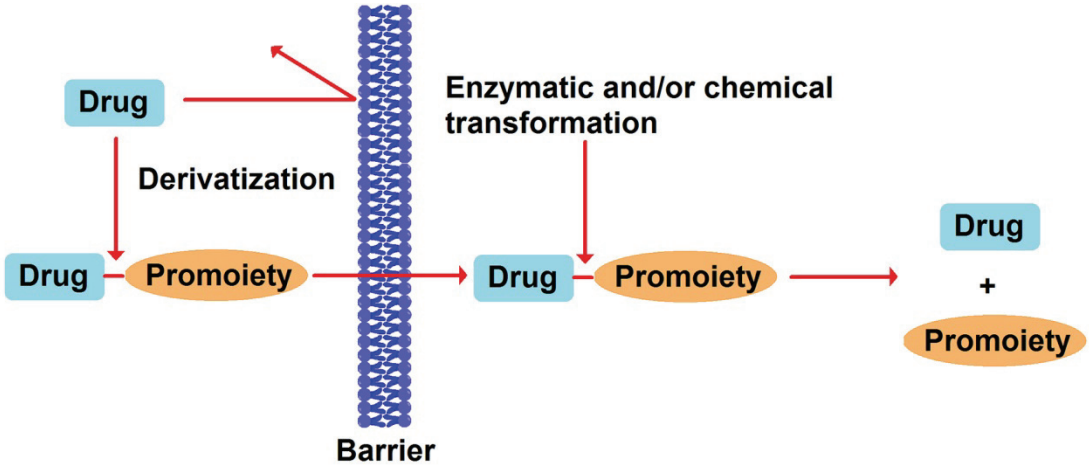

Figure 1-1. An illustration of the prodrug concept (modified from Rautio et al.[34]).

1.2.2 Membrane transporters in drug transport

One of the promising means of prodrug strategies is carrier-mediated transport[24]. Drug transporters are now widely acknowledged as important determinants governing drug absorption, excretion, and in many cases, extent of drug entry into target organs[35]. There are more than 400 membrane transporters belonging to two major superfamilies of membrane transporters: the ATP-binding cassette (ABC) and the solute carrier (SLC) families[36]. There have been many attempts to improve drug absorption by targeting specific membrane transporters (Fig. 1-2). The frequently exploited transporters include peptide, amino acid and glucose transporters.

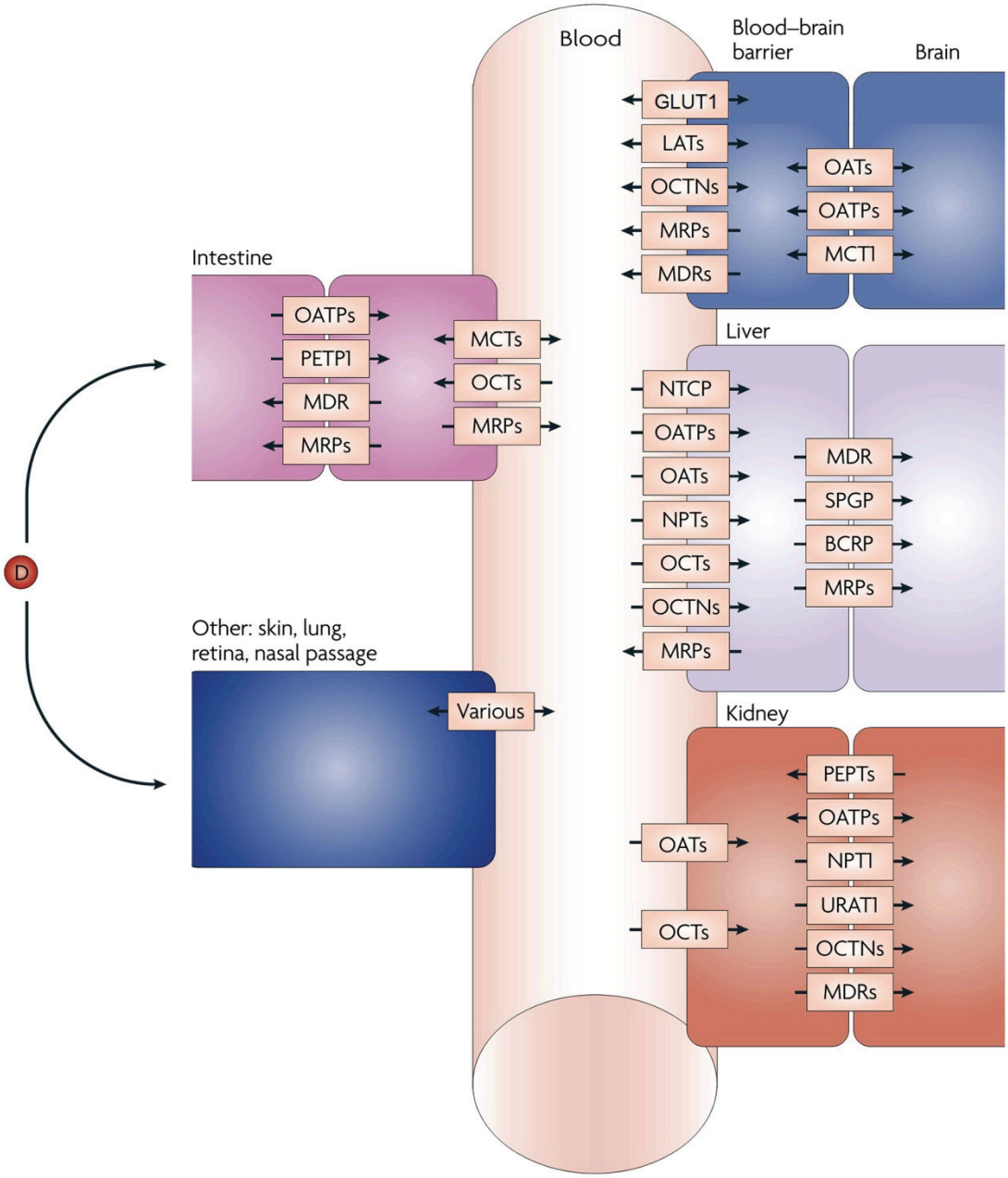

Figure 1-2. Multiple drug carriers in different tissues (modified from Ref.[37]). LATs, L-type amino-acid transporters; MCT1, monocarboxylate transporter 1 (also known as SLC16A1); MDR, multidrug-resistant; MRPs, multidrug-resistance-related proteins; NPT1, sodium phosphate transporter 1 (also known as SLC17A1), NTCP, sodium-dependent taurocholate co-transporter (also known as SLC10A1); OATs, ornithine aminotransferases; OATPs, organic anion transporting polypeptides; OCTs/OCTNs, organic cation transporters; PETP1, peptide transporter 1 (also known as SLC15A1); SPGP, sister P-glycoprotein (also known as ABCB11); URAT1, urate anion exchanger 1 (also known as SLC22A12); GLUT1, glucose transporter 1.

1.2.2.1 Peptide Transporters: PEPT1 and PEPT2

Members of the oligopeptide transporter family (SLC15) mediate proton-coupled co-transport of many diverse peptide and peptidomimetic compounds. Two family members, PEPT1 (SLC15A1) and PEPT2 (SLC15A2), have been identified in mammals[38]. The expression of both transporters in different mammalian tissues was summarized by Brandsch and co-workers[39]. PEPT1 is mainly expressed in the small intestine and PEPT2 in the kidney[37]. Almost all 400 dipeptides and 8000 tripeptides derived from the 20 proteinogenic L-α-amino acids are the substrates for both transporters, despite large differences in molecular size, net charge and hydrophobicity [38,40]. Peptides that contain D-enantiomers at the N-terminal position have good affinities and are transported with high rates[38].

Because of their broad substrate specificity, particular affinity and high capacity, peptide transporters represent attractive targets for the delivery of pharmacologically active compounds[37,38,41-43]. Several β-lactam antibiotics and a number of amino acid and dipeptide prodrugs are transported by peptide transporters. Amoxicillin and cefaclor (Fig 1-3A and Fig 1-3B) are two of the widely prescribed β-lactam antibiotics exhibiting good oral bioavailability[44]. Uptake study revealed that both antibiotics were transported by PEPT1 and PEPT2, suggesting that peptide transporters are responsible for the renal reabsorption and intestinal absorption of the two antibiotics[45]. Bestatin (Fig 1-3C), a dipeptide-like anticancer drug, is well absorbed following oral administration[46]. Further study demonstrated that Bestatin is actively transported by PEPT1 and PEPT2[42,47]. The oral absorption and systemic availability of 5'-amino acid ester prodrugs of acyclovir and zidovudine can be improved via the PEPT1 transporter (Fig 1-3D) [48]. Valganciclovir (Fig 1-3E), a valine ester prodrug of ganciclovir, is actively transported by PEPT1, which results in the enhancement of bioavailability with 10-fold higher than that of ganciclovir (60.9 vs 5.6%)[49]. Valganciclovir has also been shown to be a substrate of the PEPT2 transporter[50].

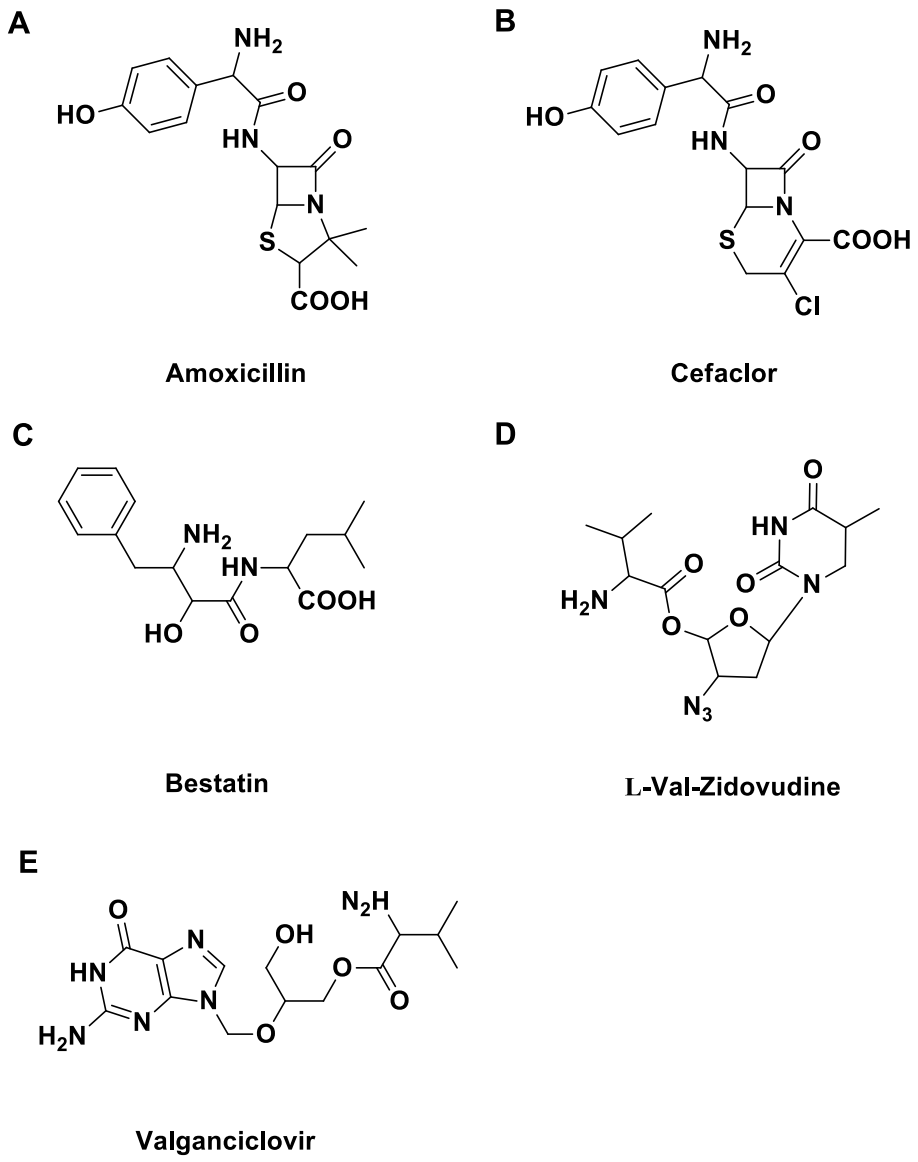

Figure 1-3. Examples of prodrug uptake by peptide transporters[44,46,49,50].

1.2.2.2 L-type amino acid transporter 1 (LAT1)

The L-type amino acid transporters 1 (LAT1) is a sodium-independent exchanger, which was first cloned in 1998[51,52]. LAT1 has various tissues distribution, including brain, intestine and kidney[53,54]. More specifically, LAT1 is highly expressed in the luminal and abluminal membranes of the blood–brain barrier (BBB) [52,55,56]. The supply of large neutral amino acids across the BBB is regulated by LAT1[55]. In addition, LAT1 prefers neutral L-amino acids with bulky or branched side chains for its substrates[57].

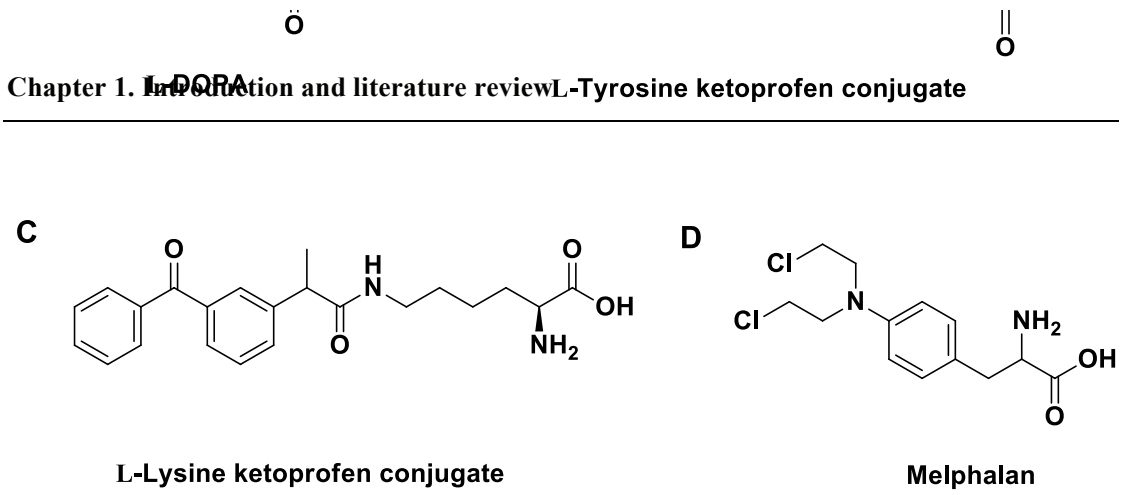

Figure 1-4. Chemical structures of amino acid-related drugs[54,58,59].

The exploitation of the LAT1 with prodrug strategy could be an attractive way to enhance BBB penetration and improve the delivery of amino acid-related drugs to the central nervous system without increasing non-specific tissue binding[54,58-60]. L-DOPA (Fig 1-4A), a widely used prodrug in the treatment of Parkinson's disease, is a straightforward example of compounds transported into the brain via the LAT1[57,61,62]. However, its parent compound dopamine is poorly transported across the blood-brain barrier due to its hydrophilic nature. A recent example of the successful application of LAT1 mediated uptake is an L-tyrosine prodrug of keto-profen[58]. This is the first demonstration of an amino acid-drug conjugate (Fig 1-4B), which is not only recognized but also actively transported across the BBB by LAT1. Similarly, a ketoprofen–lysine prodrug (Fig 1-4C) was also able to cross the BBB via LAT1-mediated uptake, which further proved that carrier-mediated transport is a fea-sible way to deliver small molecular weight drugs into brain[59]. Melphalan is a polar alkylating agent used to treat brain cancer (Fig 1-4D), which was transported across the rat blood-brain barrier via BBB large neutral amino-acid carrier[63]. Recently, amino acid prodrugs have been successfully applied to improve poor permeability, intravenous delivery, drug targeting and metabolic stability[64].

1.2.2.3 Glucose Transporter (GLUT1)

Glucose transporter isoform 1 (GLUT1) is a sodium-independent facilitative glucose transporter[65], which has a high transport capacity[34]. GLUT1 is specifically distributed on the BBB[66]. The BBB GLUT1 transports not only D-glucose, but also transports other hexoses including mannose, galactose, deoxyglucose, and 3-*O*-methylglucose[67].

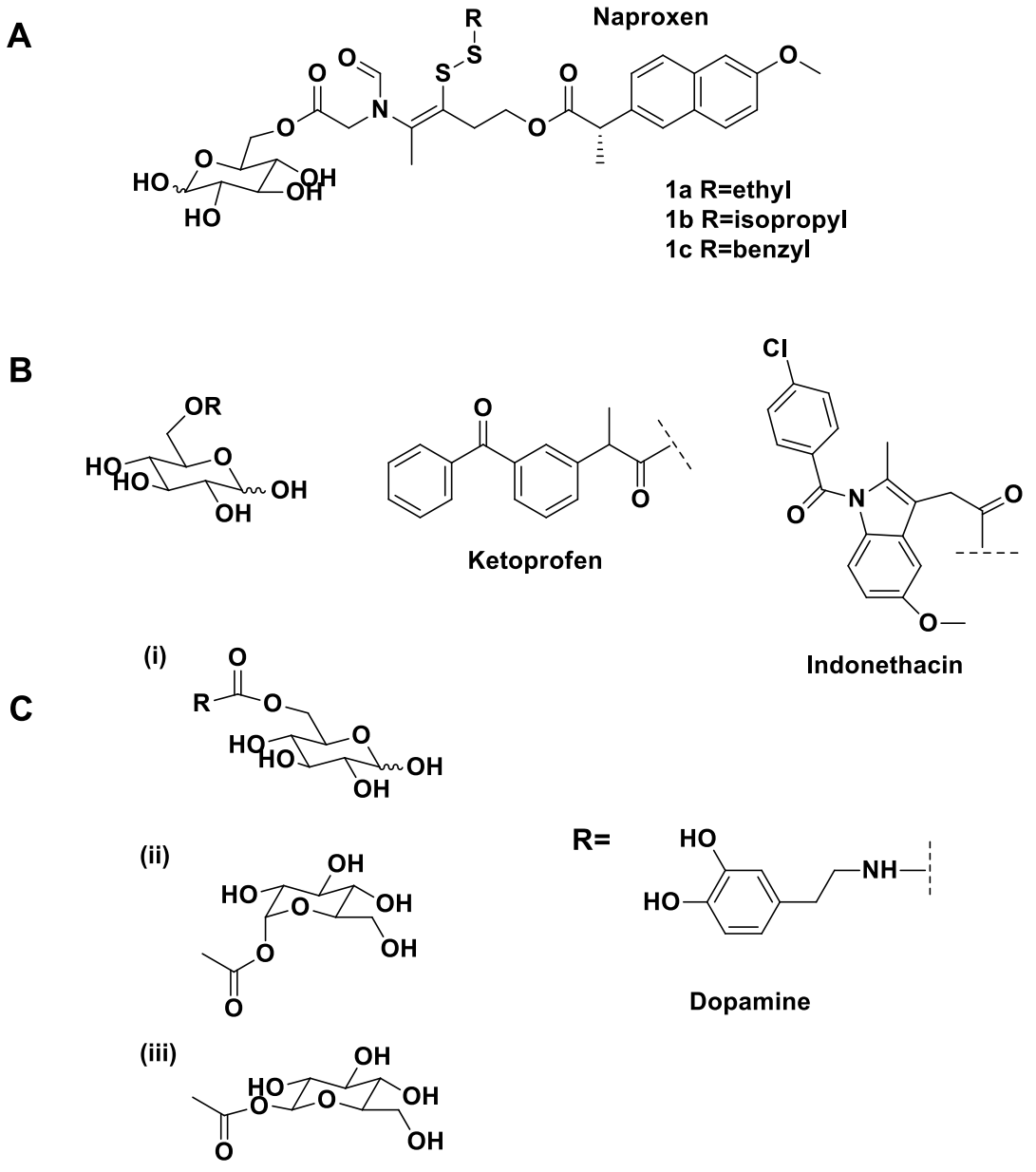

Figure 1-5. D-glucose conjugates as substrates for the blood-brain barrier GLUT1 transporter. A, Naproxen conjugates[68]. B, Glucose conjugates with ketoprofen and indomethacin[69]. C, Dopamine conjugates at the C-1, C-3, and C-6 positions[70].

Recently, several studies have shown that GLUT1 may participate in the transport of some glycosyl prodrugs through the blood-brain barrier. Glycosylation strategy has been used to deliver naproxen, a nonsteroidal anti-inflammatory drug, into the central nervous system[68]. The glucosyl thiamine disulfide prodrugs of naproxen (Fig 1-5A) exhibited excellent transport ability across the BBB after systemic injection. Gynther and co-workers[69] prepared two conjugates of ketoprofen and indomethacin with glucose (Fig 1-5B), and both glycosyl prodrugs were able to significantly inhibit the uptake of [^{14}C]D-glucose into rat brain. Furthermore, the two produced prodrugs were capable to cross the BBB in a temperature-dependent manner, suggesting that the brain uptake of the prodrugs is carrier-mediated. Fernandez *et al*. [70] synthesized a series of glycosyl dopamine derivatives with different linkers at the C-1, C-3, and C-6 positions of D-glucose (Fig 1-5C). The results of glucose uptake inhibition using human erythrocytes showed that the C-6 substituted derivative displayed the best affinity for GLUT1.

1.3 Uptake and transport of pesticide in plants

1.3.1 Basics of pesticide systemicity

Systemicity can be defined as the ability of a pesticide to be absorbed into plants and translocated in the vascular system[71]. In other words, systemic compound is able to enter a plant to reach the conducting tissues and to be transported away from the site of application via xylem and/or phloem. The uptake of systemic pesticides mainly rely on foliar or root application. In addition, the systemic distribution of pesticide in plants can be achieved following two pathways of movement (Fig 1-6) termed apoplastic (or apoplasmic) and symplastic (or symplasmic) [72,73]. The symplast (or symplasm) is the living part of the plant that is enclosed by the plasma membrane. Short-distance cell-to-cell transport through plasmodesmata as well as long-distance transport in the phloem is symplastic. The apoplast (or apoplasm) is the "nonliving" part of the plant that is outside the plasma membrane. Short distance movement through the intercellular spaces as well as long-distance movement in the xylem vessels is apoplastic.

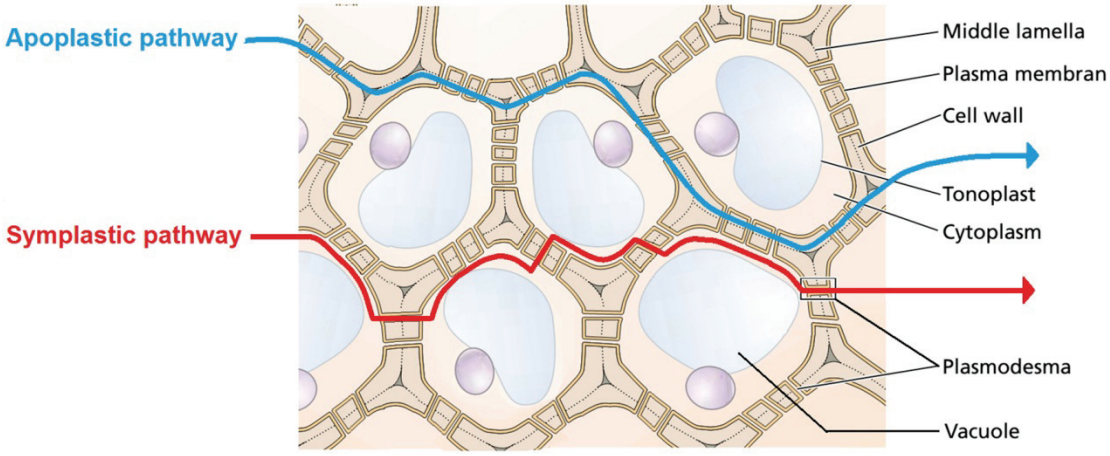

Figure 1-6. The apoplast and symplast pathways (modified form [74])

There are four basic types of systemicity, depending on the different degrees of uptake and transport in plants[75]. The first is local systemicity, which means absorption from the leaf surface and penetration into the leaf with limited diffusion around the site of application. The second is translaminar systemicity, which is used to de-

scribe a compound being applied to the upper leaf surface and then exerting its effect on the lower leaf surface after penetration (Fig 1-7A). The third is xylem systemicity, which means long-distance translocation in the same direction as the transpiration stream from roots to shoots (acropetal) (Fig 1-7B). The fourth is phloem systemicity. In this case, the movement can be both downwards from the leaves to roots (basipetal) and upwards from the shoots to apical sink tissues via the phloem sieve (acropetal) (Fig 1-7B). In addition, the pesticides which can be transported both in the xylem and the phloem are termed ambimobile pesticides[72]. In many cases, pesticides considered as systemic are only those which are able to move in the vascular system.

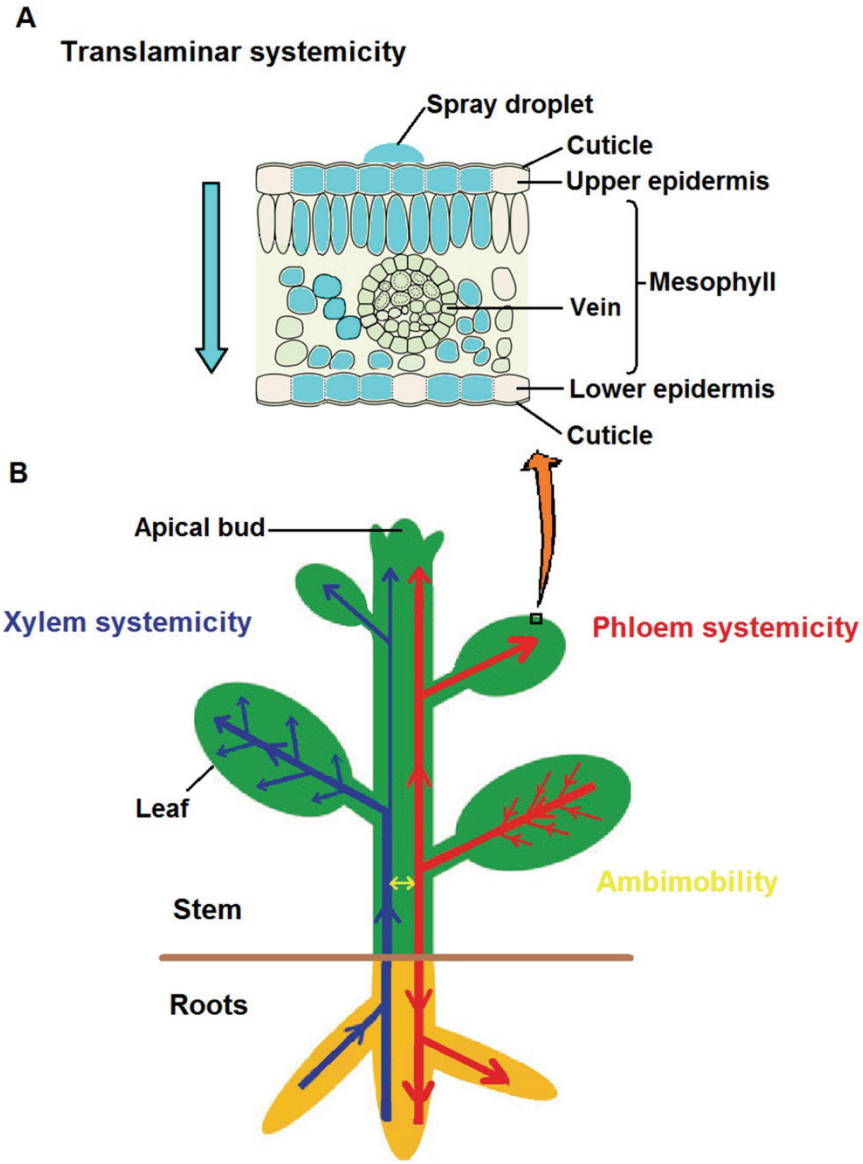

Figure 1-7. The different types of systemicity (modified from [76])

Systemic property is important to the expression of biological activity of pesticides, and can also lead to a strategy to improve the biological performance. Because of less wash-off by rain, the compounds which have local or translaminar systemicity can provide longer-term residual activity after penetrating into leaf tissues. Therefore, systemic pesticides are able to considerably reduce not only the dose rate but also the number of applications for each season. Also, translaminar systemicity is important property for both fungicide and insecticide. Some fungi and insects live on the lower leaf surface, but pesticide is often applied as sprays to the upper leaf surface. Cotton aphid (*Aphis gossypii* Glover) usually feed on the lower surface of cotton leaves, and can cause leaf malformations of young plant like leaf curling or rolling. Insecticides acetamiprid with translaminar systemicity can provide good control of cotton aphid[77]. When acetamiprid was applied to the upper leaf surface of cotton, its translaminar residual activity provided 100% mortality of aphids feeding on the lower leaf surface within 12 days[77]. Further, translaminar systemic insecticide can be used to control insects that feed inside the leave such as leafminer and tomato pinworm[75]. Long-distance transport of pesticide is mainly carried through xylem and/or phloem, and a compound can be transported to the different parts of plants after soil or foliar application. The sulfonylurea herbicides, which inhibit acetolactate synthase, need to be translocated in the phloem to all parts of the plant[78]. Ambimobile insecticide spirotetramat is used to control hidden and soil living sucking pests, such as aphids and whiteflies[79]. In addition, long-distance movement enables pesticide to protect newly emerging shoots.

1.3.2 Passive processes of systemicity

In general, the uptake and transport of most pesticides are passive diffusion determined by physicochemical properties, including penetration into the plant surface and translocation within the plants via xylem and/or phloem[80-82]. Both soil and foliar application are the most popular pesticide application techniques, which require pesticides to be taken up by plant roots or leaves, respectively.

Soil-applied pesticides, which can be absorbed by roots, move in the direction of the transpiration steam. Root Concentration Factor (RCF) and Transpiration Stream Concentration Factor (TSCF) can be used to described the root absorption and the subsequent translocation to shoots, defined as the following equations[83]:

$$\text{RCF}=\frac{\text{Concentration in roots}}{\text{Concentration in the nutrient solution}}$$

$$\text{TSCF}=\frac{\text{Concentration in xylem sap}}{\text{Concentration in nutrient solution}}$$

Bromilow and co-workers[84] established the correlation of the RCF and TSCF to lipophlicity of pesticides (estimated using *K*ow, 1-octanol/water partition coeffi-cient). After study the absorption of 18 non-ionized compounds in barley macerated roots, the results showed that accumulation in roots and transport to shoots could be related to *K*ow by the following equations:

$$\text{Log}\,(\text{RCF-0.82}\,) = 0.77 \log K_{ow}\text{-1.52}$$

$$\text{TSCF} = 0.784 \exp\text{-}[(\log K_{ow}\text{-1.78})^2/2.44]$$

It was found that the values (RCF) increased with increasing lipophilicity (log *K*ow) in barley roots[84]. However, an optimum lipophilicity (log *K*ow = 1.8) was observed in hydroponically grown barley roots for maximum translocation to shoots, and slightly lower than that reported in soybean roots at a log *K*ow value of 3[71,84]. The xylem mobility of very lipophilic compounds with log *K*ow > 4.5 was very small[84].

Unlike nonionized compounds, root uptake and translocation to shoot of many weak acids increased as the pH of the bathing solution decreased. The uptake of 2,4-D into barley roots from nutrient solution was 36 times greater at pH 4.0 than at pH 7.0 for 24 h[85]. Meanwhile, the translocation of 2,4-D following uptake by roots in-creased about 4 times as the pH was reduced from 7.0 to 4.0[85]. Xylem systemicity

of ionized compounds in plants can be predicted from pKa and log *K*ow values. Bromilow and co-workers established a general rule, lipophilic weak acid compounds (log *K*ow >4) are non-systemic, while compounds of intermediate lipophilicity (log *K*ow form -0.5 to +3.5) can move in the xylem[86].

The pathway of a foliar-applied systemic pesticide involves circular permeation, long-distance translocation within the vascular tissues (phloem and then possibly xylem), and unloading to sink regions[82]. The plant cuticle, a protecting film covering the epidermis of leaves, is the first barrier to foliar uptake[87]. The absorption of lipophilic compounds by plant cuticles can be predicted with good precision from log *K*ow values of the compounds[88]. Generally, cuticle penetration tends to increase with increasing lipophilicity of the chemicals within certain range[21]. But greater lipophilicity was associated with decreased translaminar activity[89].

Kleier and co-workers[90] developed a mathematical model for the plant vascular system that enables the prediction of phloem systemicity as a function of its *K*ow and pKa. This model was built on the intermediate permeability hypothesis by incorporating the effect of acid dissociation[91]. The compounds with a log *K*ow value of 0 to 3 and a pKa value of 3 to 6 are predicted to have an optimum permeability for phloem mobility[92]. Moreover, modification of chemical structure of oxamyl based on Kleier model by adding glucuronide was proved to be a good way to confer phloem mobility to poorly mobile pesticide[93]. Satchivi and co-workers[94-96] designed a dynamic, nonlinear simulation model (ERMESSE) for whole plant transport and allocation of foliar-applied xenobiotics, which is a comprehensive model integrating physicochemical properties (lipophilicity, acid dissociation constant and molar volume) of the active ingredient with plant parameters. The results from the ERMESSE model showed that an optimum absorption and translocation can be expected for weak acid molecules ($4.0 \leq pKa \leq 8.0$) with a molar volume of 225–300 cm^3 mol^{-1} and an intermediate lipophilicity ($-0.5 \leq \log Kow \leq 2.5$)[82].

Weak acid compounds can be accumulated in sieve tubes by the ion-trap mecha-

nism, because of the pH difference between apoplast and symplast[97,98]. At the pH of the apoplast, about 5.5, weak acids are partly ionized. The undissociated form easily diffuses through the plasma membrane of the sieve tube-companion cell complex to reach the phloem symplast (pH 7.5 to 8.0) where it will be substantially ionized. Since the anions cannot easily pass back through the lipophilic membrane, they will be trapped (Fig 1-8).

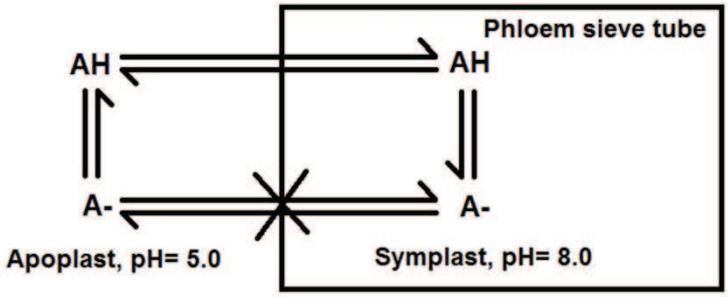

Figure 1-8. Accumulation of weak acids within cells by the ion-trap effect (adapt from [78]).

Ester-acid-toxophore hypothesis has been applied in to improve circular uptake. Once inside the plant, the ester form will be hydrolyzed and the acid form will be loaded into the phloem by ion-trap mechanism[97]. Herbicides containing carboxylic acid functions are generally applied to plants as ester derivatives, such as diclofop, 2,4-D and triclopyr[99]. They will be rapidly activated within the plant by hydrolysis to the corresponding free acids. In addition, the ion-trap strategy can be extended to other pesticide families. For example, fenpiclonil is a non-systemic phenylpyrrole fungicide, but acidic derivetives of fenpiclonil can be detected in phloem sap after attaching a carboxylic acid group[100,101].

1.3.3 Carrier-mediated processes influencing systemicity

Plant plasma membrane is a hydrophobic diffusion barrier to separate the cytoplasm from external environment. But at the same time, plasma membrane must facilitate and continuously regulate the exchanges of information and substances between the cell and its environment. Larger-scale transport between plant and environment, or between leaves and roots, is regulated by membrane transporters at the cellular level

[74]. Channel transporters are able to enhance ions and water diffusion across the plasma membranes of higher plants[102]. For example, water-channel proteins are transmembrane proteins that enable water to cross biological membranes at rates faster than the rates of diffusion through the lipid bilayer[103]. Unlike channels, carrier proteins do not have pores that extend completely across the membrane, and they bind the specific substances on one side of the membrane and release it on the other side[74]. Plant sugar transporters comprising sucrose and monosaccharide transporters mediate long distance transport of sugar from source to sink organs and play a central role in active phloem loading and unloading processes of sucrose[104-107]. Amino acids can be translocated from the site of biosynthesis to sink tissues via the phloem and xylem, in which plant amino acid transporters are essential participants in the loading and unloading steps[108]. The various plant transport systems provide potential targets as carriers for agrochemical molecules (Fig. 1-9).

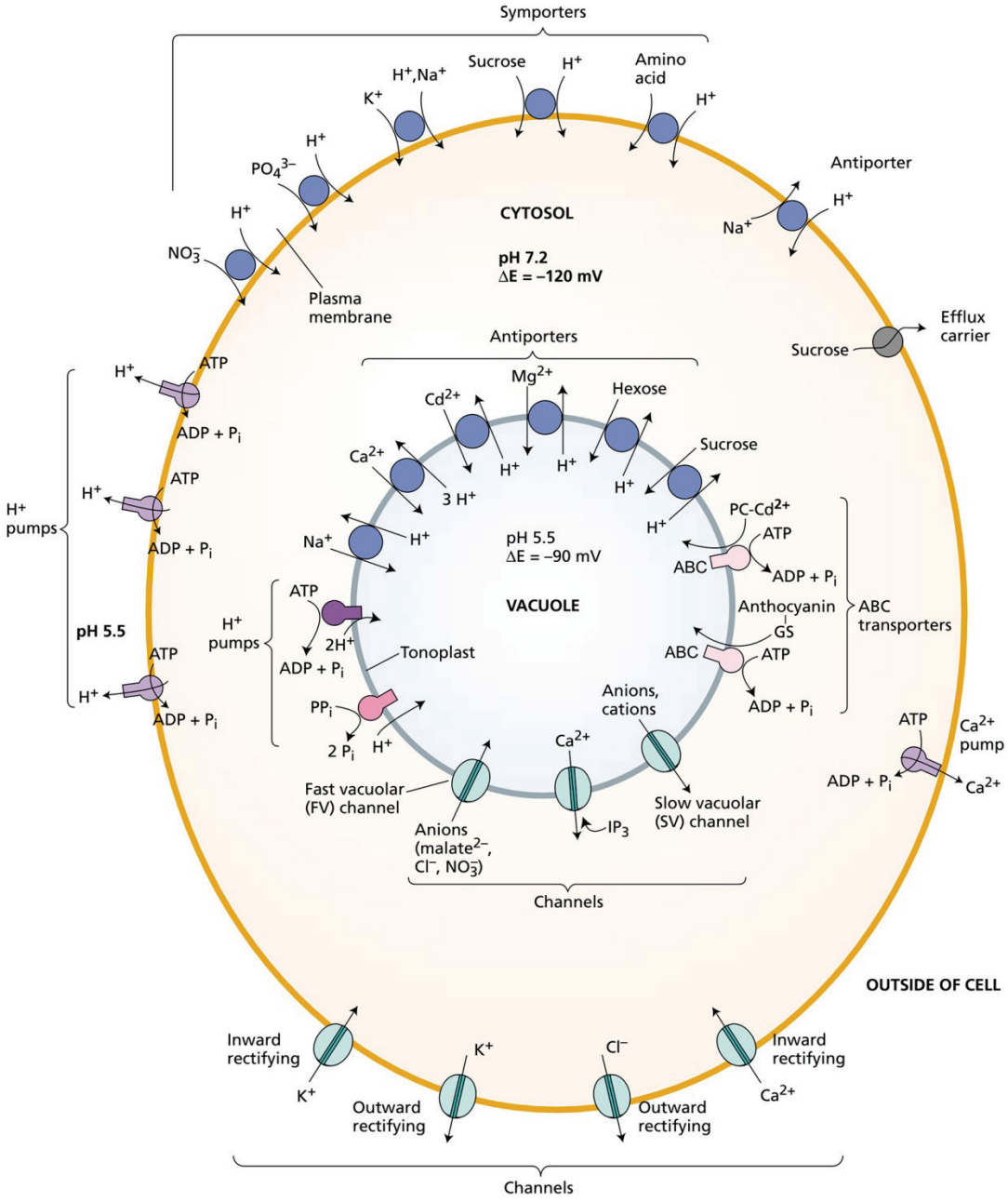

Figure 1-9. Overview of the various transport processes on the plasma membrane and the vacuolar membrane (tonoplast) of plant cells (From Ref. [74]).

In modern pharmaceutical research, it is generally accepted that both passive transcellular diffusion and carrier-mediated processes coexist and contribute to drug transport across biological membranes[24]. However, passive diffusion was considered as the only way for uptake and transport of pesticides in plants for a long time. The functions of potential plant pesticide transporters have been overlooked. The researches on the carrier-mediated processes of pesticide transport are seldom con-

cerned in the literatures.

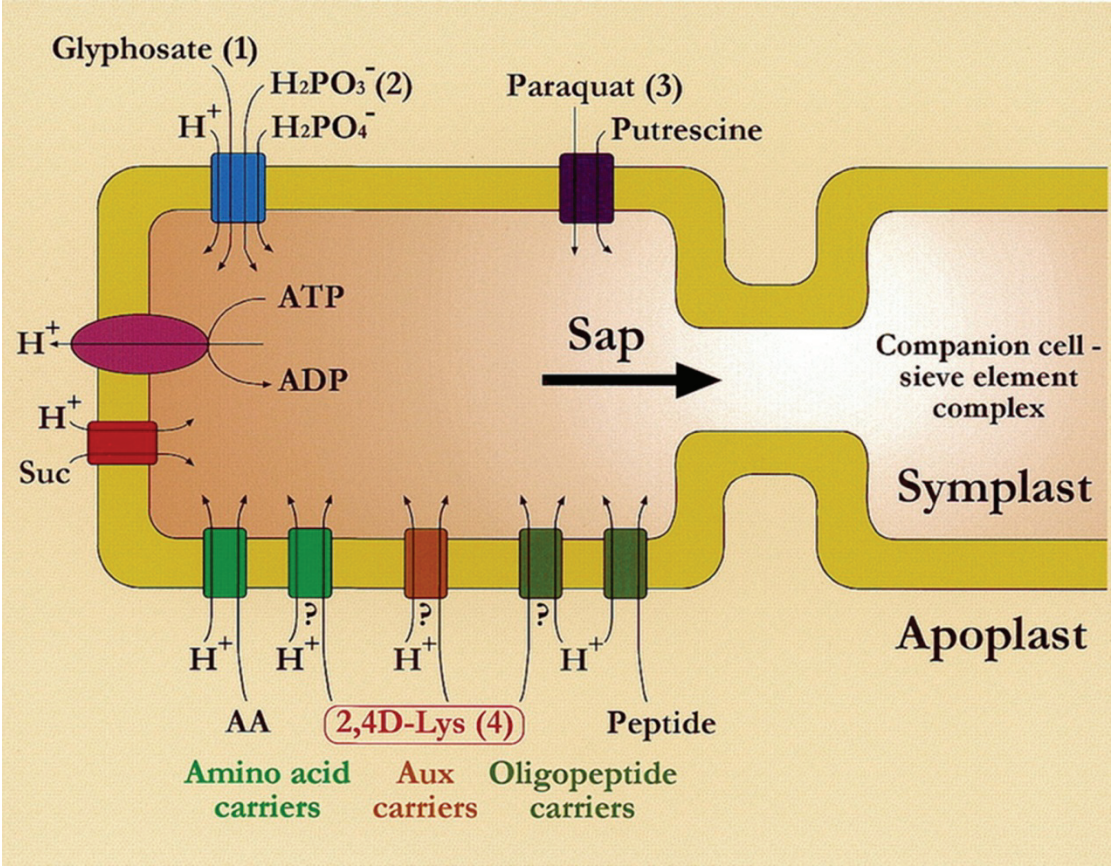

Figure 1-10. Possible carriers involved in the uptake of xenobiotics by the phloem tissue (adopted from Ref. [109])

But as research progressed in the last two decades, some pesticides have been found to be transported by identified plant carrier systems (Fig 1-10). Glyphosate, a widely used herbicide, is transported to root and shoot growing tissues via phloem after foliar application[110,111]. Carrier-mediated uptake of glyphosate at low concentration has been reported in leaf protoplasts of broad bean via a phosphate transporter[112]. In maize and soybean cell suspensions, active uptake of glyphosate was inhibited by carbonylcyanidem-chlorophenyl hydrazine (CCCP), orthovanadate, diethylstilbestrol (DES), phosphate, and phosphonoformic acid (PFA), suggesting that the glyphosate carrier is a phosphate transporter energized by the plant plasmalemma ATPase[113]. One possible glyphosate-resistance mechanism is the non-recognition of the active uptake system, because the inhibition of glyphosate-loading into the phloem was observed in some glyphosate-resistant weeds[114]. Furthermore, a recent

study shows L-type amino acid transporters (LAT1/LAT2) play major roles in the uptake of glyphosate across mammalian epithelial tissues[115].

Two other herbicides have been shown to exhibit carrier-mediated processes. The uptake of paraquat into maize root cells and maize suspension cells was competitively inhibited by putrescine, indicating that a polyamine transporter is responsible for the uptake of paraquat[116,117]. In addition, Xi *et al.*[118] identified AtPDR11, a plasma membrane-localized ABC transporter, as a paraquat transporter from the paraquat-tolerant mutant *pqt24-1*. The enhanced paraquat tolerance of *pqt24-1* is due to loss of AtPDR11, which results in less accumulation of paraquat in plant cells. The uptake of the auxinic herbicide 2, 4-D in maize root protoplasts occurs by two mechanisms, the ion-trap mechanism and active transport mediated by an auxin carrier[119]. Moreover, the ANT1 amino acid transporter can also transport 2, 4-D through the plasma membrane[120].

Over the last two decades, there have been several attempts to improve pesticide phloem mobility or translocation to root system utilizing plasma membrane sugar or amino acid transporters. Two synthesized α-amino acid conjugates, lysine-2, 4 D and aspartic acid-triazolyl alcohol (Fig 1-11A), markedly and specifically inhibited threonine uptake and phloem loading by leaf tissues, suggesting that both conjugates can be recognized by amino acids transporter[121]. Further study using several derivatives of phenoxyalkanecarboxylic acid and L-lysine (Fig 1-11B) demonstrated that the plant amino acid carrier system is able to recognize a wide range of conjugates of various sizes, structures and *K*ow[122]. Interestingly, the distribution of conjugate lysine-2, 4 D and parent compound (2, 4-D) on broad bean showed a significantly difference in certain plant organs[109]. As a non-metabolized conjugate, the accumulation of lysine-2, 4D in the root system is 5 to 10 times greater than that of parent compound (2, 4-D)[109]. Another study demonstrated that introduction of a glycinyl moiety to fipronil can improve the phloem systemicity of parent compound in intact soybean seedling[123].

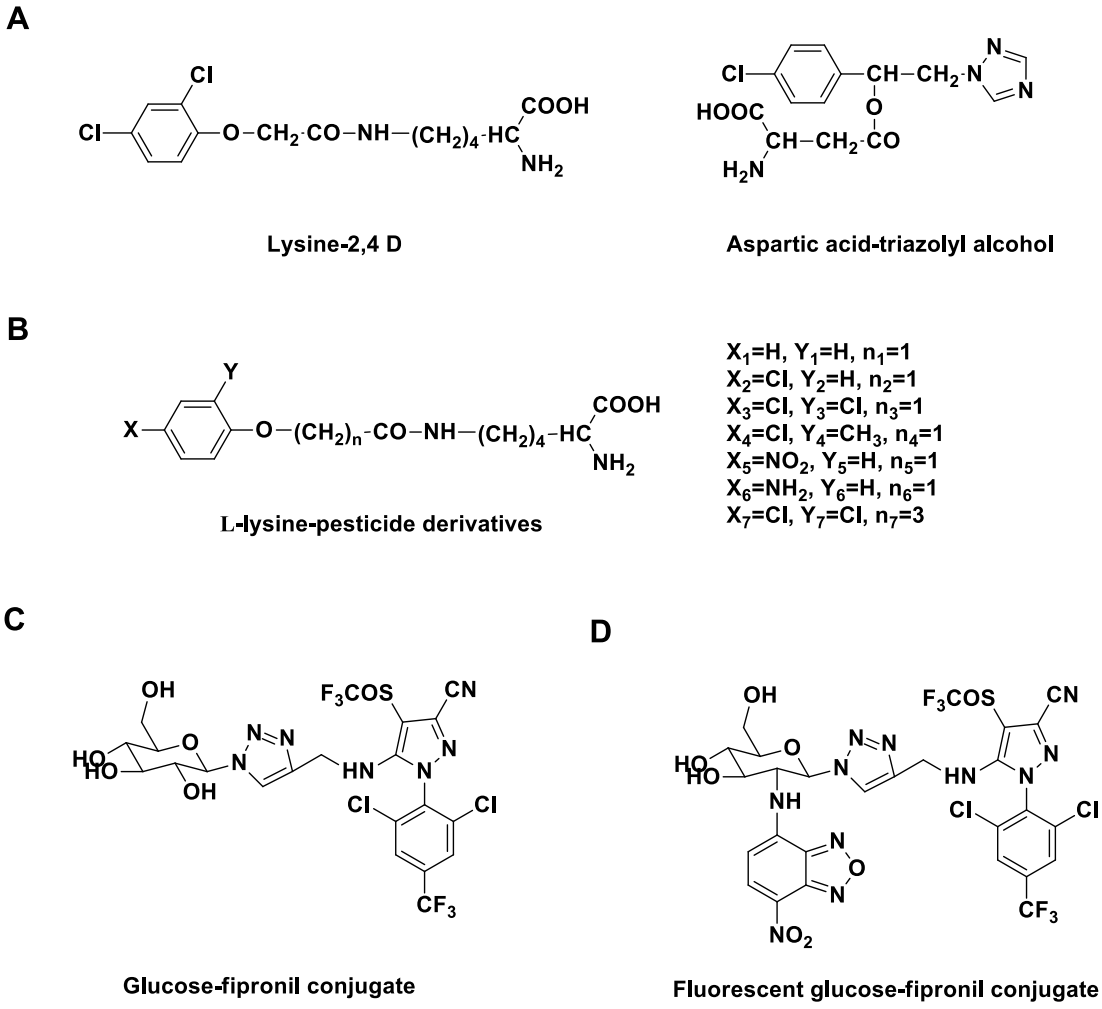

Figure 1-11. Examples of pesticide-amino acid conjugate or pesticide-sugar conjugate

Conjugation of pesticide with glucose also has been explored to develop phloem-mobile insecticide. Yang and co-workers[124] synthesized a glucose-fipronil conjugate (Fig 1-11C) and tested its phloem mobility in castor bean seedling. The result showed that glucose-fipronil conjugate was detected in phloem sap. Further study for the mechanism of systemicity showed that uptake of glucose-fipronil by cotyledon discs was markedly inhibited by CCCP and phloridzin as well as the phloem mobility was inhibited by phloridzin, revealing that a carrier-mediated mechanism was involved in uptake and phloem loading[125]. In order to visualize the movement of glucose-fipronil conjugate, a fluorescent glucose-fipronil conjugate (Fig 1-11D), which exhibited comparable phloem mobility, was synthesized via click chemistry[126]. Fluorescence photographs displayed that the fluorescent conjugate

can be loaded into sieve tubes after entering into epidermal cells and mesophyll cells and then translocate from cotyledon to hypocotyl via the phloem in castor bean seedlings (Fig 1-12). Yuan *et al*.[127] synthesized of a series of monosaccharide-fipronil conjugates including hexose, pentose, or deoxysugar moieties. Phloem mobility test in castor bean seedlings indicated that L-Rhamnose fipronil and D-fucose fipronil conjugate displayed the highest phloem mobility among all of the tested conjugates.

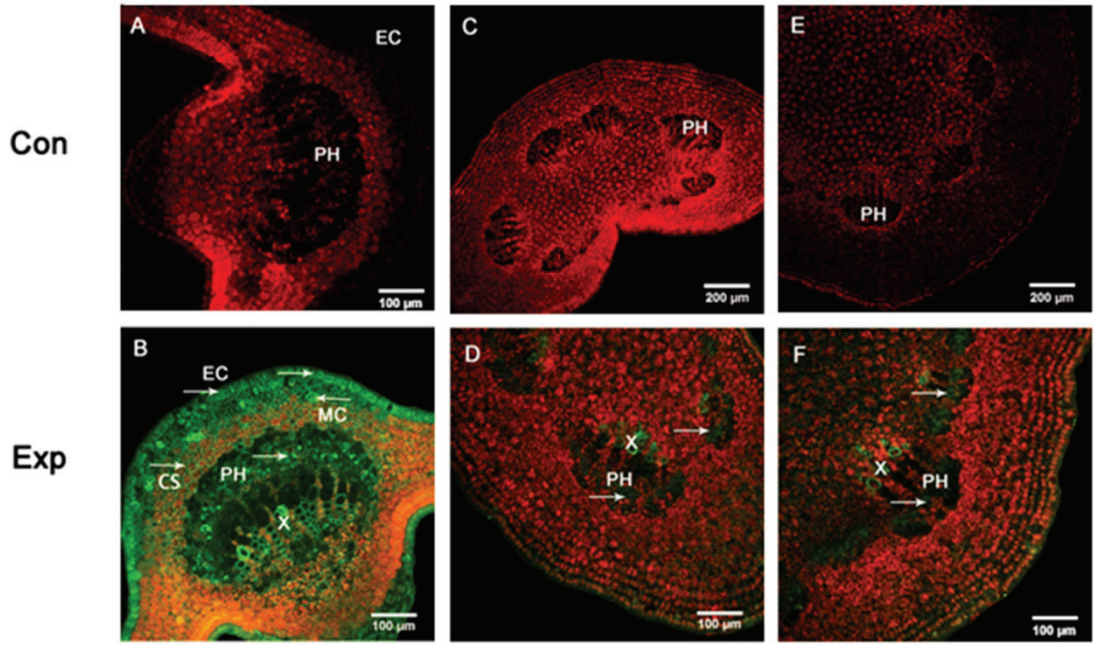

Figure 1-12. Translocation of fluorescent glucose-fipronil conjugate in caster bean (Ricinus communis L.) seedlings: The cotyledons were incubated in a buffered solution containing 100 μM conjugate. (A, B) cross sections of mid-veins of cotyledons, (C, D) cross sections of petioles of cotyledons, (E, F) cross sections of hypocotyl of caster bean seedlings. All photos were observed by confocal laser scanning microscope (Zeiss780, Germany). The presence of fluorescent xenobiotics was indicated by arrowheads. EC: epidermal cells, CS: cell wall space, MC: mesophyll cells, PH: phloem, X: xylem. (From Ref. [126])

Any pesticides transported in the vascular system must cross the plasma membrane and enter the symplast[71,98]. Therefore, the mechanism of entry into the symplast will be an important factor in influencing both its long-distance transport and distribution throughout the plant. The classical approach to improve systemicity can be achieved through optimizing the physicochemical properties to increase passive membrane penetration[92]. However, several attempts mentioned above suggest that

the targeted prodrug approach by conjugating with nutrient moiety is also a feasible approach to confer systemic properties to pesticide.

1.3.4 Methods used to investigate systemic pesticides

In the early-stage of pesticide discovery or systemicity screening, several simple rule-based models and mathematical plant uptake models have already been developed to predict and assess the plant uptake and translocation of agrochemicals based on physicochemical properties. There are two widely used models that predict the systemicity of pesticides, Bromilow model and Kleier model[128,129]. These models can be used to both nonionized and acidic compounds by combination of acid dissociation constant (*K*a) and 1-octanol-water partition coefficient (*K*ow) (Fig 1-13). In drug discovery, oral bioavailability or passive membrane permeability of drug candidates can be predicted using Lipinski's rule of five (Ro5) and other simplified rule established by Veber[130,131]. The same approach was adopted to profile agrochemicals [132-134], which can be applied to pesticide screening and optimization. In the screening process, molecules that are predicted to have acceptable absorption or permeation properties in term of appropriate physicochemical properties can be defined as 'drug-like'[131]. Hao *et al*.[135] established some rules for pesticide-likeness derived from drug-likeness, including molecular weight ≤ 435 Da, calculated octanol/water partition coefficient (ClogP) ≤ 6, number of H-bond acceptors (HBA) ≤ 6, number of H-bond donors (HBD) ≤ 2, number of rotatable bonds (ROB) ≤ 9, and number of aromatic bonds (ARB) ≤ 17. These models mentioned above are able to give useful information of the systemic ability of compounds rather than accurate predictions[136]. It should be noted that all those rule-based and mathematical models are passive diffusion models and not applicable to active carrier-mediated molecules.

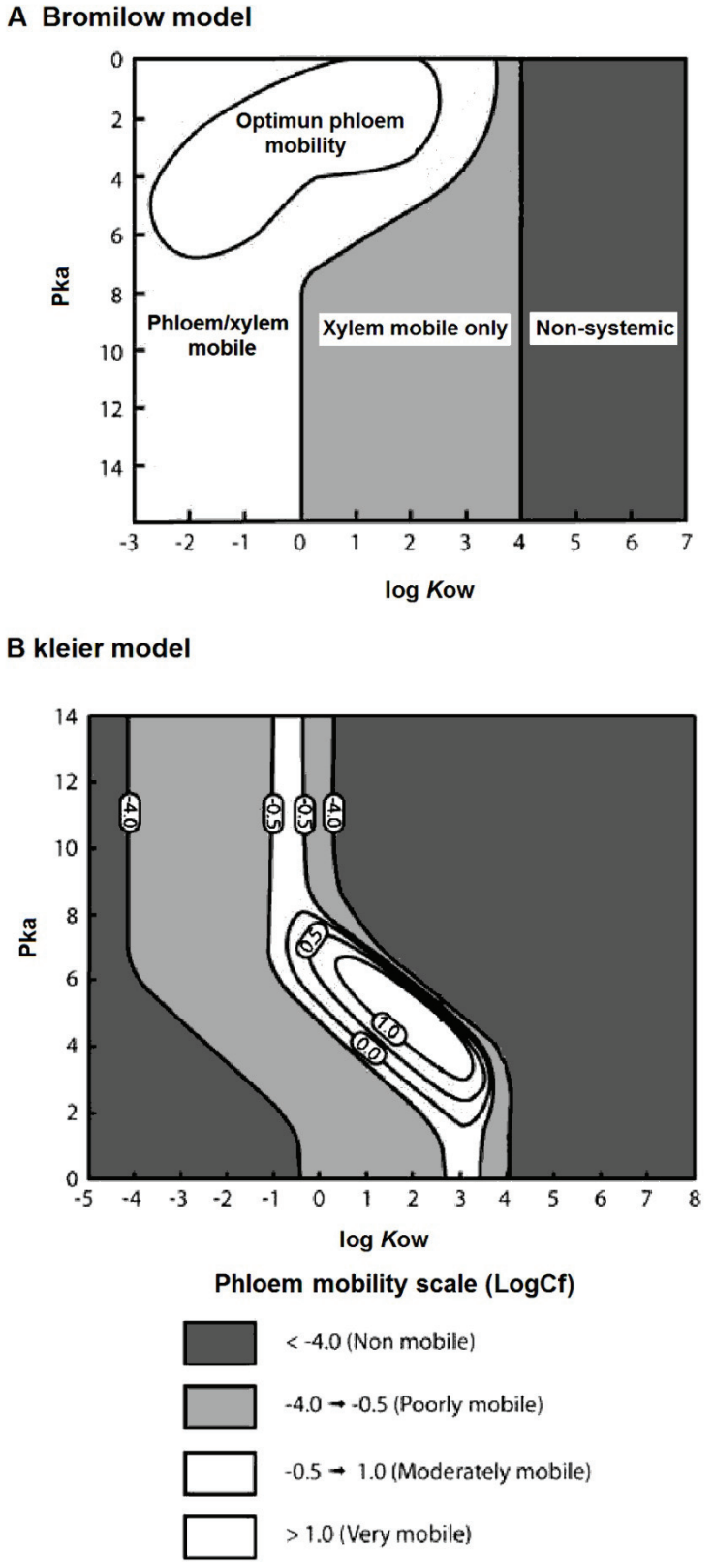

Figure 1-13. Plant uptake models for prediction of systemicity of weak acids and nonionized compounds (Modified from Ref. [137]).

There are various approaches to measure transport of pesticides in plants. The

most popular approach is the application of radioactive compounds, especially for ^{14}C-labeled pesticides. [^{14}C]-Chlordecone was used to study the uptake and distribution in radish and maize shoots after growing in contaminated soils[138,139]. To demonstrate the phloem transfer of insecticide fipronil, sunflower culture seed was coated with [^{14}C]-compound[140]. Autoradiographs of [^{14}C]-glyphosate treated plant can be used to observe its distribution and investigate its uptake and transport[80,141]. Moreover, fluorescence labeling is also a widely used approach. To simulate the foliar uptake process of pesticides, the uptake of two model fluorescent xenobiotics in broad bean leaves was visualized by confocal laser scanning microscopy[142]. Two-photon excitation microscopy technique provides a nonintrusive tool for visualizing and tracking the movement, storage locations and degradation of organic chemicals using only plant and compound autofluorescence[143]. Two bioactive fluorescent gibberellic acid compounds were synthesized and applied to study their distribution in *Arabidopsis thaliana* roots to investigate the hormone transport[144]. In addition, most of extraction and determination methods of pesticide residues can also be used to study the systemicity of pesticide at the cellular, organ and whole-body level. The plant extracts containing non-labelled compound can be analysed by chromatographic techniques, such as GC, HPLC, LC/MS[123,145,146].

The investigation of xylem or phloem systemicity is often necessary for more specialized systems of study. To study the root-to-shoot translocation of non-radiolabelled pesticides, the pressure-chamber technique was applied in detopped soybean roots to obtain a considerable volume of xylem sap[71,147]. Chollet and co-workers[148] recently developed a non-destructive method to assess root-to-shoot translocation of soil-applied pesticides. This method relied on a dwarf tomato, which can exude an abundant apoplastic fluid through large stomata for several months. Furthermore, the collection of relatively pure phloem sap has been experimentally challenging. The current methods for sampling and analysis of phloem sap were summarized by Dinant and Kehr[149]. Generally, one approach is to obtain sieve-tube

exudate from the exuding stylets of phloem-feeding insects[150,151]. Aphids are the most commonly "natural syringe" to collect pure phloem exudates[152], but it is a time-consuming and work-intensive technique. In addition, a few plant species exude phloem sap from wounds after severing sieve elements, which are possible to be used in pesticide research, such as excised leaves of legumes and excised flowering stems of yucca[78]. Cucumber and pumpkin are two other plants that are widely used to collect phloem sap from the wounds in the stems[153,154].

Castor bean (*Ricinus communis* L.) is a widely used model plant to study the phloem translocation of nutrients as well as xenobiotics[155-159]. *Ricinus* seedlings offers the unique chance to obtain pure phloem sap for several hours after cutting the hypocotyl hook, because in contrast to nearly all other plants it lacks an efficient sieve-tube plugging-mechanism[157]. The cotyledons of the *Ricinus* seedling are embedded in the endosperm, which provides germination nutrients. Duo to the absence of cuticle (or very thin), the apoplast of the cotyledons is in direct contact with the external space so that it can be considered as an "open apoplast" [160]. This feature enables *Ricinus* seedlings to be an ideal model plant to study the uptake and phloem loading characteristics of the cotyledons. After the endosperm is removed, chemicals can be added to incubation solution bathing the cotyledon and phloem sap can be sampled from the upper part of severed hypocotyl[100,109,124].

1.4 Systemic fungicides and its significance

1.4.1 Brief history to systemic fungicides

The control of crop diseases with fungicides has already had a successful history for almost 200 years. The discovery of Bordeaux mixture in France in 1882 was an important milestone of chemical fungicides. Up until the 1940s, chemical disease control was mainly dependent on inorganic chemical preparations[161]. Inorganic fungicides are generally contact fungicides with multiple sites of action and protective activity against various diseases. Two decades from 1940 to 1960 witnessed a start of a new era for organic fungicides. During this period, the introduction of the dithiocarbamates and later the phthalimides was a major improvement over previously used inorganic fungicides for plant disease control[162]. From 1960 to 1970, the benzimidazoles, carboxamides, and early sterol biosynthesis inhibitors (SBIs) were introduced as the first fungicides with a specific mode of action[163]. The 1970s saw significant advances in crop protection chemistry. Dicarboximides, triazoles, phenylamides and host defense inducers were commercialized[161,162]. During the 1980s, second generation SBIs, amines, carboxylic acid amides and melanin biosynthesis inhibitors entered the market[164]. With the continually growing agrochemical markets, strobilurins, anilinopyrimidines, phenypyrrole and new triazoles were launched in the following 10 year period from 1990 to 2000. Since the year 2000, major new products consisted of “new generation” triazoles and strobilurins, and several molecules with new modes of action such as metrafenone, mandipropamid and boscalid[165]. Over the past 70 years, many novel fungicides in various chemical groups were discovered. Meanwhile chemical fungicides gradually became a central part of disease control. However, with the increasing regulatory requirements and the emergence of resistance to fungicides, the rate of introduction of new products slowed dramatically in recent years.

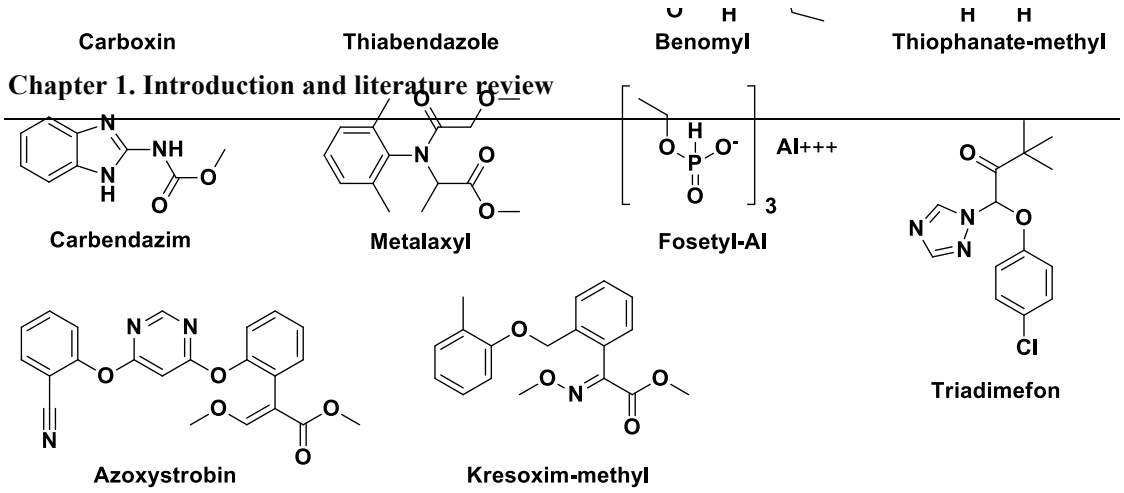

Figure 1-14. Some milestone systemic fungicides.

The invention of systemic fungicides was regarded as a significant progress in crop protection. Systemic properties offered new opportunities in disease control and fungicide application. The development of systemic fungicides leads to reduce both the dose rate and the application frequency. There were some milestone systemic fungicides in development history (Fig 1-14). Carboxin, introduced in 1966, was first systemic fungicide as a cereal seed treatment. Carboxin was used to effectively control smuts and bunts but it was inactive on other fungal groups and not effective as a foliar spray. Systemic test in lemon seedlings suggested that carboxin has only xylem systemicity[166]. Thiabendazole was the first broad-spectrum foliar systemic fungicide, which belongs to benzimidazole fungicide. One successful member of the same group was benomyl, which was launched by DuPont in 1970. Because of a good systemic property, the benzimidazole fungicides including thiophanate-methyl (1970) and carbendazim (1976) achieved great success in controlling a wide range of disease of fruits and vegetables. As most systemic fungicides, the benzimidazole fungicides can be taken up by roots and translocated upward via the xylem to the leaves, but their downward translocation in most plants is usually small[167]. In addition, Translaminar systemicity of benomyl and thiabendazole has been detected by protecting sugar-

beet leaf form infection when the opposite surface was treated with the fungicides[168]. The phenylamide fungicide Metalaxyl, which was introduced in 1977, was a successful systemic compound to control oomycete. Fosetyl-Al, a phosphonate fungicide also introduced in 1977, is the unique ambimobile fungicide available in the market.

First triazole fungicide triadimefon, launched by Bayer in 1976, was a milestone for systemic fungicide. The triazole fungicides displayed both curative and protective activity as well as good redistribution in plant. They have been reported to be transported in both the xylem and the phloem, but tanslocation in the xylem is still a predominant pathway[169-171]. A translocation study between the ears and the flag leaves of wheat showed that the basipetal translocation of tebuconazole and prothioconazole was more expressed than the acropetal translocation, but the rate seldom exceeded 5%[171]. Using cucumber powdery mildew as a biological indicator to examine translaminar activity of 61 fungicides, all 7 commercialized triazole fungicides (tebuconazole, propiconazole, cyproconazole, myclobutanil, epoxiconazole, fenbuconazole and prothioconazole) exhibited moderate or strong translaminar activity[89]. After three decades of expansion, the triazole fungicides have been the largest fungicide group in global market. At present, the second largest fungicide group is the strobilurins, which are systemic fungicides with protective, curative and eradicant activity. The first strobilurin fungicides were azoxystrobin and kresoxim-methyl, which entered the market in 1996[172]. The systemic properties of several strobilurins (azoxystobin, trifloxystrobin, kresoxim-methyl, metominostrobin, pyraclostrobin and picoxystrobin) were summarized by Bartlett and co-workers[172]. In general, azoxystobin showed translaminar and xylem systemicity, while no selected fungicides was phloem mobile. Until recently, systemic fungicides have become the dominant of overall fungicide market.

1.4.2 Systemic fungicides for management of vascular diseases

1.4.2.1 Problems in chemical control of Grapevine trunk diseases

Vascular diseases are the most destructive plant disease worldwide and cause huge economic losses for annual crops as well as woody perennials. These diseases are generally caused by soil-borne bacteria, fungi and oomycetes that infect the xylem vessels of plants, and as a result, interfering with the translocation of water and minerals to the crown of the plant[173]. Infection of xylem vessels can induce symptoms of vascular wilts and/or cankers, which are two types of vascular diseases[174]. Vascular wilt-causing fungi enters and colonizes the plant vascular (xylem) system, disrupting water transport, and leading to wilting, browning and dying of leaves followed by death of the whole plant[174]. Cankers may cause some reduction in the translocation of water, but generally, do not kill plants unless the cankers are big or numerous enough to encircle the plant[174]. Over the last 10-15 years, some xylem-inhabiting fungi in vineyards gradually became the major concern for the global wine industry. Eutypa dieback, esca and botryosphaeria dieback are three significant grapevine trunk diseases (GTDs), which are caused by one or several xylem-inhabiting fungi[175]. The symptoms of the GTDs (Fig 1-15) include woody necrosis and decay, chlorosis and deformation on leaves and berries and the eventual vine death[176,177].

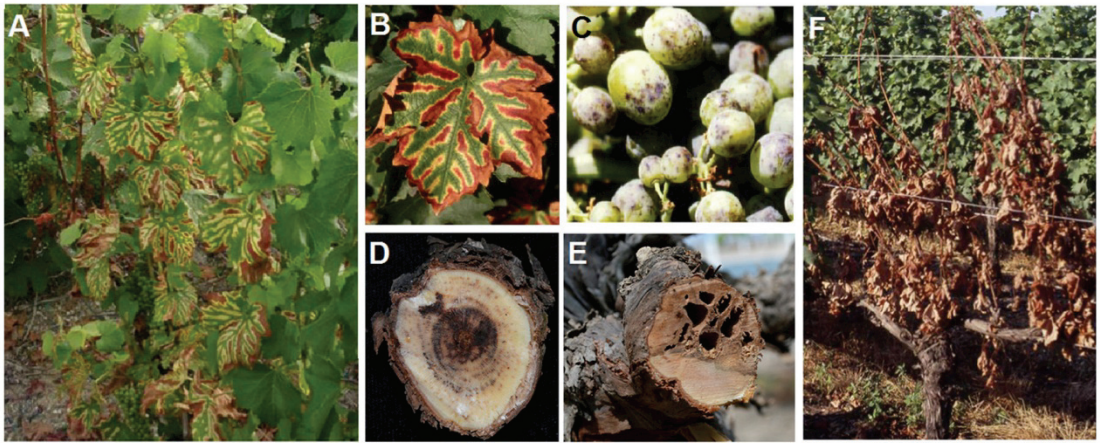

Figure 1-15. Disease symptoms of grapevine trunk diseases. (A, B) typical tiger-like necrosis and chlorosis on leaves. (C) spotting on grappe berries. (D) cross section of a grapevine wood spur infected with esca. (E) cross section of a grapevine cordon infected with eutypa dieback. (F)

symptoms of apoplexy, characterized by dieback of one or more shoots. (picture A, B, C, F from Fontaine et al. [177] and picture D, E from Pouzoulet et al[178].)

At present, there are no efficient treatments available for the management of the GTDs [175]. Before 2000, sodium arsenite was once the only potentially effective treatment to control GTDs, but this inorganic fungicide was banned in 2001 because of its carcinogenic effect on to humans[175,177,179]. Subsequently, a wide range of methods, including chemical, biological and cultural, are continually attempting to control GTDs. Today, chemical treatments are usually used to avoid grapevine infection and limit fungal expansion by protecting pruning wonuds[175]. Several fungicides have been reported to effectively reduce mycylial growth and conidial germination of grapevine pathogens *in vitro* experiments. Bester *et al*. [180] investigated the efficacy of ten fungicides on mycelial inhibition of the four pathogenic species of Botryosphaeriaceae (*Diplodia seriata*, *Lasiodiplodia theobromae*, *Neofusicoccum australe*, *Neofusicoccum parvum*). The result showed that benomyl, tebuconazole, prochloraz manganese chloride and flusilazole were the most effective fungicides in inhibiting the mycelial growth on potato dextrose agar (PDA) medium. Pitt *et al*. [181] reported that mycelial growth of four species of Botryosphaeriaceae was inhibited by fludioxonil, carbendazim, fluazinam, tebuconazole, flusilazole, penconazole, procymidone, iprodione, myclobutanil and pyraclostrobin with EC_{50} values less than 1.0 mg/L. Amponsah *et al*.[182] showed that nine fungicides were effective at reducing mycelial growth and/or conidial germination of *Neofusicoccum australe*, *Neofusicoccum luteum* and *Diplodia mutila*. Sosnowski *et al*.[183] evaluated the effect of 13 fungicides on mycelial growth and ascospore germination of *Eutypa lata in vitro* experiments. They reported that tebuconazole and fenarimol were the most effective fungicides to reduce ascospore germination, and mycelial growth was completely inhibited by carbendazim and cyprodinil + fludioxionil.

However, the efficacy of the same fungicides selected from *in vitro* experiments was usually variable under field conditions[181-183]. More importantly, no efficient fungicide was reported to cure infected plants. The different effect of the same fungi-

cide between *in vitro* and in the field is probably because the grapevine trunk pathogens live deep in the interior of the host plants. This leads to the failure of fungicides to reach the targeted pathogens under natural conditions. Thus, whether the fungicides can be delivered into vascular tissues may be a limiting factor for both protective and curative activity against GTDs.

1.4.2.2 The need for new fungicides

Most vascular pathogens enter and colonize the xylem vessels, which is composed of dead tracheary elements with relatively low osmotic pressure[173]. Furthermore, xylem systemicity is the primary route for pesticides translocation, and most of the systemic fungicides move with the transpiration stream in plants. However, xylem-mobile fungicides are not effective for the control of most vascular pathogens. Because, xylem-mobile fungicides are usually translocated and accumulated at the sites of high transpiration (e.g. leaf tips and margins), leaving the vascular tissues relatively unprotected[184]. Such wedge-shaped distribution pattern (also named "apoplastic wedge") is usually observed in dicotyledonous plants, in which xylem-mobile compounds will move increasingly to the leaf margins[78]. In addition, the disruption of water flows due to embolism or occlusion of vessels in infected plants diverts the transpiration stream and therefore the fungicides are away from the pathogen and transported to the leaves[184].

Hence, phloem-mobile fungicide is more promising in control. In the primary infection processes, the pathogen penetrates the root surface, traverses tissues and stay in the prevascular phase[185]. Phloem-mobile symplastic translocation enables fungicides to be applied to leaves then pass to roots and also to new growth tissues[184]. Thus many infections can be eliminated in the prevascular phase after the redistribution of fungicide through the plant. Additionally, the phloem-mobile fungicide which can be translocated to the vascular tissues is likely to cure infected plants. Unfortunately, the phloem-mobile fungicide for effectively controlling the GTDs is not available at present. The only ambimobile fungicide fosetyl-Al provided no interest-

ing effect in GTDs control[179]. To effectively and economiclly control GTDs, it is necessary to find a new phloem mobile fungicide with good activity against grapevine trunk disease pathogens.

1.5 Objectives of the thesis

The percentage of prodrugs among the successful drugs is steadily increasing. One of attractive prodrug approach is to conjugate a drug with endogenous substrates, and the resulting molecules can be recognized and transported by membrane transporters. Carrier-mediated uptake can not only be utilized to enhance drug delivery with suitable prodrug formation, but also be a crucial factor in the pharmacokinetic, safety and efficacy profiles of drugs.

Prodrug strategy has achieved great success in pharmaceutical industry, while it is rarely used in agrochemical industry. Prodrug approaches for enhancing the bioavailability of drugs have set good examples for agrochemicals. In order to increase the pesticide efficiency and reduce pesticide use, the same strategy can provide a new possible way for pesticide design. In particular, carrier-mediated processes will be a promising approach to enhance the uptake of pesticide and improve the site-targeted distribution by utilizing plant membrane transporters. For example, phloem systemic fungicides are supposed to be foliar applied to control vascular or root diseases where the most existed fungicides are unable to access..

Both amino acid and glucose conjugates have been successfully exploited as carried-mediated propesticides, respectively[109,122,124]. But there is no comparative study of the effect of amino acid and glucose moitry for phloem systemic profungicide. Furthermore, it is unclear that whether the carrier-mediated propesticide strategy can be improved by optimizing the spacer arm structure. These structure-activity studies can promote the understanding of carrier-mediated processes of agrochemcals.

Therefore, there are four objectives for this thesis:

1. To compare the effect of amino acid and glucose promoiety on phloem transport of fungicide conjugates.
2. To investigate the carrier-mediated mechanism of amino acid-fungicide conjugate and glucose-fungicide conjugate and their possible effect on nutrient transport and compartmentation.

3. To examine the influence of different spacer structures on phloem systemicity of the conjugates.
4. To compare the conjugate ability to be loaded in the phloem with that of acidic derivatives already described[101].

References

1. United Nations, Department of Economic and Social Affairs, *World Population Prospects: The 2012 Revision, Highlights and Advance Tables*, in *Working Paper No. ESA/P/WP.228*. 2013: Population Division.
2. Tilman, D., Balzer, C., Hill, J., and Befort, B. L., *Global Food Demand and the Sustainable Intensification of Agriculture.* Proceedings of the National Academy of Sciences of the United States of America, 2011. **108**(50): p. 20260-20264.
3. Tscharntke, T., Clough, Y., Wanger, T. C., Jackson, L., Motzke, I., Perfecto, I., Vandermeer, J., and Whitbread, A., *Global Food Security, Biodiversity Conservation and the Future of Agricultural Intensification.* Biological Conservation, 2012. **151**(1): p. 53-59.
4. Godfray, H. C. J., Beddington, J. R., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F., Pretty, J., Robinson, S., Thomas, S. M., and Toulmin, C., *Food Security: The Challenge of Feeding 9 Billion People.* Science, 2010. **327**(5967): p. 812-818.
5. Bebber, D. P., Ramotowski, M. A. T., and Gurr, S. J., *Crop Pests and Pathogens Move Polewards in a Warming World.* Nature Climate Change, 2013. **3**(11): p. 985-988.
6. Fears, R., Aro, E. M., Pais, M. S., and ter Meulen, V., *How Should We Tackle the Global Risks to Plant Health?* Trends in Plant Science, 2014. **19**(4): p. 206-208.
7. Flood, J., *The Importance of Plant Health to Food Security.* Food Security, 2010. **2**(3): p. 215-231.
8. Popp, J., Peto, K., and Nagy, J., *Pesticide Productivity and Food Security. A Review.* Agronomy for Sustainable Development, 2013. **33**(1): p. 243-255.
9. Oerke, E. C., *Crop Losses to Pests.* Journal of Agricultural Science, 2006. **144**: p. 31-43.
10. Abrol, D. P. and Shankar, U., *History, Overview and Principles of Ecologically-Based Pest Management*, in *Integrated Pest Management: Principles and Practice*, Abrol, D. P. and Shankar, U., Editors. 2012, CABI. p. 1-26.
11. Bebber, D. P., Holmes, T., and Gurr, S. J., *The Global Spread of Crop Pests and Pathogens.* Global Ecology and Biogeography, 2014. **23**(12): p. 1398-1407.
12. Chandler, D., Bailey, A. S., Tatchell, G. M., Davidson, G., Greaves, J., and Grant, W. P., *The Development, Regulation and Use of Biopesticides for Integrated Pest Management.* Philosophical Transactions of the Royal Society B-Biological Sciences, 2011. **366**(1573): p. 1987-1998.
13. Pretty, J., *Agricultural Sustainability: Concepts, Principles and Evidence.* Philosophical Transactions of the Royal Society B-Biological Sciences, 2008. **363**(1491): p. 447-465.
14. Naranjo, S. E. and Ellsworth, P. C., *Fifty Years of the Integrated Control Concept: Moving the Model and Implementation Forward in Arizona.* Pest Management Science, 2009. **65**(12): p. 1267-1286.

15. Ehler, L. E., *Integrated Pest Management (Ipm): Definition, Historical Development and Implementation, and the Other Ipm.* Pest Management Science, 2006. **62**(9): p. 787-789.
16. van der Werf, H. M. G., *Assessing the Impact of Pesticides on the Environment.* Agriculture Ecosystems & Environment, 1996. **60**(2-3): p. 81-96.
17. Pimentel, D., *Environmental and Economic Costs of the Application of Pesticides Primarily in the United States*, in *Integrated Pest Management: Innovation-Development Process: Volume 1*, Peshin, R. and Dhawan, A. K., Editors. 2009, Springer Netherlands: Dordrecht. p. 89-111.
18. European Parliament, Council of the European Union, *Directive 2009/128/Ec of the European Parliament and of the Council of 21 October 2009 Establishing a Framework for Community Action to Achieve the Sustainable Use of Pesticides*. 2009: Official Journal of the European Union.
19. Chollet, J. F., Couderchet, M., and Bonnemain, J. L., *Crop Protection: New Strategies for Sustainable Development.* Environmental Science and Pollution Research, 2014. **21**(7): p. 4793-4796.
20. Ministère de l'Agriculture, de l'Agroalimentaire et de la Forêt, *Plan Ecophyto II*. 2015; 1-67. Available from: http://agriculture.gouv.fr/sites/minagri/files/151022_ecophyto.pdf.
21. Wang, C. J. and Liu, Z. Q., *Foliar Uptake of Pesticides - Present Status and Future Challenge.* Pesticide Biochemistry and Physiology, 2007. **87**(1): p. 1-8.
22. Devarajan, P. V. and Jain, S., *Targeted Drug Delivery: Concepts and Design*. 2015: Springer International Publishing.
23. van de Waterbeemd, H. and Testa, B., *Drug Bioavailability: Estimation of Solubility, Permeability, Absorption and Bioavailability*, ed. Mannhold, R., Kubinyi, H., and Folkers, G. Vol. 40. 2009: Wiley-VCH.
24. Sugano, K., Kansy, M., Artursson, P., Avdeef, A., Bendels, S., Di, L., Ecker, G. F., Faller, B., Fischer, H., Gerebtzoff, G., Lennernaes, H., and Senner, F., *Coexistence of Passive and Carrier-Mediated Processes in Drug Transport.* Nature Reviews Drug Discovery, 2010. **9**(8): p. 597-614.
25. Aungst, B. J., *Absorption Enhancers: Applications and Advances.* Aaps Journal, 2012. **14**(1): p. 10-18.
26. Hirtz, J., *The Gastrointestinal Absorption of Drugs in Man - a Review of Current Concepts and Methods of Investigation.* British journal of clinical pharmacology, 1985. **19**: p. S77-S83.
27. Ensign, L. M., Cone, R., and Hanes, J., *Oral Drug Delivery with Polymeric Nanoparticles: The Gastrointestinal Mucus Barriers.* Advanced Drug Delivery Reviews, 2012. **64**(6): p. 557-570.
28. Begley, D. J., *Understanding and Circumventing the Blood-Brain Barrier.* Acta Paediatrica, 2003. **92**: p. 83-91.

29. Ballabh, P., Braun, A., and Nedergaard, M., *The Blood-Brain Barrier: An Overview - Structure, Regulation, and Clinical Implications.* Neurobiology of Disease, 2004. **16**(1): p. 1-13.
30. Fan, W., Yan, C. Y., Qian, S., Yao, N., Tang, L., and Wu, Y., *Design, Synthesis and in Vivo Evaluation of Sulfhydryl Beta-D-Glucose Cholesterols as Ligands for Brain Targeting Liposomes.* Letters in Drug Design & Discovery, 2010. **7**(4): p. 281-289.
31. Albert, A., *Chemical Aspects of Selective Toxicity.* Nature, 1958. **182**(4633): p. 421-423.
32. Stella, V. J., Borchardt, R. T., Hageman, M. J., Oliyai, R., Maag, H., and Tilley, J. W., *Prodrugs: Challenges and Rewards*. 2007: Springer Science & Business Media.
33. Zawilska, J. B., Wojcieszak, J., and Olejniczak, A. B., *Prodrugs: A Challenge for the Drug Development.* Pharmacological Reports, 2013. **65**(1): p. 1-14.
34. Rautio, J., Laine, K., Gynther, M., and Savolainen, J., *Prodrug Approaches for Cns Delivery.* Aaps Journal, 2008. **10**(1): p. 92-102.
35. DeGorter, M. K., Xia, C. Q., Yang, J. J., and Kim, R. B., *Drug Transporters in Drug Efficacy and Toxicity*, in *Annual Review of Pharmacology and Toxicology, Vol 52*, Insel, P. A., Amara, S. G., and Blaschke, T. F., Editors. 2012, Annual Reviews: Palo Alto. p. 249-273.
36. Giacomini, K. M., Huang, S. M., Tweedie, D. J., Benet, L. Z., Brouwer, K. L. R., Chu, X. Y., Dahlin, A., Evers, R., Fischer, V., Hillgren, K. M., Hoffmaster, K. A., Ishikawa, T., Keppler, D., Kim, R. B., Lee, C. A., Niemi, M., Polli, J. W., Sugiyama, Y., Swaan, P. W., Ware, J. A., Wright, S. H., Yee, S. W., Zamek-Gliszczynski, M. J., Zhang, L., and International, T., *Membrane Transporters in Drug Development.* Nature Reviews Drug Discovery, 2010. **9**(3): p. 215-236.
37. Dobson, P. D. and Kell, D. B., *Carrier-Mediated Cellular Uptake of Pharmaceutical Drugs: An Exception or the Rule?* Nature Reviews Drug Discovery, 2008. **7**(3): p. 205-220.
38. Rubio-Aliaga, I. and Daniel, H., *Mammalian Peptide Transporters as Targets for Drug Delivery.* Trends in Pharmacological Sciences, 2002. **23**(9): p. 434-440.
39. Brandsch, M., Knutter, I., and Bosse-Doenecke, E., *Pharmaceutical and Pharmacological Importance of Peptide Transporters.* Journal of Pharmacy and Pharmacology, 2008. **60**(5): p. 543-585.
40. Leibach, F. H. and Ganapathy, V., *Peptide Transporters in the Intestine and the Kidney.* Annual review of nutrition, 1996. **16**: p. 99-119.
41. Balimane, P. V., Tamai, I., Guo, A. L., Nakanishi, T., Kitada, H., Leibach, F. H., Tsuji, A., and Sinko, P. J., *Direct Evidence for Peptide Transporter (Pept1)-Mediated Uptake of a Nonpeptide Prodrug, Valacyclovir.* Biochemical and biophysical research communications, 1998. **250**(2): p. 246-251.

42. Sala-Rabanal, M., Loo, D. D. F., Hirayama, B. A., Turk, E., and Wright, E. M., *Molecular Interactions between Dipeptides, Drugs and the Human Intestinal H+-Oligopeptide Cotransporter Hpept1.* Journal of Physiology-London, 2006. **574**(1): p. 149-166.
43. Brandsch, M., *Drug Transport Via the Intestinal Peptide Transporter Pept1.* Current Opinion in Pharmacology, 2013. **13**(6): p. 881-887.
44. Meyers, B. A., *Cefaclor Revisited.* Clinical Therapeutics, 2000. **22**(2): p. 154-166.
45. Li, M., Anderson, G. D., Phillips, B. R., Kong, W., Shen, D. D., and Wang, J., *Interactions of Amoxicillin and Cefaclor with Human Renal Organic Anion and Peptide Transporters.* Drug Metabolism and Disposition, 2006. **34**(4): p. 547-555.
46. Hori, R., Tomita, Y., Katsura, T., Yasuhara, M., Inui, K., and Takano, M., *Transport of Bestatin in Rat Renal Brush-Border Membrane-Vesicles.* Biochemical Pharmacology, 1993. **45**(9): p. 1763-1768.
47. Saito, H., Terada, T., Okuda, M., Sasaki, S., and Inui, K., *Molecular Cloning and Tissue Distribution of Rat Peptide Transporter Pept2.* Biochimica Et Biophysica Acta-Biomembranes, 1996. **1280**(2): p. 173-177.
48. Han, H. K., de Vrueh, R. L. A., Rhie, J. K., Covitz, K. M. Y., Smith, P. L., Lee, C. P., Oh, D. M., Sadee, W., and Amidon, G. L., *5 '-Amino Acid Esters of Antiviral Nucleosides, Acyclovir, and Azt Are Absorbed by the Intestinal Pept1 Peptide Transporter.* Pharmaceutical Research, 1998. **15**(8): p. 1154-1159.
49. Brown, F., Banken, L., Saywell, K., and Arum, I., *Pharmacokinetics of Valganciclovir and Ganciclovir Following Multiple Oral Dosages of Valganciclovir in Hiv- and Cmv-Seropositive Volunteers.* Clinical Pharmacokinetics, 1999. **37**(2): p. 167-176.
50. Sugawara, M., Huang, W., Fei, Y. L., Leibach, F. H., Ganapathy, V., and Ganapathy, M. E., *Transport of Valganciclovir, a Ganciclovir Prodrug, Via Peptide Transporters Pept1 and Pept2.* Journal of Pharmaceutical Sciences, 2000. **89**(6): p. 781-789.
51. Kanai, Y., Segawa, H., Miyamoto, K., Uchino, H., Takeda, E., and Endou, H., *Expression Cloning and Characterization of a Transporter for Large Neutral Amino Acids Activated by the Heavy Chain of 4f2 Antigen (Cd98).* Journal of Biological Chemistry, 1998. **273**(37): p. 23629-23632.
52. Geier, E. G., Schlessinger, A., Fan, H., Gable, J. E., Irwin, J. J., Sali, A., and Giacomini, K. M., *Structure-Based Ligand Discovery for the Large-Neutral Amino Acid Transporter 1, Lat-1.* Proceedings of the National Academy of Sciences of the United States of America, 2013. **110**(14): p. 5480-5485.
53. Prasad, P. D., Wang, H. P., Huang, W., Kekuda, R., Rajan, D. P., Leibach, F. H., and Ganapathy, V., *Human Lat1, a Subunit of System L Amino Acid Transporter: Molecular Cloning and Transport Function.* Biochemical and biophysical research communications, 1999. **255**(2): p. 283-288.

54. del Amo, E. M., Urtti, A., and Yliperttula, M., *Pharmacokinetic Role of L-Type Amino Acid Transporters Lat1 and Lat2.* European Journal of Pharmaceutical Sciences, 2008. **35**(3): p. 161-174.
55. Boado, R. J., Li, J. Y., Nagaya, M., Zhang, C., and Pardridge, W. M., *Selective Expression of the Large Neutral Amino Acid Transporter at the Blood-Brain Barrier.* Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(21): p. 12079-12084.
56. Duelli, R., Enerson, B. E., Gerhart, D. Z., and Drewes, L. R., *Expression of Large Amino Acid Transporter Lat1 in Rat Brain Endothelium.* Journal of Cerebral Blood Flow and Metabolism, 2000. **20**(11): p. 1557-1562.
57. Uchino, H., Kanai, Y., Kim, D. K., Wempe, M. F., Chairoungdua, A., Morimoto, E., Anders, M. W., and Endou, H., *Transport of Amino Acid-Related Compounds Mediated by L-Type Amino Acid Transporter 1 (Lat1): Insights into the Mechanisms of Substrate Recognition.* Molecular pharmacology, 2002. **61**(4): p. 729-737.
58. Gynther, M., Laine, K., Ropponen, J., Leppanen, J., Mannila, A., Nevalainen, T., Savolainen, J., Jarvinen, T. T., and Rautio, J., *Large Neutral Amino Acid Transporter Enables Brain Drug Delivery Via Prodrugs.* Journal of Medicinal Chemistry, 2008. **51**(4): p. 932-936.
59. Gynther, M., Jalkanen, A., Lehtonen, M., Forsberg, M., Laine, K., Ropponen, J., Leppanen, J., Knuuti, J., and Rautio, J., *Brain Uptake of Ketoprofen-Lysine Prodrug in Rats.* International Journal of Pharmaceutics, 2010. **399**(1-2): p. 121-128.
60. Ohtsuki, S. and Terasaki, T., *Contribution of Carrier-Mediated Transport Systems to the Blood-Brain Barrier as a Supporting and Protecting Interface for the Brain; Importance for Cns Drug Discovery and Development.* Pharmaceutical Research, 2007. **24**(9): p. 1745-1758.
61. Wade, L. A. and Katzman, R., *Synthetic Amino Acids and the Nature of L‐Dopa Transport at the Blood‐Brain Barrier.* Journal of neurochemistry, 1975. **25**(6): p. 837-842.
62. Gomes, P. and Soares-da-Silva, P., *L-Dopa Transport Properties in an Immortalised Cell Line of Rat Capillary Cerebral Endothelial Cells, Rbe 4.* Brain Research, 1999. **829**(1-2): p. 143-150.
63. Cornford, E. M., Young, D., Paxton, J. W., Finlay, G. J., Wilson, W. R., and Pardridge, W. M., *Melphalan Penetration of the Blood-Brain-Barrier Via the Neutral Amino-Acid Transporter in Tumor-Bearing Brain.* Cancer Research, 1992. **52**(1): p. 138-143.
64. Vig, B. S., Huttunen, K. M., Laine, K., and Rautio, J., *Amino Acids as Promoieties in Prodrug Design and Development.* Advanced Drug Delivery Reviews, 2013. **65**(10): p. 1370-1385.
65. Guo, X. L., Meiyu, G., and Guanhua, D., *Glucose Transporter 1, Distribution in the Brain and in Neural Disorders: Its Relationship with Transport of Neuroactive Drugs through the Blood-Brain Barrier.* Biochemical Genetics, 2005. **43**(3-4): p. 175-187.

66. Farrell, C. L. and Pardridge, W. M., *Blood-Brain-Barrier Glucose Transporter Is Asymmetrically Distributed on Brain Capillary Endothelial Lumenal and Ablumenal Membranes - an Electron-Microscopic Immunogold Study.* Proceedings of the National Academy of Sciences of the United States of America, 1991. **88**(13): p. 5779-5783.
67. Pardridge, W. M. and Oldendorf, W. H., *Kinetics of Blood-Brain-Barrier Transport of Hexoses.* Biochimica et biophysica acta, 1975. **382**(3): p. 377-392.
68. Fan, W., Wu, Y., Li, X. K., Yao, N., Li, X., Yu, Y. G., and Hai, L., *Design, Synthesis and Biological Evaluation of Brain-Specific Glucosyl Thiamine Disulfide Prodrugs of Naproxen.* European Journal of Medicinal Chemistry, 2011. **46**(9): p. 3651-3661.
69. Gynther, M., Ropponen, J., Laine, K., Leppanen, J., Haapakoski, P., Peura, L., Jarvinen, T., and Rautio, J., *Glucose Promoiety Enables Glucose Transporter Mediated Brain Uptake of Ketoprofen and Indomethacin Prodrugs in Rats.* Journal of Medicinal Chemistry, 2009. **52**(10): p. 3348-3353.
70. Fernandez, C., Nieto, O., Fontenla, J. A., Rivas, E., de Ceballos, M. L., and Fernandez-Mayoralas, A., *Synthesis of Glycosyl Derivatives as Dopamine Prodrugs: Interaction with Glucose Carrier Glut-1.* Organic & Biomolecular Chemistry, 2003. **1**(5): p. 767-771.
71. Sicbaldi, F., Sacchi, G. A., Trevisan, M., and DelRe, A. A. M., *Root Uptake and Xylem Translocation of Pesticides from Different Chemical Classes.* Pesticide Science, 1997. **50**(2): p. 111-119.
72. Peterson, C. A., de Wildt, P. P. Q., and Edgington, L. V., *A Rationale for the Ambimobile Translocation of the Nematicide Oxamyl in Plants.* Pesticide Biochemistry and Physiology, 1978. **8**(1): p. 1-9.
73. Jachetta, J. J., Appleby, A. P., and Boersma, L., *Apoplastic and Symplastic Pathways of Atrazine and Glyphosate Transport in Shoots of Seedling Sunflower.* Plant physiology, 1986. **82**(4): p. 1000-1007.
74. Taiz, L. and Zeiger, E., *Plant Physiology,Third Edition*. 2002: Sinauer Associates.
75. Donovan, S. F., *Physical Property Requirements of Agrochemicals*, in *Synthesis and Chemistry of Agrochemicals Vii*, Lyga, J. W. and Theodoridis, G., Editors. 2007, American Chemical Society. p. 7-22.
76. Lucas, W. J., Groover, A., Lichtenberger, R., Furuta, K., Yadav, S. R., Helariutta, Y., He, X. Q., Fukuda, H., Kang, J., Brady, S. M., Patrick, J. W., Sperry, J., Yoshida, A., Lopez-Millan, A. F., Grusak, M. A., and Kachroo, P., *The Plant Vascular System: Evolution, Development and Functions.* Journal of Integrative Plant Biology, 2013. **55**(4): p. 294-388.
77. Buchholz, A. and Nauen, R., *Translocation and Translaminar Bioavailability of Two Neonicotinoid Insecticides after Foliar Application to Cabbage and Cotton.* Pest Management Science, 2002. **58**(1): p. 10-16.

78. Bromilow, R. H. and Chamberlain, K., *Pathways and Mechanisms of Transport of Herbicides in Plants*, in *Target Sites for Herbicide Action*, Kirkwood, R. C., Editor. 1991, Springer US: Boston, MA. p. 245-284.
79. Bruck, E., Elbert, A., Fischer, R., Krueger, S., Kuhnhold, J., Klueken, A. M., Nauen, R., Niebes, J. F., Reckmann, U., Schnorbach, H. J., Steffens, R., and van Waetermeulen, X., *Movento (R), an Innovative Ambimobile Insecticide for Sucking Insect Pest Control in Agriculture: Biological Profile and Field Performance.* Crop Protection, 2009. **28**(10): p. 838-844.
80. Bromilow, R. H. and Chamberlain, K., *The Herbicide Glyphosate and Related Molecules: Physicochemical and Structural Factors Determining Their Mobility in Phloem.* Pest Management Science, 2000. **56**(4): p. 368-373.
81. Trapp, S., *Plant Uptake and Transport Models for Neutral and Ionic Chemicals.* Environmental Science and Pollution Research, 2004. **11**(1): p. 33-39.
82. Satchivi, N. M., Stoller, E. W., Wax, L. M., and Briskin, D. P., *A Nonlinear, Dynamic, Simulation Model for Transport, and Whole Plant Allocation of Systemic Xenobiotics Following Foliar Application. Iv: Physicochemical Properties Requirements for Optimum Absorption and Translocation.* Pesticide Biochemistry and Physiology, 2006. **84**(2): p. 83-97.
83. Shone, M. G. T. and Wood, A. V., *Comparison of Uptake and Translocation of Some Organic Herbicides and a Systemic Fungicide by Barley .1. Absorption in Relation to Physicochemical Properties.* Journal of Experimental Botany, 1974. **25**(85): p. 390-400.
84. Briggs, G. G., Bromilow, R. H., and Evans, A. A., *Relationships between Lipophilicity and Root Uptake and Translocation of Non-Ionized Chemicals by Barley.* Pesticide Science, 1982. **13**(5): p. 495-504.
85. Briggs, G. G., Rigitano, R. L. O., and Bromilow, R. H., *Physicochemical Factors Affecting Uptake by Roots and Translocation to Shoots of Weak Acids in Barley.* Pesticide Science, 1987. **19**(2): p. 101-112.
86. Cobb, A. H. and Reade, J. P. H., *Herbicide Uptake and Movement*, in *Herbicides and Plant Physiology*. 2010, Wiley-Blackwell. p. 50-69.
87. Kirkwood, R. C., *Recent Developments in Our Understanding of the Plant Cuticle as a Barrier to the Foliar Uptake of Pesticides.* Pesticide Science, 1999. **55**(1): p. 69-77.
88. Kerler, F. and Schonherr, J., *Accumulation of Lipophilic Chemicals in Plant Cuticles - Prediction from Octanol Water Partition-Coefficients.* Archives of Environmental Contamination and Toxicology, 1988. **17**(1): p. 1-6.
89. Klittich, C. J. R. and Ray, S. L., *Effects of Physical Properties on the Translaminar Activity of Fungicides.* Pesticide Biochemistry and Physiology, 2013. **107**(3): p. 351-359.

90. Kleier, D. A., *Phloem Mobility of Xenobiotics .1. Mathematical-Model Unifying the Weak Acid and Intermediate Permeability Theories.* Plant physiology, 1988. **86**(3): p. 803-810.
91. Grayson, B. T. and Kleier, D. A., *Phloem Mobility of Xenobiotics .4. Modeling of Pesticide Movement in Plants.* Pesticide Science, 1990. **30**(1): p. 67-79.
92. Kleier, D. A. and Francis, C. H., *Phloem Mobility of Xenobiotics. Vii. The Design of Phloem Systemic Pesticides.* Weed Science, 1996. **44**(3): p. 749-756.
93. Hsu, F. C., Sun, K. M., Kleier, D. A., and Fielding, M. J., *Phloem Mobility of Xenobiotics .6. A Phloem-Mobile Pro-Nematicide Based on Oxamyl Exhibiting Root-Specific Activation in Transgenic Tobacco.* Pesticide Science, 1995. **44**(1): p. 9-19.
94. Satchivi, N. M., Stoller, E. W., Wax, L. M., and Briskin, D. P., *A Nonlinear Dynamic Simulation Model for Xenobiotic Transport and Whole Plant Allocation Following Foliar Application - I. Conceptual Foundation for Model Development.* Pesticide Biochemistry and Physiology, 2000. **68**(2): p. 67-84.
95. Satchivi, N. M., Stoller, E. W., Wax, L. M., and Briskin, D. P., *A Nonlinear Dynamic Simulation Model for Xenobiotic Transport and Whole Plant Allocation Following Foliar Application - Ii. Model Validation.* Pesticide Biochemistry and Physiology, 2000. **68**(2): p. 85-95.
96. Satchivi, N. M., Stoller, E. W., Wax, L. M., and Briskin, D. P., *A Nonlinear Dynamic Simulation Model for Xenobiotic Transport and Whole Plant Allocation Following Foliar Application. Iii. Influence of Chemical Properties, Plant Characteristics, and Environmental Parameters on Xenobiotic Absorption and Translocation.* Pesticide Biochemistry and Physiology, 2001. **71**(2): p. 77-87.
97. Edgington, L. V., *Structural Requirements of Systemic Fungicides.* Annual Review of Phytopathology, 1981. **19**: p. 107-124.
98. Delrot, S. and Bonnemain, J. L., *Le Transport à Longue Distance Des Herbicides Dans La Plante.* Les herbicides mode d'action et principes d'utilisation, 1991: p. 51-77.
99. Owen, W. J., *Herbicide Metabolism as a Basis for Selectivity*, in *Target Sites for Herbicide Action*, Kirkwood, R. C., Editor. 1991, Springer US: Boston, MA. p. 285-314.
100. Chollet, J. F., Rocher, F., Jousse, C., Deletage-Grandon, C., Bashiardes, G., and Bonnemain, J. L., *Synthesis and Phloem Mobility of Acidic Derivatives of the Fungicide Fenpiclonil.* Pest Management Science, 2004. **60**(11): p. 1063-1072.
101. Chollet, J. F., Rocher, F., Jousse, C., Deletage-Grandon, C., Bashiardes, G., and Bonnemain, J. L., *Acidic Derivatives of the Fungicide Fenpiclonil: Effect of Adding a Methyl Group to the N-Substituted Chain on Systemicity and Fungicidal Activity.* Pest Management Science, 2005. **61**(4): p. 377-382.
102. Assmann, S. M. and Haubrick, L. L., *Transport Proteins of the Plant Plasma Membrane.* Current Opinion in Cell Biology, 1996. **8**(4): p. 458-467.

103. Chrispeels, M. J. and Agre, P., *Aquaporins - Water Channel Proteins of Plant and Animal-Cells.* Trends in Biochemical Sciences, 1994. **19**(10): p. 421-425.
104. Williams, L. E., Lemoine, R., and Sauer, N., *Sugar Transporters in Higher Plants - a Diversity of Roles and Complex Regulation.* Trends in Plant Science, 2000. **5**(7): p. 283-290.
105. Kuhn, C. and Grof, C. P. L., *Sucrose Transporters of Higher Plants.* Current Opinion in Plant Biology, 2010. **13**(3): p. 287-298.
106. Doidy, J., Grace, E., Kuhn, C., Simon-Plas, F., Casieri, L., and Wipf, D., *Sugar Transporters in Plants and in Their Interactions with Fungi.* Trends in Plant Science, 2012. **17**(7): p. 413-422.
107. Lemoine, R., La Camera, S., Atanassova, R., Dédaldéchamp, F., Allario, T., Pourtau, N., Bonnemain, J. L., Laloi, M., Coutos-Thévenot, P., Maurousset, L., Faucher, M., Girousse, C., Lemonnier, P., Parrilla, J., and Durand, M., *Source-to-Sink Transport of Sugar and Regulation by Environmental Factors.* Frontiers in Plant Science, 2013. **4**: p. 21.
108. Ortiz-Lopez, A., Chang, H. C., and Bush, D. R., *Amino Acid Transporters in Plants.* Biochimica Et Biophysica Acta-Biomembranes, 2000. **1465**(1-2): p. 275-280.
109. Deletage-Grandon, C., Chollet, J. F., Faucher, M., Rocher, F., Komor, E., and Bonnemain, J. L., *Carrier-Mediated Uptake and Phloem Systemy of a 350-Dalton Chlorinated Xenobiotic with an Alpha-Amino Acid Function.* Plant physiology, 2001. **125**(4): p. 1620-1632.
110. Claus, J. S. and Behrens, R., *Glyphosate Translocation and Quackgrass Rhizome Bud Kill.* Weed Science, 1976. **24**(2): p. 149-152.
111. Gougler, J. A. and Geiger, D. R., *Uptake and Distribution of N-Phosphonomethylglycine in Sugar-Beet Plants.* Plant physiology, 1981. **68**(3): p. 668-672.
112. Denis, M. H. and Delrot, S., *Carrier-Mediated Uptake of Glyphosate in Broad Bean (Vicia-Faba) Via a Phosphate Transporter.* Physiologia plantarum, 1993. **87**(4): p. 569-575.
113. Hetherington, P. R., Marshall, G., Kirkwood, R. C., and Warner, J. M., *Absorption and Efflux of Glyphosate by Cell Suspensions.* Journal of Experimental Botany, 1998. **49**(320): p. 527-533.
114. Shaner, D. L., *Role of Translocation as a Mechanism of Resistance to Glyphosate.* Weed Science, 2009. **57**(1): p. 118-123.
115. Xu, J. Q., Li, G., Wang, Z. Y., Si, L. Q., He, S. J., Cai, J. L., Huang, J. G., and Donovan, M. D., *The Role of L-Type Amino Acid Transporters in the Uptake of Glyphosate across Mammalian Epithelial Tissues.* Chemosphere, 2016. **145**: p. 487-494.
116. Hart, J. J., Ditomaso, J. M., Linscott, D. L., and Kochian, L. V., *Transport Interactions between Paraquat and Polyamines in Roots of Intact Maize Seedlings.* Plant physiology, 1992. **99**(4): p. 1400-1405.

117. Hart, J. J., Ditomaso, J. M., and Kochian, L. V., *Characterization of Paraquat Transport in Protoplasts from Maize (Zea-Mays L) Suspension Cells.* Plant physiology, 1993. **103**(3): p. 963-969.

118. Xi, J., Xu, P., and Xiang, C. B., *Loss of Atpdr11, a Plasma Membrane-Localized Abc Transporter, Confers Paraquat Tolerance in Arabidopsis Thaliana.* Plant Journal, 2012. **69**(5): p. 782-791.

119. Kasai, F. and Bayer, D. E., *Effects of 2,4-Dichlorophenoxyacetic Acid, Antiauxins, and Metabolic Perturbations on Cytoplasmic and Vacuolar Ph of Corn Root-Tips Measured by in-Vivo P-31-Nmr.* Pesticide Biochemistry and Physiology, 1995. **51**(3): p. 161-169.

120. Chen, L. S., Ortiz-Lopez, A., Jung, A., and Bush, D. R., *Ant1, an Aromatic and Neutral Amino Acid Transporter in Arabidopsis.* Plant physiology, 2001. **125**(4): p. 1813-1820.

121. Dufaud, A., Chollet, J. F., Rudelle, J., Miginiac, L., and Bonnemain, J. L., *Derivatives of Pesticides with an Alpha-Amino-Acid Function - Synthesis and Effect on Threonine Uptake.* Pesticide Science, 1994. **41**(4): p. 297-304.

122. Chollet, J. F., Deletage, C., Faucher, M., Miginiac, L., and Bonnemain, J. L., *Synthesis and Structure-Activity Relationships of Some Pesticides with an Alpha-Amino Acid Function.* Biochimica Et Biophysica Acta-General Subjects, 1997. **1336**(2): p. 331-341.

123. Jiang, D. X., Lu, X. L., Hu, S., Zhang, X. B., and Xu, H. H., *A New Derivative of Fipronil: Effect of Adding a Glycinyl Group to the 5-Amine of Pyrazole on Phloem Mobility and Insecticidal Activity.* Pesticide Biochemistry and Physiology, 2009. **95**(3): p. 126-130.

124. Yang, W., Wu, H. X., Xu, H. H., Hu, A. L., and Lu, M. L., *Synthesis of Glucose-Fipronil Conjugate and Its Phloem Mobility.* Journal of Agricultural and Food Chemistry, 2011. **59**(23): p. 12534-12542.

125. Wu, H. X., Yang, W., Zhang, Z. X., Huang, T., Yao, G. K., and Xu, H. H., *Uptake and Phloem Transport of Glucose-Fipronil Conjugate in Ricinus Communis Involve a Carrier-Mediated Mechanism.* Journal of Agricultural and Food Chemistry, 2012. **60**(24): p. 6088-6094.

126. Wang, J., Lei, Z. W., Wen, Y. J., Mao, G. L., Wu, H. X., and Xu, H. H., *A Novel Fluorescent Conjugate Applicable to Visualize the Translocation of Glucose-Fipronil.* Journal of Agricultural and Food Chemistry, 2014. **62**(35): p. 8791-8798.

127. Yuan, J. G., Wu, H. X., Lu, M. L., Song, G. P., and Xu, H. H., *Synthesis of a Series of Monosaccharide-Fipronil Conjugates and Their Phloem Mobility.* Journal of Agricultural and Food Chemistry, 2013. **61**(18): p. 4236-4241.

128. Bromilow, R. H., Chamberlain, K., and Evans, A. A., *Physicochemical Aspects of Phloem Translocation of Herbicides.* Weed Science, 1990. **38**(3): p. 305-314.

129. Hsu, F. C. and Kleier, D. A., *Phloem Mobility of Xenobiotics Viii. A Short Review.* Journal of Experimental Botany, 1996. **47**: p. 1265-1271.

130. Veber, D. F., Johnson, S. R., Cheng, H. Y., Smith, B. R., Ward, K. W., and Kopple, K. D., *Molecular Properties That Influence the Oral Bioavailability of Drug Candidates.* Journal of Medicinal Chemistry, 2002. **45**(12): p. 2615-2623.
131. Lipinski, C. A., Lombardo, F., Dominy, B. W., and Feeney, P. J., *Experimental and Computational Approaches to Estimate Solubility and Permeability in Drug Discovery and Development Settings.* Advanced Drug Delivery Reviews, 2012. **64**: p. 4-17.
132. Tice, C. M., *Selecting the Right Compounds for Screening: Does Lipinski's Rule of 5 for Pharmaceuticals Apply to Agrochemicals?* Pest Management Science, 2001. **57**(1): p. 3-16.
133. Tice, C. M., *Selecting the Rights Compounds for Screening: Use of Surface-Area Parameters.* Pest Management Science, 2002. **58**(3): p. 219-233.
134. Avram, S., Funar-Timofei, S., Borota, A., Chennamaneni, S. R., Manchala, A. K., and Muresan, S., *Quantitative Estimation of Pesticide-Likeness for Agrochemical Discovery.* Journal of Cheminformatics, 2014. **6**: p. 11.
135. Hao, G. F., Dong, Q. J., and Yang, G. F., *A Comparative Study on the Constitutive Properties of Marketed Pesticides.* Molecular Informatics, 2011. **30**(6-7): p. 614-622.
136. Rocher, F., Chollet, J. F., Legros, S., Jousse, C., Lemoine, R., Faucher, M., Bush, D. R., and Bonnemain, J. L., *Salicylic Acid Transport in Ricinus Communis Involves a Ph-Dependent Carrier System in Addition to Diffusion.* Plant physiology, 2009. **150**(4): p. 2081-2091.
137. Rocher, F., Chollet, J. F., Jousse, C., and Bonnemain, J. L., *Salicylic Acid, an Ambimobile Molecule Exhibiting a High Ability to Accumulate in the Phloem.* Plant physiology, 2006. **141**(4): p. 1684-1693.
138. Letondor, C., Pascal-Lorber, S., and Laurent, F., *Uptake and Distribution of Chlordecone in Radish: Different Contamination Routes in Edible Roots.* Chemosphere, 2015. **118**: p. 20-28.
139. Pascal-Lorber, S., Letondor, C., Liber, Y., Jamin, E. L., and Laurent, F., *Chlordecone Transfer and Distribution in Maize Shoots.* Journal of Agricultural and Food Chemistry, 2016. **64**(2): p. 409-415.
140. Aajoud, A., Raveton, M., Azrou-Isghi, D., Tissut, M., and Ravanel, P., *How Can the Fipronil Insecticide Access Phloem?* Journal of Agricultural and Food Chemistry, 2008. **56**(10): p. 3732-3737.
141. Feng, P. C. C., Tran, M., Chiu, T., Sammons, R. D., Heck, G. R., and CaJacob, C. A., *Investigations into Glyphosate-Resistant Horseweed (Conyza Canadensis): Retention, Uptake, Translocation, and Metabolism.* Weed Science, 2004. **52**(4): p. 498-505.
142. Liu, Z. Q. and Gaskin, R. E., *Visualisation of the Uptake of Two Model Xenobiotics into Bean Leaves by Confocal Laser Scanning Microscopy: Diffusion Pathways and Implication in Phloem Translocation.* Pest Management Science, 2004. **60**(5): p. 434-439.

143. Wild, E., Dent, J., Barber, J. L., Thomas, G. O., and Jones, K. C., *A Novel Analytical Approach for Visualizing and Tracking Organic Chemicals in Plants.* Environmental Science & Technology, 2004. **38**(15): p. 4195-4199.
144. Shani, E., Weinstain, R., Zhang, Y., Castillejo, C., Kaiserli, E., Chory, J., Tsien, R. Y., and Estelle, M., *Gibberellins Accumulate in the Elongating Endodermal Cells of Arabidopsis Root.* Proceedings of the National Academy of Sciences of the United States of America, 2013. **110**(12): p. 4834-4839.
145. Juraske, R., Castells, F., Vijay, A., Munoz, P., and Anton, A., *Uptake and Persistence of Pesticides in Plants: Measurements and Model Estimates for Imidacloprid after Foliar and Soil Application.* Journal of Hazardous Materials, 2009. **165**(1-3): p. 683-689.
146. Tanner, G. and Czerwenka, C., *Lc-Ms/Ms Analysis of Neonicotinoid Insecticides in Honey: Methodology and Residue Findings in Austrian Honeys.* Journal of Agricultural and Food Chemistry, 2011. **59**(23): p. 12271-12277.
147. Hsu, F. C., Marxmiller, R. L., and Yang, A. Y. S., *Study of Root Uptake and Xylem Translocation of Cinmethylin and Related-Compounds in Detopped Soybean Roots Using a Pressure Chamber Technique.* Plant physiology, 1990. **93**(4): p. 1573-1578.
148. Guglielmi, M. A., Rocher, F., Legros, S., Bonnemain, J. L., and Chollet, J. F., *A Non-Destructive Method for Testing Two Components of the Behaviour of Soil-Applied Agricultural Chemicals over a Long Period.* Pest Management Science, 2012. **68**(6): p. 897-905.
149. Dinant, S. and Kehr, J., *Sampling and Analysis of Phloem Sap*, in *Plant Mineral Nutrients: Methods and Protocols*, Maathuis, F. J. M., Editor. 2013, Humana Press: Totowa, NJ. p. 185-194.
150. Fisher, D. B. and Frame, J. M., *A Guide to the Use of the Exuding-Stylet Technique in Phloem Physiology.* Planta, 1984. **161**(5): p. 385-393.
151. Hayashi, H. and Chino, M., *Collection of Pure Phloem Sap from Wheat and Its Chemical-Composition.* Plant and Cell Physiology, 1986. **27**(7): p. 1387-1393.
152. Gaupels, F., Buhtz, A., Knauer, T., Deshmukh, S., Waller, F., van Bel, A. J. E., Kogel, K. H., and Kehr, J., *Adaptation of Aphid Stylectomy for Analyses of Proteins and Mrnas in Barley Phloem Sap.* Journal of Experimental Botany, 2008. **59**(12): p. 3297-3306.
153. Walz, C., Giavalisco, P., Schad, M., Juenger, M., Klose, J., and Kehr, J., *Proteomics of Curcurbit Phloem Exudate Reveals a Network of Defence Proteins.* Phytochemistry, 2004. **65**(12): p. 1795-1804.
154. Hu, L. P., Meng, F. Z., Wang, S. H., Sui, X. L., Li, W., Wei, Y. X., Sun, J. L., and Zhang, Z. X., *Changes in Carbohydrate Levels and Their Metabolic Enzymes in Leaves, Phloem Sap and Mesocarp During Cucumber (Cucumis Sativus L.) Fruit Development.* Scientia Horticulturae, 2009. **121**(2): p. 131-137.

155. Kriedema.P and Beevers, H., *Sugar Uptake and Translocation in Castor Bean Seedling .I. Characteristics of Transfer in Intact and Excised Seedlings.* Plant physiology, 1967. **42**(2): p. 161-&.

156. Bromilow, R. H., Rigitano, R. L. O., Briggs, G. G., and Chamberlain, K., *Phloem Translocation of Non-Ionized Chemicals in Ricinus-Communis.* Pesticide Science, 1987. **19**(2): p. 85-99.

157. Kallarackal, J., Orlich, G., Schobert, C., and Komor, E., *Sucrose Transport into the Phloem of Ricinus-Communis L Seedlings as Measured by the Analysis of Sieve-Tube Sap.* Planta, 1989. **177**(3): p. 327-335.

158. Bromilow, R. H., Chamberlain, K., and Patil, S. G., *A Rapid Method Using Ricinus-Communis for the Estimation of Phloem Translocation of Xenobiotics.* Pesticide Science, 1990. **30**(1): p. 1-12.

159. Ye, W. L., Wood, B. A., Stroud, J. L., Andralojc, P. J., Raab, A., McGrath, S. P., Feldmann, J., and Zhao, F. J., *Arsenic Speciation in Phloem and Xylem Exudates of Castor Bean.* Plant physiology, 2010. **154**(3): p. 1505-1513.

160. Komor, E., Orlich, G., and Bauer-Ruckdeschel, H., *Relationship between Apoplastic Nutrient Concentrations and the Long-Distance Transport of Nutrients in the Ricinus Communis L. Seedling*, in *The Apoplast of Higher Plants: Compartment of Storage, Transport and Reactions: The Significance of the Apoplast for the Mineral Nutrition of Higher Plants*, Sattelmacher, B. and Horst, W. J., Editors. 2007, Springer Netherlands: Dordrecht. p. 231-249.

161. Russell, P. E., *A Century of Fungicide Evolution.* Journal of Agricultural Science, 2005. **143**: p. 11-25.

162. Morton, V. and Staub, T., *A Short History of Fungicides*, in *APSnet Features*. 2008.

163. Kuck, K.-H. and Gisi, U., *Frac Mode of Action Classification and Resistance Risk of Fungicides*, in *Modern Crop Protection Compounds*. 2007, Wiley-VCH Verlag GmbH. p. 415-432.

164. Hirooka, T. and Ishii, H., *Chemical Control of Plant Diseases.* Journal of General Plant Pathology, 2013. **79**(6): p. 390-401.

165. Leadbeater, A. and Gisi, U., *The Challenges of Chemical Control of Plant Diseases*, in *Recent Developments in Management of Plant Diseases*, Gisi, U., Chet, I., and Gullino, M. L., Editors. 2009, Springer Netherlands: Dordrecht. p. 3-17.

166. Solel, Z. and Pinkas, J., *Bioassay Evaluation of the Systemic Properties of Carboxin in Lemon Seedlings.* Pesticide Science, 1972. **3**(2): p. 149-151.

167. Erwin, D. C., *Systemic Fungicides - Disease Control, Translocation, and Mode of Action.* Annual Review of Phytopathology, 1973. **11**: p. 389-422.

168. Solel, Z., *Performance of Benzimidazole Fungicides in Control of Cercospora Leaf Spot of Sugarbeet.* Journal of the American Society of Sugar Beet Technologists, 1970. **16**(2): p. 93-&.
169. Holderness, M., *Control of Vascular-Streak Dieback of Cocoa with Triazole Fungicides and the Problem of Phytotoxicity.* Plant Pathology, 1990. **39**(2): p. 286-293.
170. Witchard, M., *Paclobutrazol Is Phloem Mobile in Castor Oil Plant (Ricinus Communis L.).* Journal of Plant Growth Regulation, 1997. **16**(4): p. 215-217.
171. Lehoczki-Krsjak, S., Varga, M., Szabo-Hever, A., and Mesterhazy, A., *Translocation and Degradation of Tebuconazole and Prothioconazole in Wheat Following Fungicide Treatment at Flowering.* Pest Management Science, 2013. **69**(11): p. 1216-1224.
172. W. Bartett, D., M. Clough, J., R. A. Godfrey, C., R. Godwin, J., A. Hall, A., P. Heaney, S., and J. Maund, S., *Understanding the Strobilurin Fungicides.* Pesticide Outlook, 2001. **12**(4): p. 143-148.
173. Yadeta, K. and Thomma, B., *The Xylem as Battleground for Plant Hosts and Vascular Wilt Pathogens.* Frontiers in Plant Science, 2013. **4**: p. 12.
174. Agrios, G. N., *Plant Pathology (Fifth Edition)*. 2005, San Diego: Elsevier Academic Press.
175. Bertsch, C., Ramirez-Suero, M., Magnin-Robert, M., Larignon, P., Chong, J., Abou-Mansour, E., Spagnolo, A., Clement, C., and Fontaine, F., *Grapevine Trunk Diseases: Complex and Still Poorly Understood.* Plant Pathology, 2013. **62**(2): p. 243-265.
176. Andolfi, A., Mugnai, L., Luque, J., Surico, G., Cimmino, A., and Evidente, A., *Phytotoxins Produced by Fungi Associated with Grapevine Trunk Diseases.* Toxins, 2011. **3**(12): p. 1569-1605.
177. Fontaine, F., Pinto, C., Vallet, J., Clement, C., Gomes, A. C., and Spagnolo, A., *The Effects of Grapevine Trunk Diseases (Gtds) on Vine Physiology.* European Journal of Plant Pathology, 2016. **144**(4): p. 707-721.
178. Pouzoulet, J., Pivovaroff, A. L., Santiago, L. S., and Rolshausen, P. E., *Can Vessel Dimension Explain Tolerance toward Fungal Vascular Wilt Diseases in Woody Plants? Lessons from Dutch Elm Disease and Esca Disease in Grapevine.* Frontiers in Plant Science, 2014. **5**: p. 1-11.
179. Di Marco, S., Mazzullo, A., Calzarano, F., and Cesari, A., *The Control of Esca: Status and Perspectives.* Phytopathologia Mediterranea, 2000. **39**(1): p. 232-240.
180. Bester, W., Crous, P. W., and Fourie, P. H., *Evaluation of Fungicides as Potential Grapevine Pruning Wound Protectants against Botryosphaeria Species.* Australasian Plant Pathology, 2007. **36**(1): p. 73-77.
181. Pitt, W. M., Sosnowski, M. R., Huang, R. J., Qiu, Y., Steel, C. C., and Savocchia, S., *Evaluation of Fungicides for the Management of Botryosphaeria Canker of Grapevines.* Plant Disease, 2012. **96**(9): p. 1303-1308.

182. Amponsah, N. T., Jones, E., Ridgway, H. J., and Jaspers, M. V., *Evaluation of Fungicides for the Management of Botryosphaeria Dieback Diseases of Grapevines.* Pest Management Science, 2012. **68**(5): p. 676-683.
183. Sosnowski, M. R., Loschiavo, A. P., Wicks, T. J., and Scott, E. S., *Evaluating Treatments and Spray Application for the Protection of Grapevine Pruning Wounds from Infection by Eutypa Lata.* Plant Disease, 2013. **97**(12): p. 1599-1604.
184. Tjamos, E. C., *Problems and Prospects in Controlling Verticillium Wilt*, in *Vascular Wilt Diseases of Plants: Basic Studies and Control*, Tjamos, E. C. and Beckman, C. H., Editors. 1989, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 441-456.
185. Diener, A., *Visualizing and Quantifying Fusarium Oxysporum in the Plant Host.* Molecular Plant-Microbe Interactions, 2012. **25**(12): p. 1531-1541.

Chapter 2. Materials and methods

2.1 Chemicals

2.1.1 Profungicides

Fenpiclonil, which is a phenylpyrrole fungicide from chemical modifications of a bacterial metabolite pyrrolnitrin, was selected as a model compound in this study. We modified its chemical structure by adding glucose or amino acid moiety (Fig 2-1). All fenpiclonil conjugates were synthesized in our laboratory (Synthesis was shown in following chapters).

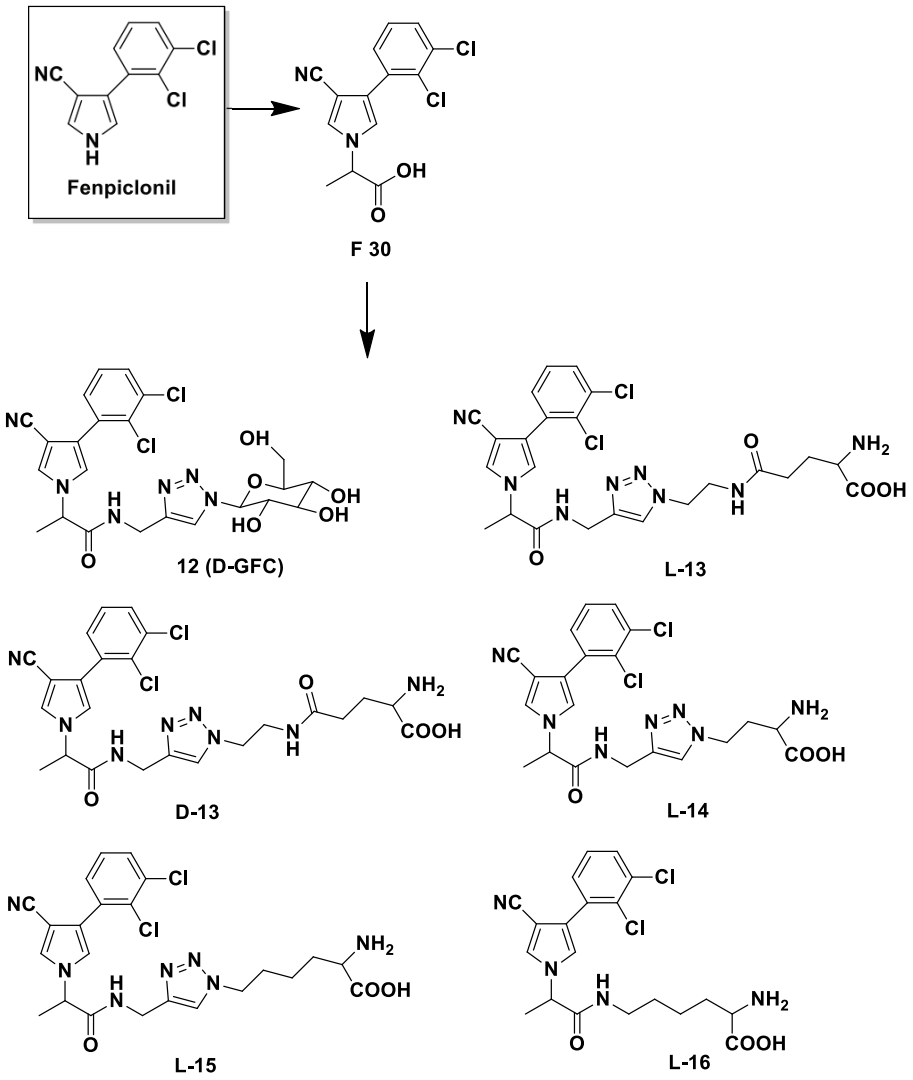

Figure 2-1. Chemical structure of fenpiclonil and phenylpyrrole profungicide

2.1.2 Physicochemical properties

Physicochemical properties and descriptors were predicted using the ACD/Labs Percepta 2015 release (Build 2726) software from Advanced Chemistry Development, Inc. (Toronto, Canada). 6 physicochemical properties were calculated: molecular weight (MW), number of hydrogen bond donors (HBD), number of hydrogen bond acceptors (HBA), Log D, free rotatable bonds (FRB) and polar surface area (PSA). The calculated properties were chosen according to their influence on passive membrane transport in plants[1].

2.2 Systemicity test in *Ricinus* model

2.2.1 Plant material and growth conditions

Castor bean seeds (*Ricinus communis* L. cv *Sanguineus*) were purchased from Graines Girerd et Fils (Le Thor, France). The seeds were placed in wet cotton wool for 24 h at 27°C ± 1°C and then sown in vermiculite. Seedlings were grown in a humid atmosphere (80% ± 5%) at 27°C ± 1°C with a 14/10 h light/dark cycle. After 6 days of growth in vermiculite, seedlings of average size were selected for the experiments (Fig 2-2A).

2.2.2 Incubation conditions for phloem loading via the cotyledons

The experiments were carried out in the same environment as described above. The endosperm of seedlings were carefully removed without bending or squeezing the cotyledons or the hypocotyl[2] (Fig 2-2B). Then the cotyledons were incubated in buffer solution containing 0.25 mM $MgCl_2$ and 0.5 mM $CaCl_2$. The buffers used were 20 mM MES (pH 5.0, 5.5, 6.0 and 6.5) or 20 mM HEPES (pH 7.0 and 8.0). After 30 min pre-incubation in a standard buffer solution, the cotyledons were incubated in the same buffer solution supplemented with tested compound or other compounds of interest at given concentration (Fig 2-2C).

To investigate the effect of glucose conjugate **12** on transporter systems, the cot-

yledons were incubated in buffer solution containing 0.5 mM labeled product: [^{3}H]-3-*O*-Methyl glucose (1.5 µCi/10 ml), [^{14}C]-sucrose (0.2 µCi/10 ml) or [^{3}H]-glutamine (1.5 µCi/10 ml) with or without 0.5 mM glucose conjugate **12**.

2.2.3 Phloem-sap collection and cotyledon uptake

After the cotyledons were incubated another 30 min in buffer solution containing tested compound, the hypocotyl was cut in the hook region about 1 cm from the cotyledons with a razor blade[2-4]. The exuding phloem sap was collected from the cut end of the upright hypocotyl with a micropipette (Fig 2-2, D and E). The hypocotyl can be re-cut to increase the flow again. To reduce evaporation, the sap collection was performed in a Plexiglas microcabinet laid out with wet filter paper. The collected sap was stored at −20 °C until analysis.

For the determination of radioisotope uptake, the cotyledons were rinsed (3×2min) in the standard buffer solution, wiped off with paper and weighted. Then, two cotyledons were digested overnight at 60 °C in a mixture of perchloric acid (65 %; 0.56 mL), hydrogen peroxide (33 %; 0. 27 mL), and Triton X-100 (1 g/L; 0.17 mL). Radioactivity measurements were conducted on each plant separately.

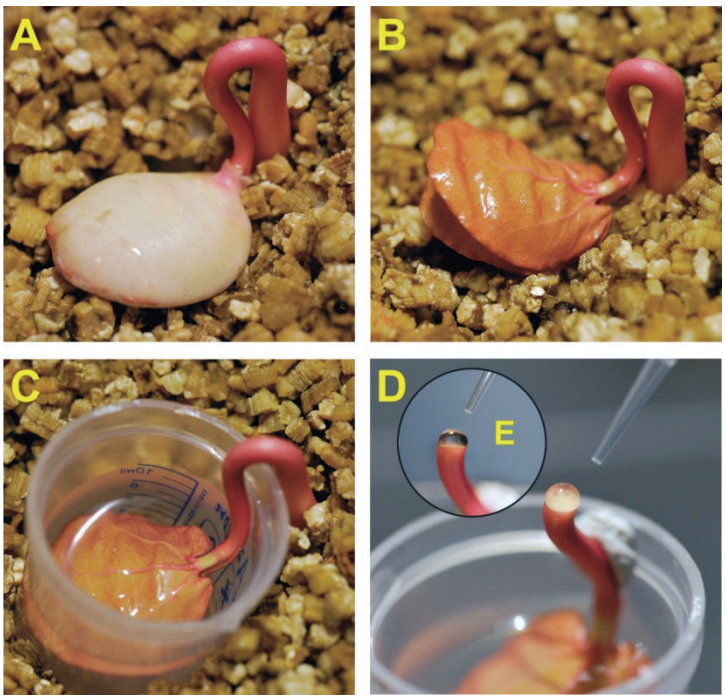

Figure 2-2. The *Ricinus* model. (A) The 6 days *Ricinus* seedlings on vermiculite. (B) The endo-

sperm was carefully removed. (C) Cotyledons that still attached to the hypocotyls were immersed in a buffered solution containing the tested product. (D)(E) After cutting the hypocotyl, phloem sap accumulated on the cross section and then was collected with a micropipette.

2.2.4 Analytical methods

All fenpiclonil derivatives in the phloem sap were analyzed by HPLC after dilution with UHQ grade water (phloem sap/ pure water, 1/9, v/v). The HPLC system consisted of an autosampler, a quaternary pump and a UV6000LP photodiode array detector. A reversible-phase Discovery C16 RP-amide column (250 × 4.6 mm, 5 µm particle size; Supelco, Bellefonte, PA) was used at a flow rate of 0.8 mL/min. The injection volume was 10 µL. The chromatograms were analyzed with PC 1000 software v3.5 from Thermo Fisher Scientific (Courtabœuf, France). Chromatographic separations were achieved using a gradient elution system (Tab 2-1).

Table 2-1. Chromatographic conditions for the determination of tested compounds

Products	Mobile Phase (gradient)			Flow Rate ($ml.min^{-1}$)	Detection UV (nm)	Retention time (min)
	Time (min)	Water + TFA 0.1 %	CH_3CN			
F 30	t=0	68 %	32 %	0.8	218	24.6
	t=30	45 %	55 %			
12	t=35	68 %	32 %			8.2
	t=40	68 %	32 %			
F 30						22.9
L-13/D-13	t=0	70 %	30 %			8.0
	t=30	40 %	60 %			
L-14	t=35	70 %	30 %	0.8	218	8.8
	t=37	70 %	30 %			
L-15						9.7
L-16						9.2

The endogenous sugars glucose, fructose and sucrose in the phloem sap were determined enzymatically using previously described methods[5]. A sucrose/D-fructose/D-glucose assay kit (K-SUFRG; Megazyme, Ltd., Bray, Ireland) was used following the manufacturer's instructions. To measure the sucrose concen-

tration, the phloem sap was diluted 100-fold with pure water. All measurements were performed by using 1/4 of the amounts of the reagents recommended by the manufacturer.

Radioactivity was measured by liquid scintillation analyzer (Tri-Carb 2910TR, PerkinElmer). Phloem sap was added to 4 mL scintillation cocktail (EcoLite, ICN Biomedicals). The digested cotyledons were mixed with 14 mL of EcoLite scintillation cocktail and counted by liquid scintillation analyzer.

2.2.5 pH transients in the incubation solution

The measurement of pH transients in the medium using *Ricinus* cotyledons was similar to that described previously[6-8]. The endosperm was removed carefully. The cotyledon still attached to the seedling was incubated in a solution (10 mL) containing 0.25 mM $MgCl_2$ and 0.5 mM $CaCl_2$ at 27°C ± 1°C. The solution was stirred continuously and the pH of the solution was monitored every 30 seconds with a pH microelectrode (Mettler Toledo, InLab Micro), which was connected to a pH meter (S220 SevenCompact pH/Ion Meter, Mettler Toledo) and a recorder. Depending on the aim of the experiment, the solution can be supplemented with tested compound at a final concentration of 0.5 or 1 mM. The pH of the solution bathing the cotyledons stabilized after 30 min at pH 4.8-5.0. Small aliquots of concentrated solutions of sucrose or tested compounds at the same pH were added after reaching a steady state. The pH was monitored continuously over 3 h.

2.3 Uptake experiments with leaf discs of Broad bean

Broad bean (*Vicia faba* cv Aguadulce) plants were grown on vermiculite as already described[9]. The experiments were performed on plants possessing five mature bifoliate leaves.

The leaf discs of broad bean were prepared as previously described[10]. After stripping off the lower epidermis, leaf discs (1.13 cm^2) were preincubated for 30 min in a buffer solution containing 0.25 mM $MgCl_2$, 0.5 mM $CaCl_2$, 250 mM mannitol

and 20 mM MES (pH 5.0). After preincubation, the discs were incubated in the same buffer solution for 30 min supplemented with 0.5 mM labeled sucrose (1 μCi/10 ml) or 3-O-methylglucose (1.5 μCi/10 ml) with or without 0.5 mM glucose conjugate. At the end of incubation the discs were rinsed in the solution similar to the preincubation medium (3×2min). Each disc was then digested overnight at 60 °C in a mixture of perchloric acid (65%; 112 μL), hydrogen peroxide (33%; 54 μL), and Triton X-100 (1 g L^{-1}; 34 μL). Radioactivity was measured by liquid scintillation analyzer mentioned above. The measurements were made on each disc separately.

2.4 Uptake experiments in yeast

Saccharomyces cerevisiae strain RS 453 cells were grown and transformed as described[11]. The *AtSUC2* coding region in plasmid *pDONR207* coding region was a generous gift from Dr F. Vilaine (Insitut Jean Pierre Bourgin, Versailles, France). The coding region was cloned by recombination into plasmid *pDR–R1-R2-HIS3*[12] derived from pDR 192[13]. The plasmid containing *AtSUC2* and the empty plasmid were inserted into *Saccharomyces cerevisiae RS453* and sucrose uptake experiments were run as described[11]. Briefly, yeast cells were grown to early logarithmic phase in YNB medium supplemented with 2% glucose. Cells were washed and resuspended with 50 mM MES buffer (pH 4.5) to reach a final OD_{600nm} value of 0.5. Aliquots (100 μL) of cell suspension were added to 100 μL of a solution containing 50 mM MES (pH 4.5), and a mixture of unlabelled and ^{14}C-labelled sucrose (concentration: 1 mM; specific activity: 0.50 mCi mmol−1) at 28 °C for 5 min. The final sucrose concentration in the medium was therefore 0.5 mM.

References

1. Wu, H., Marhadour, S., Lei, Z.-W., Yang, W., Marivingt-Mounir, C., Bonnemain, J.-L., and Chollet, J.-F., *Vectorization of Agrochemicals: Amino Acid Carriers Are More Efficient Than Sugar Carriers to Translocate Phenylpyrrole Conjugates in the Ricinus System.* Environmental Science and Pollution Research, 2016: p. 1-14. DOI:10.1007/s11356-016-8107-x
2. Kallarackal, J., Orlich, G., Schobert, C., and Komor, E., *Sucrose Transport into the Phloem of Ricinus-Communis L Seedlings as Measured by the Analysis of Sieve-Tube Sap.* Planta, 1989. **177**(3): p. 327-335.
3. Chollet, J. F., Rocher, F., Jousse, C., Deletage-Grandon, C., Bashiardes, G., and Bonnemain, J. L., *Synthesis and Phloem Mobility of Acidic Derivatives of the Fungicide Fenpiclonil.* Pest Management Science, 2004. **60**(11): p. 1063-1072.
4. Antognoni, F., Fornale, S., Grimmer, C., Komor, E., and Bagni, N., *Long-Distance Translocation of Polyamines in Phloem and Xylem of Ricinus Communis L. Plants.* Planta, 1998. **204**(4): p. 520-527.
5. Orlich, G. and Komor, E., *Phloem Loading in Ricinus Cotyledons - Sucrose Pathways Via the Mesophyll and the Apoplasm.* Planta, 1992. **187**(4): p. 460-474.
6. Komor, E., Rotter, M., and Tanner, W., *Proton-Cotransport System in a Higher Plant - Sucrose Transport in Ricinus-Communis.* Plant Science Letters, 1977. **9**(2): p. 153-162.
7. Hutchings, V. M., *Sucrose and Proton Cotransport in Ricinus Cotyledons .1. H+ Influx Associated with Sucrose Uptake.* Planta, 1978. **138**(3): p. 229-235.
8. Robinson, S. P. and Beevers, H., *Evidence for Amino-Acid-Proton Cotransport in Ricinus Cotyledons.* Planta, 1981. **152**(6): p. 527-533.
9. Lemoine, R., Delrot, S., and Auger, E., *Development of Ph Sensitivity of Sucrose Uptake During Aging of Vicia-Faba Leaf-Disks.* Physiologia plantarum, 1984. **61**(4): p. 571-576.
10. Lemoine, R. and Delrot, S., *Recognition of Phlorizin by the Carriers of Sucrose and Hexose in Broad Bean-Leaves.* Physiologia plantarum, 1987. **69**(4): p. 639-644.
11. Henry, C., Rabot, A., Laloi, M., Mortreau, E., Sigogne, M., Leduc, N., Lemoine, R., Sakr, S., Vian, A., and Pelleschi-Travier, S., *Regulation of Rhsuc2, a Sucrose Transporter, Is Correlated with the Light Control of Bud Burst in Rosa Sp.* Plant Cell and Environment, 2011. **34**(10): p. 1776-1789.
12. Cagnac, O., Leterrier, M., Yeager, M., and Blumwald, E., *Identification and Characterization of Vnx1p, a Novel Type of Vacuolar Monovalent Cation/H+ Antiporter of Saccharomyces Cerevisiae.* Journal of Biological Chemistry, 2007. **282**(33): p. 24284-24293.
13. Rentsch, D., Laloi, M., Rouhara, I., Schmelzer, E., Delrot, S., and Frommer, W. B., *Ntr1 Encodes a High Affinity Oligopeptide Transporter in Arabidopsis.* FEBS Lett, 1995. **370**(3): p. 264-268.

Chapter 3. A comparison of systemicity between D-glucose conjugate and L-amino acid conjugate

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ENVIRONMENTAL AND HUMAN HEALTH ISSUES RELATED TO PESTICIDES: FROM USAGE AND ENVIRONMENTAL FATE TO IMPACT

Vectorization of agrochemicals: amino acid carriers are more efficient than sugar carriers to translocate phenylpyrrole conjugates in the *Ricinus* system

Hanxiang Wu[1,2] · Sophie Marhadour[1] · Zhi-Wei Lei[3] · Wen Yang[3] · Cécile Marivingt-Mounir[1] · Jean-Louis Bonnemain[2] · Jean-François Chollet[1]

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Abstract Producing quality food in sufficient quantity while using less agrochemical inputs will be one of the great challenges of the twenty-first century. One way of achieving this goal is to greatly reduce the doses of plant protection compounds by improving the targeting of pests to eradicate. Therefore, we developed a vectorization strategy to confer phloem mobility to fenpiclonil, a contact fungicide from the phenylpyrrole family used as a model molecule. It consists in coupling the antifungal compound to an amino acid or a sugar, so that the resulting conjugates are handled by active nutrient transport systems. The method of click chemistry was used to synthesize three conjugates combining fenpiclonil to glucose or glutamic acid with a spacer containing a triazole ring. Systemicity tests with the *Ricinus* model have shown that the amino acid promoiety was clearly more favorable to phloem mobility than that of glucose. In addition, the transport of the amino acid conjugate is carrier mediated since the derivative of the L series was about five times more concentrated in the phloem sap than its counterpart of the D series. The systemicity of the L-derivative is pH dependent and almost completely inhibited by the protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP). These data suggest that the phloem transport of the L-derivative is governed by a stereospecific amino acid carrier system energized by the proton motive force.

Keywords Phenylpyrroles · Fungicide · Systemicity · Phloem transport · Sustainable agriculture · Carrier mediated

Hanxiang Wu and Sophie Marhadour contributed equally to the paper.

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✉ Jean-François Chollet
jean.franc.chollet@univ-poitiers.fr

[1] Institut de Chimie des Milieux et des Matériaux de Poitiers (IC2MP), Unité Mixte de Recherche CNRS 7285, Université de Poitiers, 4 rue Michel Brunet, TSA 51106, 86073 Poitiers Cedex 9, France

[2] Laboratoire Écologie et Biologie des Interactions, Unité Mixte de Recherche CNRS 7267, Université de Poitiers, 3 rue Jacques Fort, TSA 51106, 86073 Poitiers Cedex 9, France

[3] Guizhou Tea Reasearch Institute, Guizhou Academy of Agricultural Science, Guiyang, Guizhou 550009, China

Introduction

Because of the growing public concern about environmental and health hazards, there has been a clear trend of reducing pesticide use worldwide. On the other hand, reducing pesticide use is required not to affect the quantity and quality of agricultural products (Chollet et al. 2014). Therefore, how to increase the pesticide efficiency will be a great challenge for the agricultural chemical industry. A serious problem is that only a very small part of pesticides (less than 0.1%) actually reaches the sites of action, and off-target portion becomes environmental pollutant (Wang and Liu 2007).

To keep the dose at the lowest possible level and to enhance bioavailability are prime properties to both agrochemical and drug new active ingredients. As commonly defined, bioavailability implies the extent and rate at which a drug becomes available in the systemic circulation (Mannhold et al. 2009). This term can also be used in agrochemical research to describe a pesticide which exerts its biological action in certain plant organs or cells at distance from the site of uptake. Both agrochemicals and drugs require high bioavailability and targeted delivery to ensure desirable localization at the site

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of action while limiting toxicity in non-target organs or organisms.

The prodrug concept, as first introduced by Adrian Albert in 1958 (Albert 1958), can be used to improve the physicochemical properties or bioproperties of the parent drug molecule in such a way as to enhance its deliverability (Stella et al. 2007). Prodrug strategies have been used to solve the problems in oral absorption, organ distribution, metabolism, and excretion (Zawilska et al. 2013). One of the promising means of prodrug strategies is carrier-mediated transport (Sugano et al. 2010). Drug transporters are now widely acknowledged as important determinants governing drug absorption, excretion, and, in many cases, extent of drug entry into target organs (DeGorter et al. 2012). The frequently exploited transporters include peptide, amino acid, and glucose transporters (Fan et al. 2011; Giacomini et al. 2010; Gynther et al. 2008).

Carrier-mediated processes in pesticide uptake and transport are still in the initial stage. It has been known for a long time that phloem mobile pesticides are needed to control root or vascular diseases (Chollet et al. 2005; Edgington 1981), as well as piercing and sucking insects. For example, phloem mobile insecticides (Hu et al. 2010; Yang et al. 2011; Wu et al. 2012) can be a necessary complement to biological control, cultural practices, and genetic resistance utilization in controlling aphids (Bonnemain 2010; Dedryver et al. 2010; Dogimont et al. 2010). Phloem systemicity of agrochemicals can be restricted by their inability to be transported across the plasma membrane from apoplast to symplast. Over the last two decades, there have been a few attempts to improve pesticide phloem mobility or transport to root system utilizing membrane sugar or amino acid transporters (Chollet et al. 1997; Delétage-Grandon et al. 2001; Wu et al. 2012).

The purpose of the present work was to select an ideal promoiety for designing phloem systemic profungicide. Fenpiclonil is a phenylpyrrole fungicide based on chemical modifications of a bacterial metabolite pyrrolnitrin (van Pee and Ligon 2000). This non-systemic fungicide was selected as a model parent compound to test our vectorization strategy and at first to compare the ability of amino acid and sugar carrier systems to translocate large chlorinated xenobiotics. Three designed fenpiclonil conjugates were synthesized via click chemistry.

Material and methods

Synthesis

Some reactions were carried out under nitrogen. All reactions were monitored by TLC analysis using Merck silica gel 60F-254 thin-layer plates. Column chromatography was carried out on silica gel Merck 60 (0.015–0.04 mm). Melting points were determined on an Electrothermal IA 9200 melting point apparatus and are uncorrected. Optical rotations were measured at 20 °C in a 1 cm cell in the stated solvent; $[\alpha]^{20}_{D}$ values are given in 10^{-1} deg cm^2 g^{-1} (concentration c given as g/100 mL). ^{1}H and ^{13}C NMR spectra were performed in DMSO-*d6* or $CDCl_3$ using a Bruker AVANCE 400 MHz spectrometer. DEPT 135 and ^{1}H-^{13}C experiments were used to confirm the NMR peak assignments. Chemical shifts are reported as δ values in parts per million (ppm) relative to tetramethylsilane as internal standard, and coupling constants (*J*) are given in hertz (Hz). The following abbreviations are used to describe peak patterns when appropriate: singlet (s), doublet (d), triplet (t), quartet (q), and multiplet (m). High-resolution mass spectra were obtained on a Bruker Qtof Maxis Impact spectrometer.

Plant material

Castor bean seeds (*Ricinus communis* L. cv Sanguineus), obtained from Graines Girerd et Fils (Le Thor, France), were placed in wet cotton wool for 24 h at 27 °C ± 1 °C prior to sowing in vermiculite watered with tap water. Seedlings were grown in a humid atmosphere (80% ± 5%) at 27 °C ± 1 °C.

Phloem sap collection and analysis

The sap collection method was similar to that already described (Rocher et al. 2006). The phloem sap was analyzed by HPLC after dilution with UHQ-grade water (1 + 9 *v*/*v*). We employed reversed-phase chromatography using an Ascentis Express RP-amide C16 column (length 250 mm, internal diameter 4.6 mm, 5 μM) (Supelco, Bellefonte, PA) in accordance with the procedure set out in Table 1. Results were processed with PC 1000 software v3.5 from Thermo Fisher Scientific (Courtabœuf, France).

Chemicals

The compounds to be added to incubation solutions were from Acros Organics (Noisy-le-Grand, France) (4-morpholinoethanesulfonic acid [MES], 2-[4-(2-hydroxyethyl)-1-piperazine]ethanesulfonic acid [HEPES]) and from Alfa-Aesar (Karlsruhe, Germany) (carbonyl cyanide 3-chlorophenylhydrazone [CCCP]).

Physicochemical properties

Physicochemical properties and descriptors were predicted using the ACD/Labs Percepta 2015 release (Build 2726) software from Advanced Chemistry Development, Inc. (Toronto, Canada). The calculated properties (Table 2) were chosen according to their influence on passive membrane transport in plants (Rocher et al. 2016).

Table 1 Chromatographic data for tested products

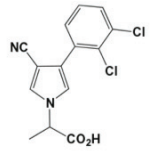
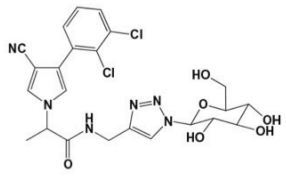
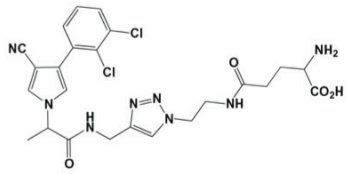
Product	Mobile phase (gradient)			Delivery (mL min^{-1})	UV detection (nm)	Retention time (min)
	Time (min)	Water + TFA 0.1%	CH_3CN			
12	$t = 0$	68	32	0.8	218	8.2
	$t = 30$	45	55			
Parent compound **9**	$t = 35$	68	32			24.6
	$t = 40$	68	32			
L-13/D-13	$t = 0$	70	30	0.8	218	8.0
	$t = 30$	40	60			
Parent compound **9**	$t = 35$	70	30			22.9
	$t = 37$	70	30			

Results and discussion

Synthesis of glucose and amino acid conjugates

Fenpiclonil was the first phenylpyrrole fungicide developed by Ciba-Geigy at the end of the 1980s for seed treatment in cereals (Nyfeler and Ackermann 1992). It is an analogue of the antifungal metabolite pyrrolnitrin, which is produced by several *Pseudomoniae*. Although no longer commercialized, fenpiclonil was selected as a model compound because of the numerous possibilities of adding substituents at various sites in the molecule, in particular to confer systemicity to this non-

Table 2 Structures, chemical descriptors, and physicochemical properties of the studied compounds computed with ACD/Labs Percepta 2015 release (Build 2726) software

No	Structure	MW	HBD	HBA	Log D (pH 5.0)	Log D (pH 8.0)	FRB	PSA (Å²)	Lipinski's rule of five violation (*)	Veber rules violation (**)
9		309,15	1	4	2,05	-0,45	4	66,02	0 / 4	0 / 2
12		551,38 (*)	5	12 (*)	0,71	0,71	8	178,68 (**)	2 / 4	1 / 2
13		561,42 (*)	5	12 (*)	0,42	0,39	13 (**)	180,95 (**)	2 / 4	2 / 2

The interpretation of the computed properties to predict crossing a biological membrane is given according to Lipinski's rule of five (MW ≤500 Da; HBD ≤5; HBA ≤10; Log *P* ≤5.0) and to Veber rule (FRB ≤10; PSA ≤140 Å^2). At biological pHs (from 5.0 to 8.0), compound **9** is predicted to be almost under its undissociated form (pKa = 3.58 ± 0.40) and compound **13** is predicted to be under its zwitterionic form (pKa = 2.13 ± 0.40)

MW molecular weight, *HBD* number of hydrogen bond donors, *HBA* number of hydrogen bond acceptors, *FRB* free rotatable bonds, *PSA* polar surface area

mobile fungicide. These substituents can be carboxylic groups to exploit the ion trap mechanism (Chollet et al. 2004, 2005).

In this work, we chose another strategy which consists in synthesizing conjugates associating the fungicide and a nutrient and in testing if the resulting compounds are recognized and manipulated by an active carrier. We have developed and validated this concept at first with an herbicide and an amino acid (Chollet et al. 1997; Delétage-Grandon et al. 2001; Dufaud et al. 1994). Then, this strategy was extended to an insecticide with a sugar moiety (Hu et al. 2010; Yang et al. 2011).

In order to possibly promote the interaction between the amino acid or the sugar and their respective carriers, a spacer was introduced between the fungicide and the nutrient. The selected spacer includes a 1,2,3-triazole ring linked to the antifungal compound with an amid bond. The 1,2,3-triazole ring has the advantage of being synthesized easily with good yields by « click chemistry » process. In 2001, Sharpless and coworkers introduce the concept of « click chemistry » in order to develop a wide range of compounds for a large scope of applications (Kolb et al. 2001). Several types of reaction fulfill all the criteria like simplicity to perform with high yield, stereospecificity, and easy removing of by-products. The classic Huisgen azide-alkyne 1,3-dipolar cycloaddition led to mixtures of the two regioisomers (Huisgen et al. 1965), whereas the copper-catalyzed reaction named CuAAC (« copper(I)-catalyzed azide-alkyne cycloaddition ») allows the synthesis of the 1,4-disubstituted regioisomers specifically (Rostovtsev et al. 2002; Tornoe et al. 2002).

In this way, for the synthesis of compounds **12** (conjugate with β-D-glucose), **L-13**, and **D-13** (conjugates with L- or D-glutamic acid), the following multistep synthesis was established according to Fig. 1.

Synthesis of azido derivatives from protected sugar or amino acids (Figs. 1 and 2; compounds **2**, **6**, **8***)*

We first started with the synthesis of azido derivatives (**2**, **6**, **8**) from commercially available protected sugar or amino acid. Pentaacetate glucose was reacted with azidotrimethylsilane in the presence of tin(IV) chloride to afford glucose derivative **2** with 86% yield (Cagnoni et al. 2011). We next decided to prepare azido derivatives from protected glutamic acid. The first step consisted of the synthesis of 2-azidoethanamine **4**, which was obtained with 83% yield by nucleophilic substitution of sodium azide on 2-bromoethylamine hydrobromide **1** in water at 75 °C (Mayer and Maier 2007). Then 2-azidoethanamine **4** was condensed with protected L- or D-glutamic acid derivatives (respectively compounds **5** and **7**) in the presence of 4-dimethylaminopyridine (DMAP) and using the condensing agent, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCl) (Sheehan et al. 1961). This latter allowed to set an amide bond between the amine function of the compound **4** and the carboxylic acid function of the compounds **5** or **7** (Sheehan and Hess 1955). The compounds **6** and **8** were obtained from moderate to good yields, 81 and 43% respectively.

Azido-6-(ethanoyloxymethyl)tetrahydro-2H-pyran-3,4,5-triyl triethanoate (compound 2) To a solution of glucose pentaacetate (5.9 g, 15.0 mmol, 1 equiv) and azidotrimethylsilane (2.98 mL, 22.5 mmol, 1.5 equiv) in anhydrous dichloromethane (150 mL), dropwise tin(IV) chloride (1.95 mL, 16.5 mmol, 1.1 equiv) was added. The reaction mixture was stirred at room temperature for 2 h and then washed with sodium bicarbonate and water. The organic layer was dried over $MgSO_4$, filtered, and concentrated under vacuum. The crude product was purified by silica gel column chromatography using pentane/ethyl acetate (8:2) as eluent to afford compound **2** as a white powder (4.9 g, 86% yield).

Rf = 0.62 (pentane/ethyl acetate 6:4); Mp = 128–129 °C (lit. mp 129 °C). ^{1}H NMR (400 MHz, DMSO-d_6): δ 5.37 (t, 1H, $^3J = {}^3J' = 9.3$ Hz, H_c), 5.18 (d, 1H, $^3J = 9.3$ Hz, H_a), 5.02 (t, 1H, $^3J = {}^3J' = 9.3$ Hz, H_d), 4.86 (t, 1H, $^3J = {}^3J' = 9.3$ Hz, H_b), 4.23–4.10 (m, 3H, H_e and H_f), 2.08, 2.07, 2.03, 1.99 (4 s, 12H, H_g). ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.03 (C=O), 169.54 (C=O), 169.25 (C=O), 169.12 (C=O), 86.23 (C_a), 72.76 (C_e), 71.77 (C_c), 70.22 (C_b), 67.75 (C_d), 61.68 (C_f), 20.47, 20.34, 20.28, 20.23 ($4C_g$). HRMS (ESI, CH_3CN): *m/z* calcd for $C_{14}H_{19}N_3O_9$ $[M + Na]^+$ 396.1019, *m/z* found 396.1013.

Azidoethanamine (compound 4) To a solution of 2-bromoethylamine hydrobromide **3** (20.4 g, 100.0 mmol, 1 equiv) in water (142 mL), sodium azide (14.2 g, 220 mmol, 2.2 equiv) was added. The reaction mixture was heated at 75 °C for 18 h. After cooling at room temperature, sodium hydroxide (4.0 g, 100.0 mmol, 1 equiv) was added portionwise to the reaction mixture. The aqueous layer was extracted twice with dichloromethane. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated under vacuum (without heating) to afford compound **4** as a colorless oil (7.1 g, 83% yield). This material was used in the next reaction without further purification.

Rf = 0.39 (ethyl acetate/methanol 6:4). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.36 (t, 2H, $^3J = 5.7$ Hz, H_a), 2.88 (t, 2H, $^3J = 5.7$ Hz, H_b), 1.24 (s, 2H, NH_2). ^{13}C NMR (100 MHz, $CDCl_3$): δ 54.85 (C_a), 41.54 (C_b).

Tert-butyl 5-(2-azidoethylamino)-2-(tert-butoxycarbonylamino)-5-oxopentanoate (compound 6) To a solution of *N*-Boc-L-glutamic acid 1-*tert*-butyl ester **5**

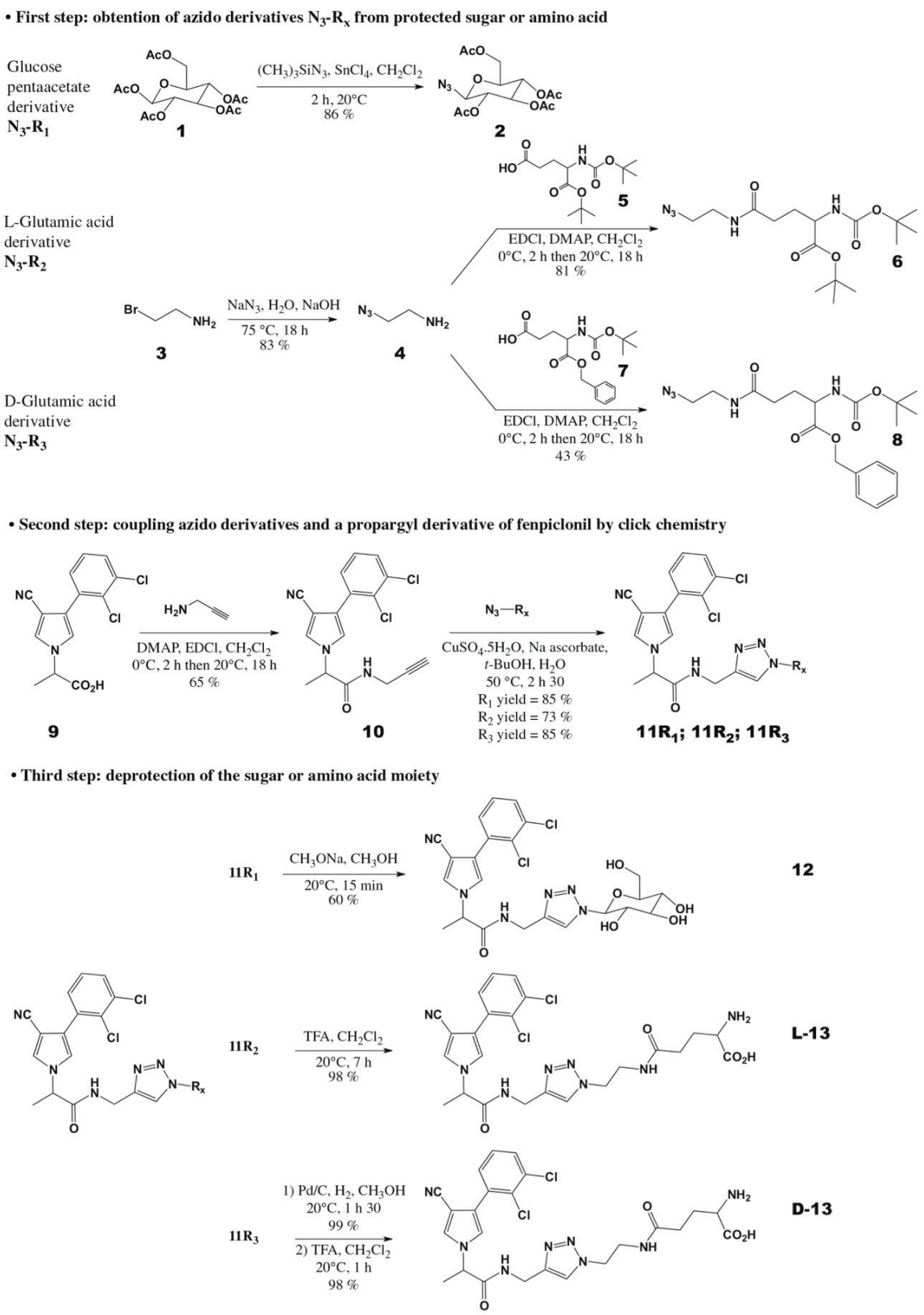


Fig. 1 General reaction scheme showing the different steps of the synthesis of the sugar-fungicide or amino acid-fungicide derivatives. *DMAP* 4-dimethylaminopyridine, *EDCI* 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, *TFA* trifluoroacetic acid

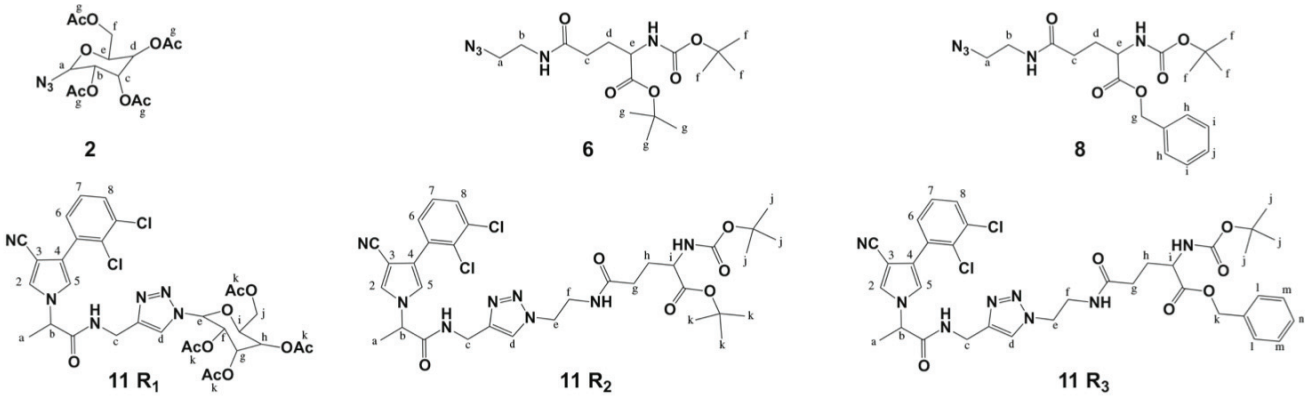


Fig. 2 Azido and fenpiclonil derivatives numbering for ^{1}H and ^{13}C assignments

(4.55 g, 15.0 mmol, 1 equiv) in anhydrous dichloromethane (45 mL) cooled to 0 °C, compound **4** (2.58 g, 30.0 mmol, 2 equiv), EDCl (6.04 g, 31.5 mmol, 2.1 equiv), and DMAP (183 mg, 1.50 mmol, 0.1 equiv) were added. The reaction mixture was purged with nitrogen through the septum and then stirred at 0 °C for 2 h. The mixture was allowed to reach room temperature and then stirred for 18 h. Water was added and the organic layer was extracted twice with dichloromethane. The combined organic layers were washed with water, dried over $MgSO_4$, filtered, and concentrated under vacuum. The crude product was purified by silica gel column chromatography using pentane/ethyl acetate (8:2) as eluent to afford compound **6** as a colorless oil (4.5 g, 81% yield).

Rf = 0.26 (pentane/ethyl acetate 6:4). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.10 (t, 1H, ^{3}J = 5.3 Hz, NH), 7.15 (d, 1H, ^{3}J = 7.8 Hz, NH), 3.84–3.74 (m, 1H, H_e), 3.38–3.24 (m, 4H, H_a and H_b), 2.18 (t, 2H, ^{3}J = 7.7 Hz, H_c), 1.97–1.71 (m, 2H, H_d), 1.43 (s, 9H, H_f), 1.42 (s, 9H, H_g). ^{13}C NMR (100 MHz, DMSO-d_6): δ 171.66 (C=O), 171.56 (C=O), 155.48 (C=O), 80.25 (C), 78.01 (C), 53.95 (C_e), 49.93 (C_a), 38.17 (C_b), 31.61 (C_c), 28.17 ($3C_g$), 27.62 ($3C_f$), 26.48 (C_d). HRMS (ESI, CH_3CN): *m/z* calcd for $C_{16}H_{29}N_5O_5$ $[M + Na]^+$ 394.2066, *m/z* found 394.2061.

Benzyl 5-(2-azidoethylamino)-2-(tert-butoxycarbonylamino)-5-oxopentanoate (compound 8) To a solution of *N*-Boc-D-glutamic acid 1-benzyl ester **7** (10.1 g, 30.0 mmol, 1 equiv) in anhydrous dichloromethane (103 mL) cooled to 0 °C, compound **4** (5.16 g, 60.0 mmol, 2 equiv), EDCl (12.1 g, 62.9 mmol, 2.1 equiv), and DMAP (366 mg, 3.00 mmol, 0.1 equiv) were added. The reaction mixture was purged with nitrogen through the septum and then stirred at 0 °C for 2 h. The mixture was allowed to reach room temperature and then stirred for 18 h. Water was added and the organic layer was extracted twice with dichloromethane. The combined organic layers were washed with water, dried over $MgSO_4$, filtered, and concentrated under vacuum. The crude product was purified by silica gel column chromatography using pentane/ethyl acetate (7:3) as eluent to afford the compound **8** as a white powder (5.2 g, 43% yield).

Rf = 0.42 (pentane/ethyl acetate 6:4); Mp = 68–69 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.10 (t, 1H, ^{3}J = 5.5 Hz, NH), 7.43–7.34 (m, 5H, H_h, H_i and H_j), 6.97 (d, 1H, ^{3}J = 8.1 Hz, NH), 5.12 (s, 2H, H_g), 4.00–3.95 (m, 1H, H_e), 3.44–3.19 (m, 4H, H_a and H_b), 2.42 (t, 2H, ^{3}J = 7.7 Hz, H_c), 2.00–1.76 (m, 2H, H_d), 1.41 (s, 9H, H_f). ^{13}C NMR (100 MHz, DMSO-d_6): δ 172.18 (C=O), 171.82 (C=O), 155.28 (C=O), 136.20 (C), 128.40 ($2C_i$), 127.95 (C_j), 127.83 ($2C_h$), 78.12 (C), 65.43 (C_g), 53.50 (C_e), 49.93 (C_a), 38.15 (C_b), 30.10 (C_c), 28.14 (3 C_f), 27.09 (C_d). HRMS (ESI, CH_3CN): *m/z* calcd for $C_{19}H_{27}N_5O_5$ $[M + Na]^+$ 428.1910, *m/z* found 428.1904.

Coupling azido derivatives and a propargyl derivative of fenpiclonil by « click chemistry » (Figs. 1 and 2; compounds $\mathbf{11R_{1-3}}$*)*

For the second step, we prepared a key intermediate of fenpiclonil, substituted with an alkyne function, allowing to consider thereafter a cycloaddition-1,3 by « click chemistry » giving the 1,2,3-triazole ring as spacer. In order to achieve this, we were interested in preparing compound **10** as a key intermediate to synthesize targeted compounds. Compound **9**, previously described (Chollet et al. 2005), reacted with propargylamine in the presence of DMAP and using EDCl as the condensing agent. The phenylpyrrole with an alkyne function **10** is obtained with 65% yield. The « click chemistry » reaction can be then considered between the alkyne **10** and the three azido derivatives prepared throughout the first step (compounds **2**, **6**, **8**). The 1,3-dipolar cycloaddition reaction is catalyzed by the active Cu(I), generated from Cu(II) salts using sodium ascorbate as the reducing agent in a mixture *tert*-butanol-water. In these conditions, the copper-catalyzed reaction allowed the synthesis of the 1,4-disubstituted regioisomers **11** specifically. We obtained the gluco-conjugate $\mathbf{11R_1}$ with 85% yield. The L-glutamic acid derivative $\mathbf{11R_2}$ and D-glutamic acid

derivative **11R$_3$** were isolated with 73 and 85% yields, respectively. We thought that this methodology could be extended to the synthesis of various amino acid derivatives, thus providing diversity for structure-activity relationship study.

(2-(3-Cyano-4-(2,3-dichlorophenyl)-1H-pyrrol-1-yl)-*N*-(prop-2-ynyl) propanamide (compound 10) To a solution of compound **9** (10.8 g, 35.0 mmol, 1 equiv) in anhydrous dichloromethane (100 mL) cooled to 0 °C, propargylamine (4.47 mL, 70.0 mmol, 2 equiv), EDCl (14.1 g, 73.4 mmol, 2.1 equiv), and DMAP (427 mg, 3.50 mmol, 0.1 equiv) were added. The reaction mixture was purged with nitrogen through the septum and then stirred at 0 °C for 2 h. The mixture was allowed to reach room temperature and then stirred for 18 h. Water was added and the organic layer was extracted twice with dichloromethane. The combined organic layers were washed with water, dried over $MgSO_4$, filtered, and concentrated under vacuum. The crude product was purified by silica gel column chromatography using pentane/ethyl acetate (6:4) as eluent to afford compound **10** as a white powder (7.9 g, 65% yield).

Rf = 0.44 (pentane/ethyl acetate 6:4); Mp = 142–143 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.77 (t, 1H, ^3J = 5.4 Hz, NH), 7.87 (d, 1H, ^4J = 2.3 Hz, H_2), 7.68 (dd, 1H, ^3J = 7.1 Hz, ^4J = 2.5 Hz, H_8), 7.49–7.43 (m, 2H, H_6 and H_7), 7.30 (d, 1H, ^4J = 2.3 Hz, H_5), 4.96 (q, 1H, ^3J = 7.1 Hz, H_b), 3.94 (dd, 2H, ^3J = 5.4 Hz, ^4J = 2.5 Hz, H_c), 3.22 (t, 1H, ^4J = 2.5 Hz, H_d), 1.64 (d, 3H, ^3J = 7.1 Hz, H_a). ^{13}C NMR (100 MHz, DMSO-d_6): δ 168.89 (C=O), 133.94 (C-Cl), 132.47 (C-Cl), 130.24 (C_7 and C), 129.73 (C_8), 129.03 (C_2), 128.22 (C_6), 122.18 (C_4), 122.09 (C_5), 116.01 (CN), 92.04 (C_3), 80.49 (C≡), 73.57 (C_d), 57.47 (C_b), 28.29 (C_c), 18.14 (C_a). HRMS (ESI, CH_3CN): *m/z* calcd for $C_{17}H_{13}Cl_2N_3O$ $[M + Na]^+$ 368.0333, *m/z* found 368.0328.

Representative coupling procedure for the synthesis of derivatives of fenpiclonil (compounds 11R$_1$–11R$_3$) To a solution of compound **10** (3.47 g, 10.0 mmol, 1 equiv) in *tert*-butanol (28 mL), compound **N$_3$-Rx** (10.0 mmol, 1 equiv) was added. Then, a solution of copper(II) sulfate pentahydrate (500 mg, 2.0 mmol, 0.2 equiv) and L-ascorbic acid sodium salt (794 mg, 4.0 mmol, 0.4 equiv) in water (28 mL) was added to the reaction mixture. The resulting solution was heated at 50 °C for 2.5 h. After cooling to room temperature, the resulting mixture was diluted with dichloromethane. The organic layer was extracted, washed with saturated ammonium chloride solution, brine, dried over $MgSO_4$, filtered, and concentrated under vacuum. The crude product was purified by silica gel column chromatography using ethyl acetate as eluent to afford compounds **11R$_1$–11R$_3$**.

2-(4-((2-(3-Cyano-4-(2,3-dichlorophenyl)-1H-pyrrol-1-yl)propanamido)methyl)-1H-1,2,3-triazol-1-yl)-6-(ethanoyloxymethyl)tetrahydro-2H-pyran-3,4,5-triyl triethanoate (compound 11R$_1$) Compound was obtained following the representative procedure, using compound **10** (3.47 g, 10.0 mmol, 1 equiv), compound **2** (3.74 g, 10.0 mmol, 1 equiv), copper(II) sulfate pentahydrate (500 mg, 2.0 mmol, 0.2 equiv), and L-ascorbic acid sodium salt (794 mg, 4.0 mmol, 0.4 equiv). The crude product was purified by silica gel column chromatography using ethyl acetate as eluent to afford compound **11R$_1$** as a white powder (6.1 g, 85% yield).

Rf = 0.62 (ethyl acetate); Mp = 107–108 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.86 (t, 1H, ^3J = 5.6 Hz, NH), 8.28 (s, 1H, H_d), 7.87 (d, 1H, ^4J = 2.2 Hz, H_2), 7.68 (dd, 1H, ^3J = 7.1 Hz, ^4J = 2.6 Hz, H_8), 7.49–7.44 (m, 2H, H_6 and H_7), 7.30 (d, 1H, ^4J = 2.2 Hz, H_5), 6.37 (d, 1H, ^3J = 9.5 Hz, H_e), 5.69 (t, 1H, $^3J = {}^3J'$ = 9.5 Hz, H_f), 5.59 (t, 1H, $^3J = {}^3J'$ = 9.5 Hz, H_g), 5.22 (t, 1H, $^3J = {}^3J'$ = 9.5 Hz, H_h), 4.98 (q, 1H, ^3J = 7.1 Hz, H_b), 4.46–4.33 (m, 3H, H_c and H_i), 4.19–4.09 (m, 2H, H_j), 2.07, 2.03, 2.00, 1.82 (4 s, 12H, H_k), 1.67 (d, 3H, ^3J = 7.1 Hz, H_a). ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.00 (C=O), 169.56 (C=O), 169.35 (C=O), 169.16 (C=O), 168.47 (C=O), 144.95 (C), 133.97 (C-Cl), 132.48 (C-Cl), 130.25 (C_7 and C), 129.71 (C_8), 129.04 (C_2), 128.20 (C_6), 122.18 (C_4), 122.11 (C_5), 121.95 (C_d), 116.05 (CN), 92.00 (C_3), 83.77 (C_e), 73.26 (C_i), 72.19 (C_g), 70.05 (C_f), 67.50 (C_h), 61.77 (C_j), 57.59 (C_b), 34.30 (C_c), 20.50, 20.38, 20.24, 19.89 (4C_k), 18.29 (C_a). HRMS (ESI, CH_3CN): *m/z* calcd for $C_{31}H_{32}Cl_2N_6O_{10}$ $[M + Na]^+$ 741.1455, *m/z* found 741.1449.

Tert-butyl 2-(tert-butoxycarbonylamino)-5-(2-(4-((2-(3-cyano-4-(2,3-dichlorophenyl)-1H-pyrrol-1-yl)propanamido)methyl)-1H-1,2,3-triazol-1-yl)ethylamino)-5-oxopentanoate (compound 11R$_2$) Compound was obtained following the representative procedure, using compound **10** (3.47 g, 10.0 mmol, 1 equiv), compound **6** (3.72 g, 10.0 mmol, 1 equiv), copper(II) sulfate pentahydrate (500 mg, 2.0 mmol, 0.2 equiv), and L-ascorbic acid sodium salt (794 mg, 4.0 mmol, 0.4 equiv). The crude product was purified by silica gel column chromatography using ethyl acetate as eluent to afford compound **11R$_2$** as a white powder (5.3 g, 73% yield).

Rf = 0.59 (ethyl acetate/methanol 9:1); Mp = 117–118 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.82 (t, 1H, ^3J = 5.1 Hz, NH), 8.04 (t, 1H, ^3J = 5.6 Hz, NH), 7.97 (s, 1H, H_d), 7.87 (d, 1H, ^4J = 2.2 Hz, H_2), 7.68 (dd, 1H, ^3J = 7.2 Hz, ^4J = 2.4 Hz, H_8), 7.48–7.42 (m, 2H, H_6 and H_7), 7.30 (d, 1H, ^4J = 2.2 Hz, H_5), 7.15 (d, 1H, ^3J = 7.7 Hz, NH), 4.98 (q, 1H, ^3J = 7.0 Hz, H_b), 4.43–4.32 (m, 4H, H_e and H_f), 3.83–3.77 (m, 1H, H_i), 3.52–3.47 (m, 2H, H_c), 2.15 (t, 2H, ^3J = 7.6 Hz, H_g), 1.95–1.70 (m, 2H, H_h), 1.65 (d, 3H, ^3J = 7.0 Hz, H_a), 1.44–1.42 (m, 18H, H_j and H_k). ^{13}C NMR (100 MHz, DMSO-d_6): δ 171.74 (C=O), 171.54 (C=O), 169.02 (C=O), 155.48 (C=O), 143.97 (C), 133.95 (C-Cl), 132.47 (C-Cl), 130.23 (C_7 and C), 129.70

(C_8), 128.98 (C_2), 128.19 (C_6), 123.18 (C_d), 122.16 (C_4), 122.05 (C_5), 116.02 (CN), 92.00 (C_3), 80.27 (C), 78.04 (C), 57.57 (C_b), 53.93 (C_i), 48.66 (C_e), 38.89 (C_c), 34.45 (C_f), 31.56 (C_g), 28.16 (3 C_k), 27.61 (3 C_j), 26.44 (C_h), 18.27 (C_a). HRMS (ESI, CH_3CN): *m/z* calcd for $C_{33}H_{42}Cl_2N_8O_6$ $[M + Na]^+$ 739.2502, *m/z* found 739.2497.

Benzyl 2-(tert-butoxycarbonylamino)-5-(2-(4-((2-(3-cyano-4-(2,3-dichlorophenyl)-1H-pyrrol-1-yl)propanamido)methyl)-1H-1,2,3-triazol-1-yl)ethylamino)-5-oxopentanoate (compound $11R_3$) Compound was obtained following the representative procedure, using compound **10** (3.47 g, 10.0 mmol, 1 equiv), compound **8** (4.06 g, 10.0 mmol, 1 equiv), copper(II) sulfate pentahydrate (500 mg, 2.0 mmol, 0.2 equiv), and L-ascorbic acid sodium salt (794 mg, 4.0 mmol, 0.4 equiv). The crude product was purified by silica gel column chromatography using ethyl acetate as eluent to afford compound $\mathbf{11R_3}$ as a white powder (6.4 g, 85% yield).

Rf = 0.18 (ethyl acetate); Mp = 156–157 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.80 (t, 1H, ^3J = 5.4 Hz, NH), 8.09 (t, 1H, ^3J = 5.7 Hz, NH), 7.96 (s, 1H, H_d), 7.87 (d, 1H, ^4J = 2.3 Hz, H_2), 7.68 (dd, 1H, ^3J = 7.2 Hz, ^4J = 2.4 Hz, H_8), 7.48–7.42 (m, 2H, H_6 and H_7), 7.40–7.33 (m, 5H, H_l, H_m and H_n), 7.30 (d, 1H, ^4J = 2.3 Hz, H_5), 7.00 (d, 1H, ^3J = 8.0 Hz, NH), 5.12 (s, 2H, H_k), 4.98 (q, 1H, ^3J = 7.0 Hz, H_b), 4.43 (t, 2H, ^3J = 6.1 Hz, H_e), 4.38–4.34 (m, 2H, H_f), 3.95–3.89 (m, 1H, H_i), 3.60–3.49 (m, 2H, H_c), 2.37 (t, 2H, ^3J = 7.1 Hz, H_g), 1.95–1.71 (m, 2H, H_h), 1.65 (d, 3H, ^3J = 7.0 Hz, H_a), 1.40 (s, 9H, H_j). ^{13}C NMR (100 MHz, DMSO-d_6): δ 172.21 (C=O), 171.94 (C=O), 169.00 (C=O), 155.34 (C=O), 143.83 (C), 136.18 (C), 133.96 (C-Cl), 132.49 (C-Cl), 130.24 (C_7 and C), 129.71 (C_8), 129.01 (C_2), 128.40 (2C_m), 128.21 (C_6), 127.95 (C_n), 127.85 (2C_l), 123.25 (C_d), 122.17 (C_4), 122.07 (C_5), 116.04 (CN), 92.00 (C_3), 78.18 (C), 65.45 (C_k), 57.57 (C_b), 53.51 (C_i), 48.54 (C_e), 38.89 (C_c), 34.43 (C_f), 30.04 (C_g), 28.15 (3 C_j), 26.97 (C_h), 18.28 (C_a). HRMS (ESI, CH_3CN): *m/z* calcd for $C_{36}H_{40}Cl_2N_8O_6$ $[M + Na]^+$ 773.2346, *m/z* found 773.2340.

Deprotection of the sugar or amino acid moiety (Fig. 1; compounds **12**, **L-13**, **D-13***)*

The third step consisted of the selective deprotection of the sugar or amino acid moiety of the compounds $\mathbf{11R_1}$, $\mathbf{11R_2}$, and $\mathbf{11R_3}$. The protecting acetyl groups of the hydroxyl functions of the β-D-glucose of $\mathbf{11R_1}$ were *O*-deacetylated by treatment with sodium methoxide, prepared in situ. Then, the obtained alkoxide was neutralized by an acid cation exchange resin (Amberlyst IRN 77) giving the final compound **12** with 60% yield. The deprotection of the alpha-amino acid function of $\mathbf{11R_2}$ was achieved by trifluoroacetic acid in dichloromethane for 7 h at room temperature and afforded the final conjugate **L-13** with 98% yield (Siebum et al. 2004). The two protecting groups of the alpha-amino acid function of $\mathbf{11R_3}$ being different, it is necessary to proceed in two steps. First, the benzyl group protecting the carboxylic acid function is hydrogenated in presence of palladium on carbon in methanol for 1.5 h at room temperature (Usuki et al. 2014). This compound is thus treated with trifluoroacetic acid in dichloromethane for 1 h at room temperature, permitting to remove the protecting *t*-butoxycarbonyl group of the amino function and led to the final compound **D-13** with 98% yield.

2-(3-Cyano-4-(2,3-dichlorophenyl)-1H-pyrrol-1-yl)-*N*-((1-(3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)-1H-1,2,3-triazol-4-yl)methyl) propanamide (compound 12) To a solution of the compound $\mathbf{11R_1}$ (2.20 g, 3.0 mmol, 1 equiv) in methanol (12 mL), sodium (169 mg, 7.3 mmol, 2.4 equiv) was added. The reaction mixture was purged with nitrogen through the septum and then stirred at room temperature for 15 min. The reaction mixture was neutralized with Amberlyst 77. The insoluble materials were separated by filtration through celite eluting with methanol. The filtrate was then concentrated under vacuum to afford the compound **12** as a white powder (1.0 g, 60% yield).

Rf = 0.12 (ethyl acetate/methanol 9:1); Mp = 138–139 °C; $[\alpha]^{20}_D$ − 3.6 (C=0.4, acetone). ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.86 (t, 1H, ^3J = 5.5 Hz, NH), 8.17 (s, 1H, H_d), 7.89 (d, 1H, ^4J = 2.2 Hz, H_2), 7.68 (dd, 1H, ^3J = 7.1 Hz, ^4J = 2.5 Hz, H_8), 7.49–7.43 (m, 2H, H_6 and H_7), 7.32 (d, 1H, ^4J = 2.2 Hz, H_5), 5.56 (d, 1H, ^3J = 9.3 Hz, H_c), 5.41 (d, 1H, ^3J = 6.0 Hz, OH), 5.34 (d, 1H, ^3J = 5.0 Hz, OH), 5.21 (d, 1H, ^3J = 5.5 Hz, OH), 4.99 (q, 1H, ^3J = 7.0 Hz, H_b), 4.68 (t, 1H, ^3J = 5.5 Hz, OH), 4.47–4.35 (m, 2H, H_c), 3.77–3.71 (m, 2H, H_f and H_j), 3.48–3.43 (m, 3H, H_g, H_i and H_j), 3.29–3.23 (m, 1H, H_h), 1.67 (d, 3H, ^3J = 7.0 Hz, H_a). ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.16 (C=O), 144.02 (C), 133.99 (C-Cl), 132.49 (C-Cl), 130.30 (C_7), 130.27 (C), 129.74 (C_8), 129.09 (C_2), 128.26 (C_6), 122.20 (C_5), 122.14 (C_4 and C_d), 116.09 (CN), 92.02 (C_3), 87.43 (C_e), 79.98 (C_i), 77.03 (C_g), 72.03 (C_f), 69.61 (C_h), 60.78 (C_j), 57.61 (C_b), 34.48 (C_c), 18.33 (C_a). HRMS (ESI, CH_3CN): *m/z* calcd for $C_{23}H_{24}Cl_2N_6O_6$ $[M + H]^+$ 551.1213, *m/z* found 551.1207.

2-Amino-5-(2-(4-((2-(3-cyano-4-(2,3-dichlorophenyl)-1H-pyrrol-1-yl)propanamido)methyl)-1H-1,2,3-triazol-1-yl)ethylamino)-5-oxopentanoic acid (compound L-13) The compound $\mathbf{11R_2}$ (2.15 g, 3.0 mmol, 1 equiv) was diluted in a mixture anhydrous dichloromethane–trifluoroacetic acid (24 mL, 1:1). The reaction mixture was purged with nitrogen through the septum and then stirred at room temperature for 7 h before being evaporated. The residue was co-evaporated with ethyl acetate and dried under vacuum to afford the compound **L-13** as a beige powder (1.6 g, 98% yield).

Rf = 0.12 (ethyl acetate/methanol 5:5); Mp = 137–138 °C; $[\alpha]^{20}_D$ − 3.2 (C=1.2, acetone). ^{1}H NMR (400 MHz, DMSO-

d_6): δ 8.87 (t, 1H, ^3J = 5.5 Hz, NH), 8.33 (s, 3H, NH_2 and OH), 8.21 (t, 1H, ^3J = 5.6 Hz, NH), 7.98 (s, 1H, H_d), 7.87 (d, 1H, ^4J = 2.3 Hz, H_2), 7.68 (dd, 1H, ^3J = 7.4 Hz, ^4J = 2.2 Hz, H_8), 7.49–7.42 (m, 2H, H_6 and H_7), 7.30 (d, 1H, ^4J = 2.3 Hz, H_5), 4.99 (q, 1H, ^3J = 7.1 Hz, H_b), 4.43 (t, 2H, ^3J = 6.2 Hz, H_e), 4.39–4.36 (m, 2H, H_f), 3.96–3.94 (m, 1H, H_i), 3.53–3.49 (m, 2H, H_c), 2.32–2.20 (m, 2H, H_g), 1.95–1.89 (m, 2H, H_h), 1.65 (d, 3H, ^3J = 7.1 Hz, H_a). ^{13}C NMR (100 MHz, DMSO-d_6): δ 172.02 (C=O), 170.82 (C=O), 169.10 (C=O), 144.11 (C), 133.97 (C-Cl), 132.50 (C-Cl), 130.26 (C_7 and C), 129.74 (C_8), 129.01 (C_2), 128.23 (C_6), 123.17 (C_d), 122.19 (C_4), 122.08 (C_5), 116.06 (CN), 92.01 (C_3), 57.58 (C_b), 51.61 (C_i), 48.63 (C_e), 38.89 (C_c), 34.47 (C_f), 30.52 (C_g), 25.83 (C_h), 18.28 (C_a). HRMS (ESI, CH_3CN): *m/z* calcd for $C_{24}H_{26}Cl_2N_8O_4$ $[M + H]^+$ 561.1532, *m/z* found 561.1527.

2-Amino-5-(2-(4-((2-(3-cyano-4-(2,3-dichlorophenyl)-1H-pyrrol-1-yl)propanamido)methyl)-1H-1,2,3-triazol-1-yl)ethylamino)-5-oxopentanoic acid (compound D-13) A solution of the compound **11R$_3$** (3.76 g, 5.00 mmol, 1 equiv) in methanol (23 mL) was treated with Pd/C (10%, 53.2 mg) and hydrogenated under balloon pressure at room temperature. After stirring for 1.5 h at room temperature, the insoluble materials were separated by filtration through celite eluting with methanol. The filtrate was then concentrated under vacuum to afford the debenzylated compound **11R$_3$'** as a white powder (3.3 g, 99% yield).

Rf = 0.14 (ethyl acetate/methanol 9:1); Mp = 153–154 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.13 (s, 1H, OH), 8.80 (t, 1H, ^3J = 5.4 Hz, NH), 8.07 (t, 1H, ^3J = 5.6 Hz, NH), 7.96 (s, 1H, H_d), 7.87 (d, 1H, ^4J = 2.2 Hz, H_2), 7.68 (dd, 1H, ^3J = 7.2 Hz, ^4J = 2.4 Hz, H_8), 7.48–7.42 (m, 2H, H_6 and H_7), 7.30 (d, 1H, ^4J = 2.2 Hz, H_5), 6.94 (d, 1H, ^3J = 7.9 Hz, NH), 4.98 (q, 1H, ^3J = 7.0 Hz, H_b), 4.43 (t, 2H, ^3J = 6.2 Hz, H_e), 4.39–4.35 (m, 2H, H_f), 3.92–3.86 (m, 1H, H_i), 3.61–3.49 (m, 2H, H_c), 2.23 (t, 2H, ^3J = 7.6 Hz, H_g), 1.86–1.64 (m, 2H, H_h), 1.62 (d, 3H, ^3J = 7.0 Hz, H_a), 1.40 (s, 9H, H_j). ^{13}C NMR (100 MHz, DMSO-d_6): δ 173.95 (C=O), 172.13 (C=O), 169.03 (C=O), 155.36 (C=O), 143.83 (C), 133.98 (C-Cl), 132.50 (C-Cl), 130.26 (C_7 and C), 129.73 (C_8), 129.02 (C_2), 128.23 (C_6), 123.28 (C_d), 122.19 (C_4), 122.09 (C_5), 116.07 (CN), 92.00 (C_3), 78.16 (C), 57.59 (C_b), 53.69 (C_i), 48.55 (C_e), 38.89 (C_c), 34.45 (C_f), 30.15 (C_g), 28.18 (3 C_j), 27.06 (C_h), 18.30 (C_a). HRMS (ESI, CH_3CN): *m/z* calcd for $C_{29}H_{34}Cl_2N_8O_6$ $[M + Na]^+$ 683.1876, *m/z* found 683.1871.

The compound **11R$_3$'** (1.98 g, 3.0 mmol, 1 equiv) was diluted in a mixture anhydrous dichloromethane–trifluoroacetic acid (10.2 mL, 1:1). The reaction mixture was purged with nitrogen through the septum and then stirred at room temperature for 1 h before being evaporated. The residue was co-evaporated with ethyl acetate and dried under vacuum to afford the compound **D-13** as a rose powder (1.6 g, 98% yield).

Phloem mobility of compounds 12, L-13, and D-13

Comparison of phloem systemicity of compounds **12** *and* **13** *in the Ricinus model*

Phloem systemicity of conjugates **12** and **13** was studied in the *Ricinus* seedlings, which was widely used to test the phloem mobility of xenobiotics (Chollet et al. 2005; Delétage-Grandon et al. 2001; Wu et al. 2012; Yang et al. 2011). One hour after the beginning of incubation of *Ricinus* cotyledons in the standard solution with compound **L-13** at 100-μM concentration, the amino acid conjugate was found in the phloem sap. Its concentration increased up to 5 h to reach about 12 μM (Fig. 3a). By contrast, the phloem systemicity of compound **12** was extremely low. Its concentration in the phloem sap was about 20 times lower than that of compound **L-13** (compare Figs. 3a and 4a). Consequently, a higher concentration of compound **12** (500 μM) in the incubation medium was required for time course experiments. Under this latter experimental condition, it could be noted that the concentration of the glucose conjugate increased up to 4 h to reach a plateau around 3 μM (Fig. 4b). Glucose is poorly present in the phloem sap of *Ricinus* seedlings (about 2 mM). However, when cotyledons were dipped in a solution containing 200 mM glucose, the same concentration was found in the sap after 2 h of incubation (Kallarackal and Komor 1989). Therefore, the extremely low systemicity of compound **12** at pH 5.0 is not due to membrane and/or metabolic mechanisms preventing glucose to enter the sieve tubes as it is the case in various species which translocate only sucrose (Barlow and Randolph 1978; Dinant et al. 2010; Girousse et al. 1991; Hayashi and Chino 1986; Liu et al. 2012). In that respect, fipronil-glucose conjugates exhibited phloem systemicity in *Ricinus* seedlings (Wu et al. 2012; Yang et al. 2011).

Mechanisms governing phloem mobility of compound **13**

In drug discovery, passive membrane permeability of drug candidates can be predicted using Lipinski's rule of five (Ro5) and another simplified rule established by Veber (Lipinski et al. 2012; Veber et al. 2002). The same approach was adopted to profile agrochemicals (Avram et al. 2014; Tice 2001). Coupling glucose or glutamic acid with the parent compound **9** modified many chemical descriptors and physicochemical properties (Table 2). Molecular weight, hydrogen bond donors, hydrogen bond acceptors, free rotatable bonds, and polar surface area values increased dramatically, whereas Log *D* value at pH 5.0 decreased. Surprisingly, the chemical descriptors and physicochemical values of the glucose conjugate **12** are very similar to those of the amino acid conjugate **13**. The computed physicochemical properties of **12** and **13** violate the Ro5 and Veber rule (Table 2), but both

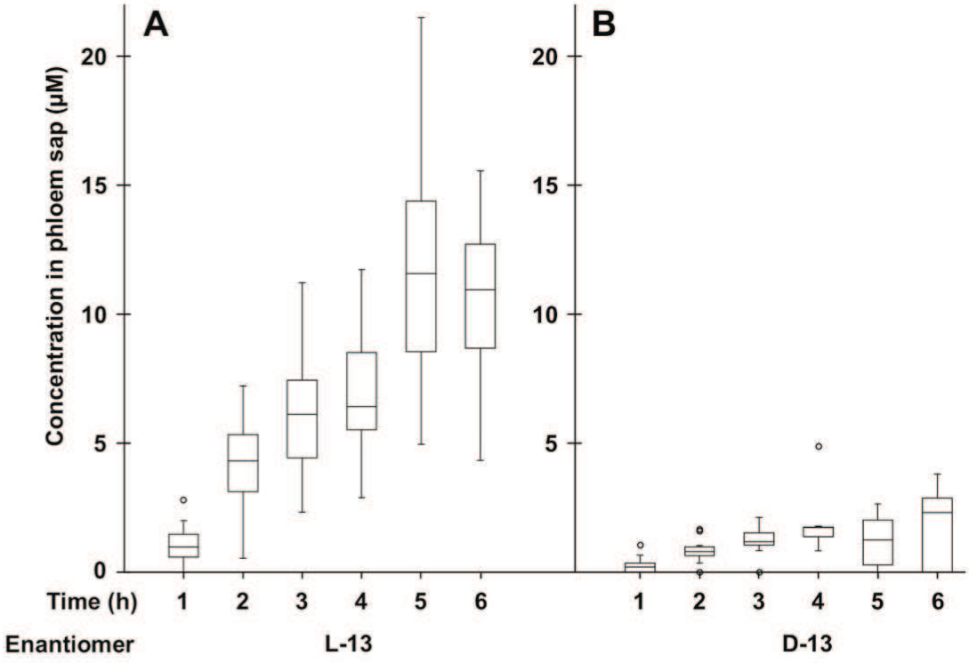


Fig. 3 Time course of amino acid conjugate **L-13** (**a**) and **D-13** (**b**) concentrations in phloem sap of *Ricinus*. Cotyledons were incubated in a standard buffered solution at pH 5.0 (Rocher et al. 2006) for 30 min, then in the same solution containing compounds **L-13** or **D-13** at 100-µM concentration. After 30 min, the hypocotyl was severed in the hook region, and then, the sap was collected every hour for 6 h. For each time, the Mann-Whitney test was used to assess statistically significant differences between the two sets at the 5% probability level. Except for $t = 1$ h where p value = 0.006, all p values <0.0001. For box plots, $n = 22$ (**a**) and $n = 10$ (**b**)

conjugates were detected in phloem sap. It should be pointed out that such prediction approaches are not applicable to actively transported compounds (Rocher et al. 2009). Taking into account the extremely poor systemicity of compound **12**, the study of transport properties of compound **9** derivatives was focused on amino acid conjugates, more especially on compound **L-13**. It has been known for a long time that the positional relationship between the alpha-amino and carboxyl groups is an important parameter in substrate recognition by amino acid carriers (Frommer et al. 1994; Li and Bush 1992). Therefore, the phloem systemicity of the amino acid conjugate **D-13** was studied and compared to that of conjugate **L-13**. The dramatic difference in the ability of **L-13** and **D-13** (Fig. 3a, b) to move in the phloem suggests that a stereospecific amino acid carrier system is involved in **L-13** uptake. In addition, the phloem transport of

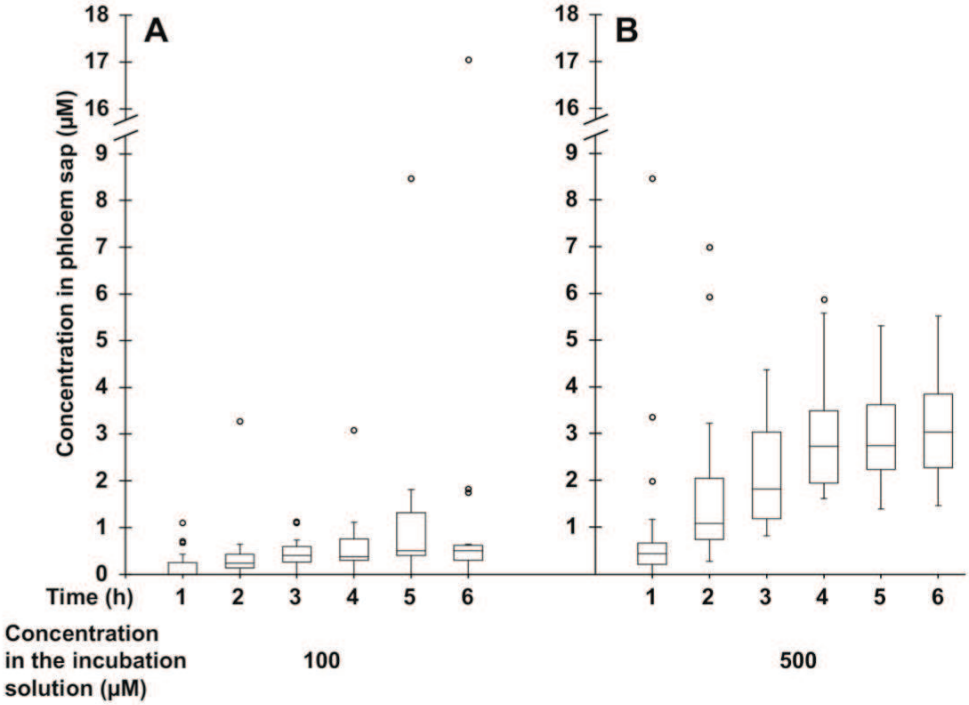


Fig. 4 Time course of glucose conjugate **12** concentration in phloem sap of *Ricinus*. Cotyledons were incubated in a standard buffered solution at pH 5.0 (Rocher et al. 2006) for 30 min, then in the same solution containing compound **12** at 100-µM (**a**) or 500-µM (**b**) concentration. After 30 min, the hypocotyl was severed in the hook region, and then, the sap was collected every hour for 6 h. For box plots, $n = 20$

L-13 was sensitive to pH changes in the incubation medium (Fig. 5). Under our experimental conditions, **L-13** systemicity was optimal at pH 5.0 and was reduced by about four times (amounts exported) and five times (concentration of phloem sap) at pH 7.0 and 8.0 (Fig. 5a, b). A similar pH dependence was noted in the past for neutral amino acid uptake in foliar tissues (Despeghel and Delrot 1983). By contrast, the phloem transport of acidic derivatives of fenpiclonil, similar (Chollet et al. 2004) or threefold higher (Chollet et al. 2005) at pH 5.0 than that of compound **L-13**, decreased dramatically at pH 6.0 and was abolished at pH 6.5. The pH values of the leaf tissue apoplast range from 5.0 to about 7.0 according to species, cell types, and environmental conditions (Husted and Schjoerring 1995; López-Millán et al. 2000; Loubet et al. 2002; Mühling and Läuchli 2000; Mühling et al. 1995; Pfanz and Dietz 1987; Pitann et al. 2009). This means that these derivatives may not be taken up by mesophyll cells and marginally loaded in the phloem.

The protonophore CCCP which dissipates the proton motive force is a potent inhibitor of sugar and amino acid uptake activities. Under our experimental conditions, it reduced phloem sap exudation and inhibited almost completely **L-13** systemicity (Fig. 6). Therefore, these data indicate that the phloem transport of compound **L-13** is governed by a stereospecific amino acid carrier system energized by the transmembrane electrochemical gradient of protons. The proton-motive force is generated by the plasma membrane H^+-ATPase, an intrinsic enzyme highly expressed in companion cells and cotyledon epidermal cells (Bouché-Pillon et al. 1994a; Bouché-Pillon et al. 1994b; DeWitt and Sussman 1995; Morsomme and Boutry 2000). In that respect, *Ricinus* cotyledon tissues, which generate high transmembrane pH and electrical gradients (Komor et al. 1980), constitute an appropriate model to study the transport properties of xenobiotics.

Conclusion

A fenpiclonil-glucose and two fenpiclonil-glutamic acid conjugates were synthesized to describe a vectorization strategy for carrier-mediated phloem systemic agrochemical. Because the –NH– function of the pyrrole ring is required for the fungicidal activity (Nyfeler and Ackermann 1992), these compounds are not expected to exhibit a biological activity without releasing the parent compound. Our work must be

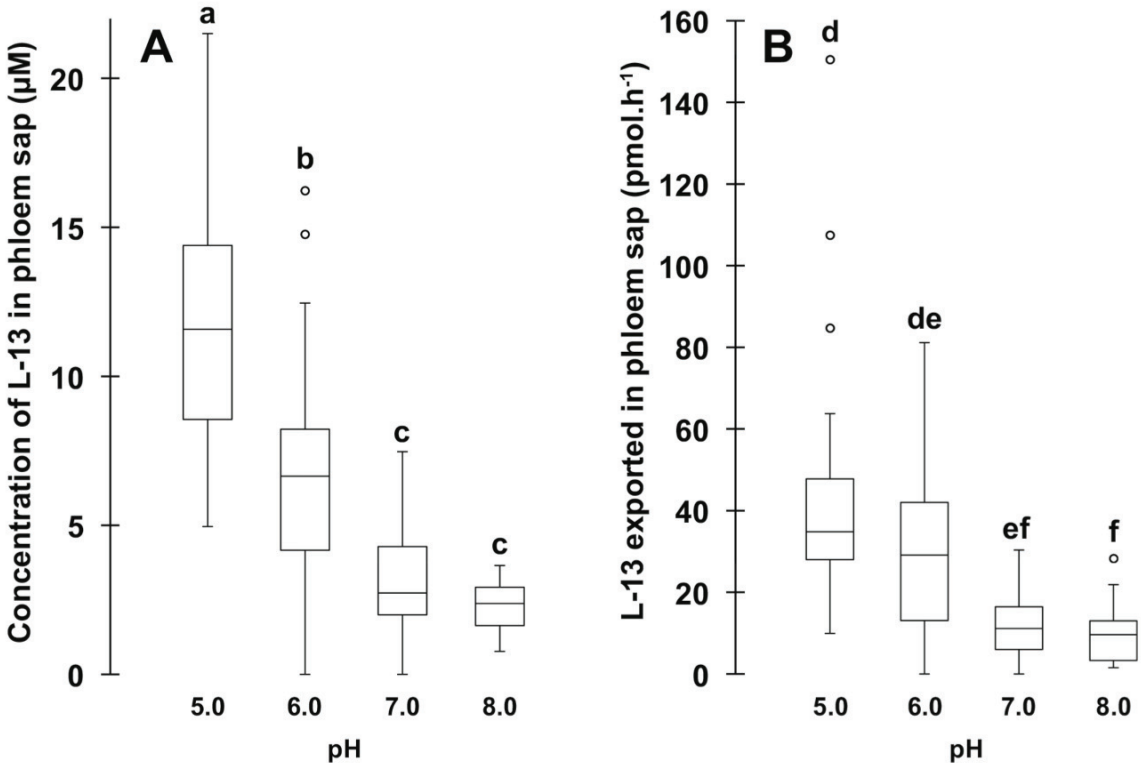


Fig. 5 Concentration (**a**) or quantity exported (**b**) of compound **L-13** in phloem sap of *Ricinus* as a function of the pH of the incubation medium. Cotyledons were incubated in a standard buffered solution at pH 5.0, 6.0, 7.0, or 8.0 (Rocher et al. 2006) for 30 min, then in the same solution containing compound **L-13** at 100-µM concentration. After 30 min, the hypocotyl was severed in the hook region, and then, the sap was collected between the fourth and fifth hours. The Kruskal-Wallis test was used to assess statistically significant differences between the different sets at the 5% probability level. A *different letter* indicates that the sets are not from the same population. For box plots, $n = 20$

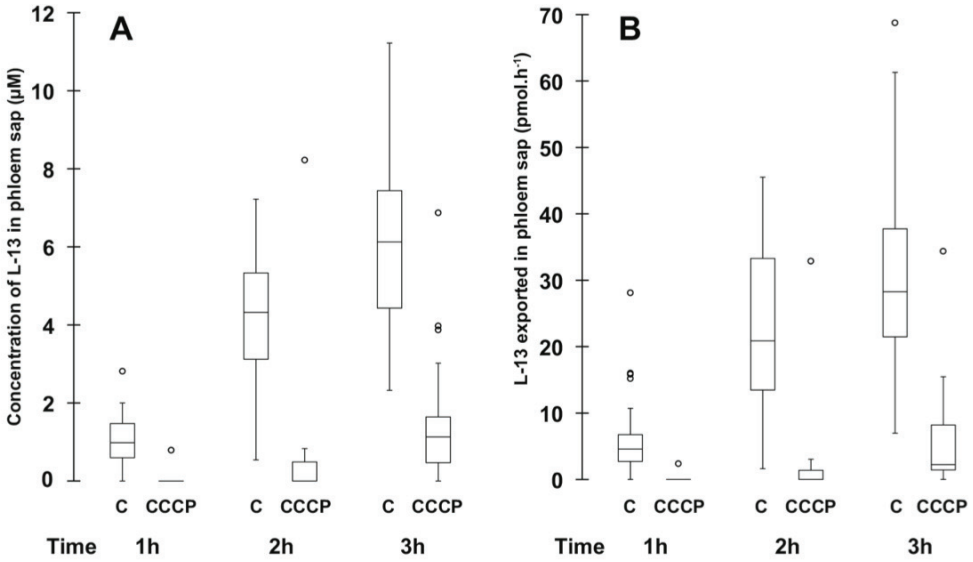


Fig. 6 Effect of CCCP on time course of conjugate **L-13** concentration (**a**) or quantity exported (**b**) in phloem sap of *Ricinus*. Cotyledons were incubated in a standard buffered solution at pH 5.0 (Rocher et al. 2006) for 30 min, then in the same solution containing compound **L-13** at 100-µM concentration without (*C* control) or with CCCP at 10-µM final concentration. After 30 min, the hypocotyl was severed in the hook region, and then, the sap was collected every hour for 3 h. For each time, the Mann-Whitney test was used to assess statistically significant differences between the two sets (with or without CCCP) at the 5% probability level. In all cases, p values <0.0001. For box plots, $n = 24$ (control) and $n = 19$ (CCCP)

considered as a new systemic profungicide approach. These large and chlorinated conjugates which violate both Lipinski and Veber rules are found in the phloem sap. However, the fenpiclonil-glutamic acid conjugate (**L-13**) showed a much more favorable phloem mobility than fenpiclonil-glucose conjugate, indicating that amino acid carriers are more promising than sugar carriers to enhance the phloem loading of phenylpyrrole conjugates. In addition, the phloem mobility of L-glutamic acid conjugate (**L-13**) was about fivefold higher than that of corresponding D-isomer (**D-13**), suggesting the involvement of a stereoselective transporter. Further investigation of mechanism displayed that compound **L-13** was pH dependent and inhibited by the protonophore CCCP. Thus, conjugation of parent pesticides with L-amino acid is a feasible strategy to confer phloem mobility to agrochemicals. The result of the present work about fenpiclonil-amino acid conjugates reinforces the previous conclusion that an amino acid transport system is able to recognize and transport chlorinated xenobiotics of larger size than its natural substrates. In our previous paper (Deletage-Grandon 2001), a halogenated xenobiotic (approximately 350 D) with an amino acid moiety (Lys 2,4-D) was proved to be recognized and translocated by an amino acid transport system. The distribution study of conjugate Lys 2,4-D and parent compound (2,4-D) showed a significantly different accumulation in certain plant organs. The parent molecule (2,4-D) is a phloem mobile herbicide, but the accumulation of Lys 2,4-D in the root system is five to ten times greater than that of 2,4-D. Therefore, the distribution pattern of amino acid conjugates of fungicides in the whole plant deserves further investigation.

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References

Albert A (1958) Chemical aspects of selective toxicity. Nature 182: 421–422

Avram S, Funar-Timofei S, Borota A, Chennamaneni SR, Manchala AK, Muresan S (2014) Quantitative estimation of pesticide-likeness for agrochemical discovery. J Cheminform 6:11. doi:10.1186/s13321-014-0042-6

Barlow CA, Randolph PA (1978) Quality and quantity of plant sap available to pea aphid (*Acyrthosiphon pisum* Harris aphididae). Ann Entomol Soc Am 71:46–48

Bonnemain JL (2010) Aphids as biological models and agricultural pests. C. R. Biol. 333:461–463. doi:10.1016/j.crvi.2010.04.002

Bouché-Pillon S, Fleurat-Lessard P, Fromont JC, Serrano R, Bonnemain JL (1994a) Immunolocalization of the plasma membrane H+-ATPase in minor veins of *Vicia faba* in relation to phloem loading. Plant Physiol 105:691–697

Bouché-Pillon S, Fleurat-Lessard P, Serrano R, Bonnemain JL (1994b) Asymmetric distribution of the plasma membrane H+-ATPase in embryos of *Vicia faba* L with special reference to transfer cells. Planta 193:392–397

Cagnoni AJ, Varela O, Gouin SG, Kovensky J, Uhrig ML (2011) Synthesis of multivalent glycoclusters from 1-thio-beta-D-galactose and their inhibitory activity against the beta-galactosidase from *E. coli*. J. Org. Chem. 76:3064–3077. doi:10.1021/jo102421e

Chollet J-F, Couderchet M, Bonnemain J-L (2014) Crop protection: new strategies for sustainable development. Environ Sci Pollut Res Int 21:4793–4796. doi:10.1007/s11356-014-2567-7

Chollet JF, Delétage C, Faucher M, Miginiac L, Bonnemain JL (1997) Synthesis and structure-activity relationships of some pesticides with an alpha-amino acid function. Biochim Biophys Acta 1336: 331–341. doi:10.1016/s0304-4165(97)00041-x

Chollet JF, Rocher F, Jousse C, Deletage-Grandon C, Bashiardes G, Bonnemain JL (2004) Synthesis and phloem mobility of acidic derivatives of the fungicide fenpiclonil. Pest Manag Sci 60:1063–1072. doi:10.1002/ps.906

Chollet JF, Rocher F, Jousse C, Deletage-Grandon C, Bashiardes G, Bonnemain JL (2005) Acidic derivatives of the fungicide fenpiclonil: effect of adding a methyl group to the N-substituted chain on systemicity and fungicidal activity. Pest Manag Sci 61: 377–382. doi:10.1002/ps.977

Dedryver CA, Le Ralec A, Fabre F (2010) The conflicting relationships between aphids and men: a review of aphid damage and control strategies. C. R. Biol. 333:539–553. doi:10.1016/j.crvi.2010.03.009

DeGorter MK, Xia CQ, Yang JJ, Kim RB (2012) Drug transporters in drug efficacy and toxicity. Annu Rev Pharmacol Toxicol 52:249–273. doi:10.1146/annurev-pharmtox-010611-134529

Delétage-Grandon C, Chollet JF, Faucher M, Rocher F, Komor E, Bonnemain JL (2001) Carrier-mediated uptake and phloem systemy of a 350-Dalton chlorinated xenobiotic with an alpha-amino acid function. Plant Physiol 125:1620–1632. doi:10.1104/pp.125.4.1620

Despeghel JP, Delrot S (1983) Energetics of amino acid uptake by *Vicia faba* leaf tissues. Plant Physiol 71:1–6. doi:10.1104/pp.71.1.1

DeWitt ND, Sussman MR (1995) Immunocytological localization of an epitope-tagged plasma membrane proton pump (H+-ATPase) in phloem companion cells. Plant Cell 7:2053–2067

Dinant S, Bonnemain JL, Girousse C, Kehr J (2010) Phloem sap intricacy and interplay with aphid feeding. C. R. Biol. 333:504–515. doi:10.1016/j.crvi.2010.03.008

Dogimont C, Bendahmane A, Chovelon V, Boissot N (2010) Host plant resistance to aphids in cultivated crops: genetic and molecular bases, and interactions with aphid populations. C R Biol 333:566–573. doi:10.1016/j.crvi.2010.04.003

Dufaud A, Chollet JF, Rudelle J, Miginiac L, Bonnemain JL (1994) Derivatives of pesticides with an alpha-amino acid function—synthesis and effect on threonine uptake. Pestic Sci 41:297–304. doi:10.1002/ps.2780410403

Edgington LV (1981) Structural requirements of systemic fungicides. Annu Rev Phytopathol 19:107–124. doi:10.1146/annurev.py.19.090181.000543

Fan W, Wu Y, Li XK, Yao N, Li X, Yu YG, Hai L (2011) Design, synthesis and biological evaluation of brain-specific glucosyl thiamine disulfide prodrugs of naproxen. Eur J Med Chem 46:3651–3661. doi:10.1016/j.ejmech.2011.05.029

Frommer WB, Kwart M, Hirner B, Fischer WN, Hummel S, Ninnemann O (1994) Transporters for nitrogenous compounds in plants. Plant Mol Biol 26:1651–1670. doi:10.1007/bf00016495

Giacomini KM et al (2010) Membrane transporters in drug development. Nat Rev Drug Discov 9:215–236. doi:10.1038/nrd3028

Girousse C, Bonnemain JL, Delrot S, Bournoville R (1991) Sugar and amino acid composition of phloem sap of *Medicago sativa*—a comparative study of 2 collecting methods. Plant Physiol Biochem 29: 41–48

Gynther M, Laine K, Ropponen J, Leppanen J, Mannila A, Nevalainen T, Savolainen J, Jarvinen TT, Rautio J (2008) Large neutral amino acid transporter enables brain drug delivery via prodrugs. J Med Chem 51:932–936. doi:10.1021/jm701175d

Hayashi H, Chino M (1986) Collection of pure phloem sap from wheat and its chemical composition. Plant Cell Physiol 27:1387–1393

Hu AL, Yang W, Xu HH (2010) Novel fluorescent conjugate containing glucose and NBD and its carrier-mediated uptake by tobacco cells. J Photochem Photobiol B 101:215–223. doi:10.1016/j.jphotobiol.2010.07.006

Huisgen R, Knorr R, Mobius L, Szeimies G (1965) 1.3-Dipolare cycloadditionen. 23. Einige beobachtungen zur addition organischer azide an cc-dreifachbindungen. Chem Ber Recl 98:4014–4021. doi:10.1002/cber.19650981228

Husted S, Schjoerring JK (1995) Apoplastic pH and ammonium concentration in leaves of *Brassica napus* L. Plant Physiol 109:1453–1460

Kallarackal J, Komor E (1989) Transport of hexoses by the phloem of *Ricinus communis* L seedlings. Planta 177:336–341. doi:10.1007/bf00403591

Kolb HC, Finn MG, Sharpless KB (2001) Click chemistry: diverse chemical function from a few good reactions. Angew Chem Int Ed 40: 2004–2021. doi:10.1002/1521-3773(20010601)40:11<2004::aid-anie2004>3.0.co;2-5

Komor E, Rotter M, Waldhauser J, Martin E, Cho BH (1980) Sucrose proton symport for phloem loading in the Ricinus seedling. Ber Dtsch bot Ges 93:211–219

Li ZC, Bush DR (1992) Structural determinants in substrate recognition by proton amino acid symports in plasma membrane vesicles isolated from sugar beet leaves. Arch Biochem Biophys 294:519–526. doi:10.1016/0003-9861(92)90719-d

Lipinski CA, Lombardo F, Dominy BW, Feeney PJ (2012) Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Del Rev 64:4–17. doi:10.1016/j.addr.2012.09.019

Liu DD, Chao WM, Turgeon R (2012) Transport of sucrose, not hexose, in the phloem. J Exp Bot 63:4315–4320. doi:10.1093/jxb/ers127

López-Millán AF, Morales F, Abadia A, Abadia J (2000) Effects of iron deficiency on the composition of the leaf apoplastic fluid and xylem sap in sugar beet. Implications for iron and carbon transport. Plant Physiol 124:873–884. doi:10.1104/pp.124.2.873

Loubet B, Milford C, Hill PW, Tang YS, Cellier P, Sutton MA (2002) Seasonal variability of apoplastic NH4+ and pH in an intensively managed grassland. Plant Soil 238:97–110. doi:10.1023/a:1014208926195

Mannhold R, Kubinyi H, Folkers G, van de Waterbeemd H, Testa B (eds) (2009) Drug bioavailability: estimation of solubility, permeability, absorption and bioavailability, 40. Wiley, Weinheim

Mayer T, Maier ME (2007) Design and synthesis of a tag-free chemical probe for photoaffinity labeling. Eur. J. Org. Chem.: 4711–4720 doi: 10.1002/ejoc.200700188

Morsomme P, Boutry M (2000) The plant plasma membrane H+-ATPase: structure, function and regulation. Biochim Biophys Acta 1465:1–16. doi:10.1016/s0005-2736(00)00128-0

Mühling KH, Plieth C, Hansen UP, Sattelmacher B (1995) Apoplastic pH of intact leaves of *Vicia faba* as influenced by light. J Exp Bot 46: 377–382. doi: 10.1093/jxb/46.4.377

Mühling KH, Läuchli A (2000) Light-induced pH and K+ changes in the apoplast of intact leaves. Planta 212:9–15. doi: 10.1007/s004250000374

Nyfeler R, Ackermann P (1992) Phenylpyrroles, a new class of agricultural fungicides related to the natural antibiotic pyrrolnitrin. ACS Symp Ser 504:395–404

Pfanz H, Dietz KJ (1987) A fluorescence method for the determination of the apoplastic proton concentration in intact leaf tissues. J Plant Physiol 129:41–48

Pitann B, Schubert S, Muhling KH (2009) Decline in leaf growth under salt stress is due to an inhibition of H+-pumping activity and increase in apoplastic pH of maize leaves. J Plant Nutr Soil Sci 172: 535–543. doi:10.1002/jpln.200800349

Rocher F, Chollet JF, Jousse C, Bonnemain JL (2006) Salicylic acid, an ambimobile molecule exhibiting a high ability to accumulate in the phloem. Plant Physiol 141:1684–1693. doi:10.1104/pp.106.082537

Rocher F, Chollet JF, Legros S, Jousse C, Lemoine R, Faucher M, Bush DR, Bonnemain JL (2009) Salicylic acid transport in *Ricinus*

communis involves a pH-dependent carrier system in addition to diffusion. Plant Physiol 150:2081–2091. doi:10.1104/pp.109.140095

Rocher F, Roblin G, Chollet J-F (2016) Modifications of the chemical structure of phenolics differentially affect physiological activities in pulvinar cells of *Mimosa pudica* L. II. Influence of various molecular properties in relation to membrane transport. Environ. Sci. Pollut. Res. Int.: In press doi: 10.1007/s11356-016-6048-z

Rostovtsev VV, Green LG, Fokin VV, Sharpless KB (2002) A stepwise Huisgen cycloaddition process: copper(I)-catalyzed regioselective "ligation" of azides and terminal alkynes. Angew Chem Int Ed 41: 2596–2599. doi:10.1002/1521-3773(20020715)41:14<2596::aid-anie2596>3.0.co;2-4

Sheehan JC, Hess GP (1955) A new method of forming peptide bonds. J Am Chem Soc 77:1067–1068. doi:10.1021/ja01609a099

Sheehan JC, Boshart GL, Cruickshank PA (1961) Convenient synthesis of water-soluble carbodiimides. J. Org. Chem. 26:2525–2528. doi:10.1021/jo01351a600

Siebum AHG, Tsang RKF, van der Steen R, Raap J, Lugtenburg J (2004) Synthesis of (epsilon-C-13-,epsilon N-15)-enriched L-lysine—establishing schemes for the preparation of all possible C-13 and N-15 isotopomers of L-lysine, L-ornithine, and L-proline. Eur. J. Org. Chem.: 4391–4396 doi: 10.1002/ejoc.200400370

Stella VJ, Borchardt RT, Hageman MJ, Oliyai R, Maag H, Tilley JW (Editors), 2007: Prodrugs challenges and rewards. Part 1. Springer-Verlag, New York, XVIII, 1464 pp

Sugano K, Kansy M, Artursson P, Avdeef A, Bendels S, Di L, Ecker GF, Faller B, Fischer H, Gerebtzoff G, Lennernaes H, Senner F (2010) Coexistence of passive and carrier-mediated processes in drug transport. Nat Rev Drug Discov 9:597–614. doi:10.1038/nrd3187

Tice CM (2001) Selecting the right compounds for screening: does Lipinski's rule of 5 for pharmaceuticals apply to agrochemicals? Pest Manage Sci 57:3–16. doi:10.1002/1526-4998(200101)57:1<3::aid-ps269>3.0.co;2-6

Tornoe CW, Christensen C, Meldal M (2002) Peptidotriazoles on solid phase: 1,2,3-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. J Org Chem 67: 3057–3064. doi:10.1021/jo011148j

Usuki T, Sugimura T, Komatsu A, Koseki Y (2014) Biomimetic chichibabin pyridine synthesis of the COPD biomarkers and elastin cross-linkers isodesmosine and desmosine. Org Lett 16:1672–1675. doi:10.1021/ol500333t

van Pee KH, Ligon JM (2000) Biosynthesis of pyrrolnitrin and other phenylpyrrole derivatives by bacteria. Nat Prod Rep 17:157–164. doi:10.1039/a902138h

Veber DF, Johnson SR, Cheng HY, Smith BR, Ward KW, Kopple KD (2002) Molecular properties that influence the oral bioavailability of drug candidates. J Med Chem 45:2615–2623. doi:10.1021/jm020017n

Wang CJ, Liu ZQ (2007) Foliar uptake of pesticides—present status and future challenge. Pestic Biochem Physiol 87:1–8. doi:10.1016/j.pestbp.2006.04.004

Wu HX, Yang W, Zhang ZX, Huang T, Yao GK, Xu HH (2012) Uptake and phloem transport of glucose-fipronil conjugate in *Ricinus communis* involve a carrier-mediated mechanism. J Agric Food Chem 60:6088–6094. doi:10.1021/jf300546t

Yang W, Wu HX, Xu HH, Hu AL, Lu ML (2011) Synthesis of glucose-fipronil conjugate and its phloem mobility. J Agric Food Chem 59: 12534–12542. doi:10.1021/jf2031154

Zawilska JB, Wojcieszak J, Olejniczak AB (2013) Prodrugs: a challenge for the drug development. Pharmacol Rep 65:1–14

Chapter 4. Investigating and exploiting the limits of the strategy

- Journal article « Use of D-Glucose–fenpiclonil Conjugate as a Potent and Specific Inhibitor of Sucrose Carriers », *Journal of Experimental Botany*. 2017, 68(20): 5599-5613, doi:10.1093/jxb/erx354.

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RESEARCH PAPER

Use of D-glucose–fenpiclonil conjugate as a potent and specific inhibitor of sucrose carriers

Hanxiang Wu[1,2], Sophie Marhadour[2], Zhi-Wei Lei[3], Émilie Dugaro[2], Cécile Gaillard[1], Benoit Porcheron[1], Cécile Marivingt-Mounir[2], Rémi Lemoine[1], Jean-François Chollet[2] and Jean-Louis Bonnemain[1,*]

[1] Laboratoire EBI (Écologie et Biologie des Interactions), UMR CNRS 7267, Équipe SEVE (Sucres, Échanges Végétaux, Environnement), Université de Poitiers, 3 rue Jacques Fort, TSA 51106, F-86073 Poitiers cedex 9, France
[2] IC2MP (Institut de Chimie des Milieux et des Matériaux de Poitiers), UMR CNRS 7285, Université de Poitiers, 4 rue Michel Brunet, TSA 51106, F-86073 Poitiers cedex 9, France
[3] Guizhou Tea Reasearch Institute, Guizhou Academy of Agricultural Science, Guiyang, Guizhou 550009, China

* Correspondence: jl.bonnemain@yahoo.fr

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Abstract

Until now, specific inhibitors of sucrose carriers were not available. This led us to study the properties of the recently synthesized D-glucose–fenpiclonil conjugate (D-GFC). This large amphiphilic glucoside exhibited an extremely low phloem systemicity in contrast to L-amino acid–fenpiclonil conjugates. Using *Ricinus* seedlings, the effect of D-GFC on 0.5 mM [^{14}C]sucrose (Suc), 3-*O*-[^{3}H]methylglucose, and [^{3}H]glutamine uptake by cotyledon tissues was compared with that of *p*-chloromercuribenzenesulfonic acid (PCMBS). D-GFC dramatically inhibited H^+–Suc symport at the same concentrations as PCMBS (0.5 and 1 mM), but in contrast to the thiol reagent, it did not affect 3-*O*-methylglucose and glutamine transport, nor the acidification of the incubation medium by cotyledon tissues. Similarly, 0.5 mM D-GFC inhibited active Suc uptake by *Vicia faba* leaf tissues and by *Saccharomyces cerevisiae* cells transformed with *AtSUC2*, a gene involved in Suc phloem loading in Arabidopsis, by approximately 80%. The data indicated that D-GFC was a potent inhibitor of Suc uptake from the endosperm and of Suc phloem loading. It is the first chemical known to exhibit such specificity, at least in *Ricinus*, and this property permitted the quantification of the two routes involved in phloem loading of endogenous sugars after endosperm removal.

Key words: Apoplasmic loaders, Arabidopsis AtSUC2, D-glucose–fenpiclonil conjugate, PCMBS, phloem loading, *Ricinus* seedlings, specific inhibition of sucrose carriers, sucrose uptake from endosperm, *Vicia faba*.

Introduction

The non-permeant or poorly permeant sulfhydryl reagent *p*-chloromercuribenzenesulfonic acid (PCMBS) has been successfully used in phloem biology, first to demonstrate that sucrose accumulates in the phloem symplasm from the vein apoplasm through Suc carriers as in *Beta vulgaris* and *Vicia faba* (Giaquinta, 1976; Delrot *et al.*, 1980; Giaquinta, 1983), and then to identify apoplasmic and symplasmic loaders (van Bel *et al.*, 1992; Turgeon and Medville, 2004; Turgeon and Ayre, 2005). PCMBS dramatically inhibits the activity of Suc carriers but because it reacts with cysteine residues of many other plasma membrane (PM) intrinsic proteins, it also affects the transport of other solutes, as demonstrated in

Abbreviations: CF, concentration factor; Gln, glutamine; D-GFC, D-glucose–fenpiclonil conjugate; 3-*O*-MeG, 3-*O*-methylglucose; PCMBS, *p*-chloromercuribenzenesulfonic acid sodium salt; PM, plasma membrane; Suc, sucrose.

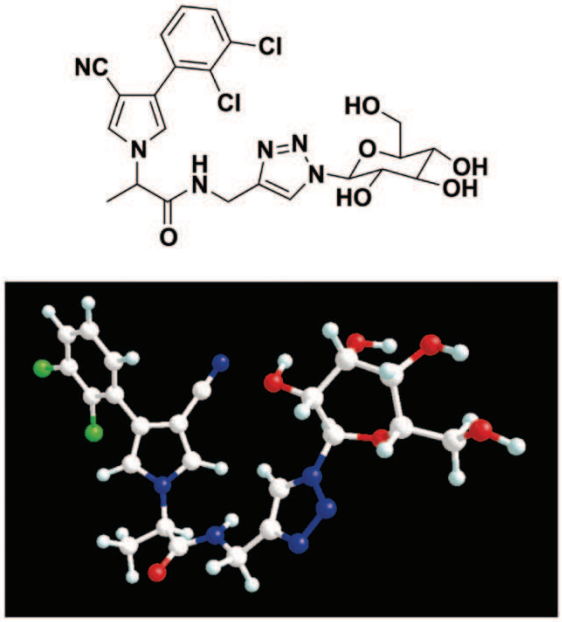

Fig. 1. Two- and three-dimensional structure of D-GFC obtained using Chem3D Pro, energy minimization with the MM2 method. Atoms are denoted by spheres in the following colours: carbon in pale grey, hydrogen in light blue, chlorine in green, oxygen in red, and nitrogen in blue. For this compound, M_r=551.38 and K_{ow}=0.71 (computed with ACD/Labs Percepta 2015 release).

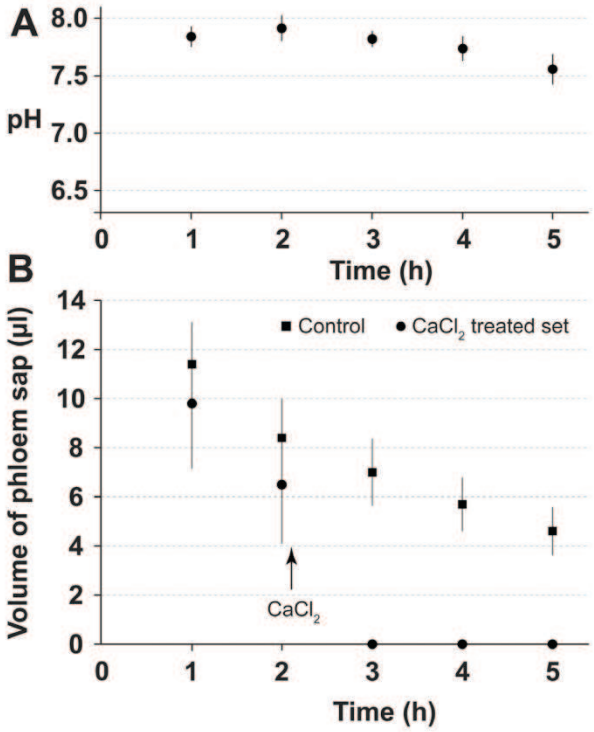

Fig. 2. Phloem sap exudation from *Ricinus* seedlings. (A) pH time course variation of the *Ricinus* phloem sap. After preincubation for 60 min in a standard medium (pH 5.0), the hypocotyls were severed, and then the phloem sap was collected each hour from the upper part for pH measurements. Each point is the mean±95% CI of three sets of five plants. (B) Effect of 1 M $CaCl_2$ on sap exudation in the presence of D-GFC. After preincubation for 30 min in a standard medium (pH 5.0), the cotyledons were dipped in the same solution containing 0.5 mM D-GFC for 30 min. Then the hypocotyls were severed and the phloem sap was collected each hour from the upper part for volume measurements. In the treated sets, 1 M $CaCl_2$ solution was applied to the hypocotyl section just after the second sap collection (arrowhead). Each point is the mean of 10 plants±95% CI.

Ricinus communis (Williams *et al.*, 1996; Rocher *et al.*, 2009; Tamas and Davies, 2016). The effect of PCMBS on the PM H^+-ATPase varies with tissues. For instance, it does not significantly affect the proton pumping activity nor the transmembrane potential difference in mature broad bean leaf tissues (Delrot *et al.*, 1980; Bourquin *et al.*, 1990), but it inhibits proton pumping by microsomal vesicles and acidification of the incubation medium by intact cotyledons of *Ricinus communis* seedlings (Williams and Hall, 1987; Williams *et al.*, 1990).

The *Ricinus* seedling has been successfully used as a model plant to study the composition of the phloem sap (Kriedemann and Beevers, 1967; Schobert and Komor, 1989; Vreugdenhil and Koot-Gronsveld, 1989; Gerendas and Schurr, 1999; Kallarackal *et al.*, 2012), the mechanisms of nutrient uptake by cotyledon tissues and phloem loading (Komor *et al.*, 1977; Komor *et al.*, 1980; Robinson and Beevers, 1981*b*; Marvier *et al.*, 1998; Williams *et al.*, 1996), as well as the long-distance transport of sugars (Kallarackal *et al.*, 1989; Metzler *et al.*, 1994; Verscht *et al.*, 1998; Kallarackal *et al.*, 2012). As in most plant species (Liu *et al.*, 2012), sucrose (Suc) is the major sugar of the *Ricinus* phloem sap, with concentrations of approximately 300 mM in intact seedlings (Kallarackal *et al.*, 1989; Verscht *et al.*, 1998). Glucose and fructose have much lower concentrations (approximately 2 and 0.6 mM, respectively) (Kallarackal and Komor, 1989). However, the phloem of *Ricinus* seedlings exhibits the peculiarity of loading exogenous glucose. When cotyledons are dipped in solutions containing from 25 to 200 mM glucose, the same concentrations as in the incubation solutions are found in the phloem sap after 2 h of incubation (Kallarackal and Komor, 1989).

The *Ricinus* seedling has also been used to study the phloem mobility of xenobiotic conjugates, i.e. compounds that associate an agrochemical and an α-amino acid (Dufaud *et al.*, 1994; Chollet *et al.*, 1997; Delétage-Grandon *et al.*, 2001; Wu *et al.*, 2016; Xie *et al.*, 2016) or a monosaccharide (Yang *et al.*, 2011; Wu *et al.*, 2012; Yuan *et al.*, 2013) in their structure. This vectorization strategy has been developed to evaluate the ability of PM carriers to translocate large and halogenated xenobiotics (Delétage-Grandon *et al.*, 2001; Yang *et al.*, 2011; Wu *et al.*, 2012; Yuan *et al.*, 2013). We have recently synthesized two types of conjugate of fenpiclonil, a contact fungicide from the phenylpyrrole family used as a model molecule, namely, a D-glucose conjugate and an L- and a D-glutamic acid conjugate (Wu *et al.*, 2016). These compounds, which violate both Lipinski's (Lipinski *et al.*, 2012) and Veber's (Veber *et al.*, 2002) rules, were predicted to have very low membrane permeability. Nevertheless, they were found in the phloem sap. Systemicity tests using the *Ricinus* model indicated that these large chlorinated conjugates exhibited dramatic differences in their ability to move in the phloem. When cotyledons were dipped in an incubation solution buffered at pH 5.0, the concentrations of the

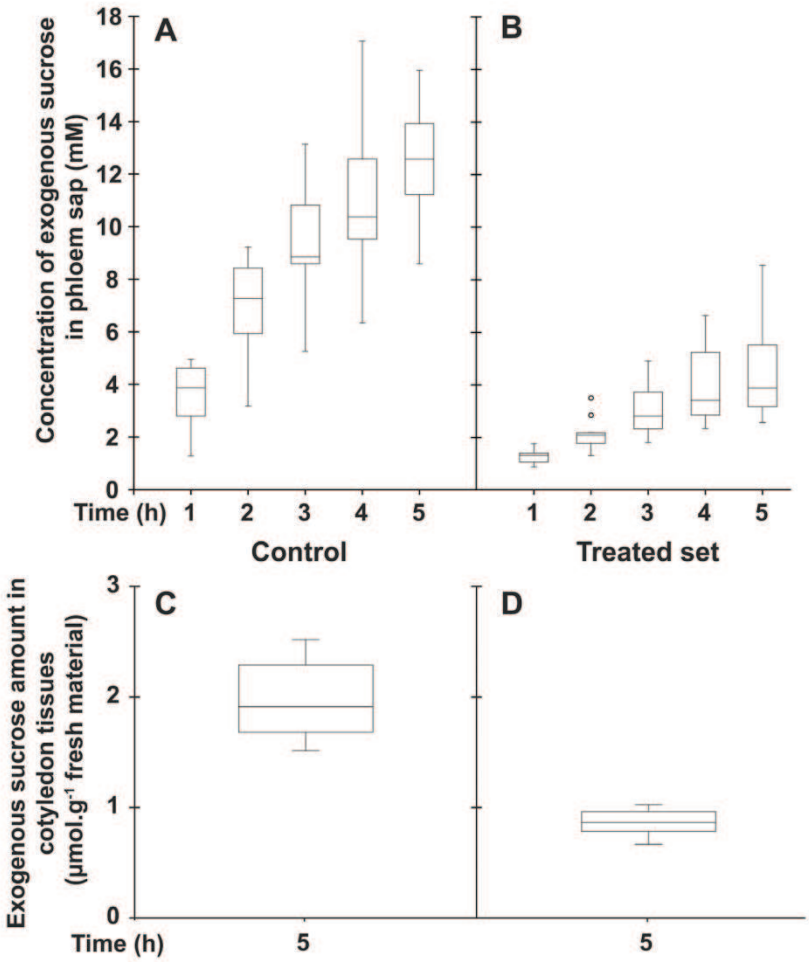

Fig. 3. Effect of D-GFC on Suc uptake in the *Ricinus* model. Cotyledons were preincubated in a standard solution buffered at pH 5.0 for 30 min and then incubated in the same solution without (control; A, C) or with (treated set; B, D) 0.5 mM D-GFC. Thirty minutes later, [^{14}C]Suc and unlabelled Suc were added to the solution to get 0.5 mM final concentration (specific activity: 0.04 mCi mmol^{-1}; 10 ml per plant). After 30 min, the hypocotyl was severed at the hook region and the sap was collected every hour for 5 h and then analysed (A, B). At the end of experiment, the amount of exogenous Suc and metabolites (in Suc equivalent) in cotyledon tissues was determined by liquid scintillation counting (C, D). The Mann–Whitney test was used to assess statistically significant differences between the two sets at the 5% probability level: (A, B) except for t=1 h where P=0.0002, all P<0.0001; (C, D) P<0.0001. For box plots, n=10.

D-glucose conjugate and the D-glutamic acid conjugate in the phloem sap were 20 and 5 times lower than that of the L-glutamic acid conjugate, respectively. The phloem systemicity of the fenpiclonil glucoside was even 30–45 times lower than that of the most recent L-amino acid–fenpiclonil conjugates synthesized (Marhadour *et al.*, 2017). Depending on their structure, natural glucosides exhibit different abilities to move in the phloem. For instance, glucosinolates are translocated long distance within the plant (Chen *et al.*, 2001; Turgeon and Wolf, 2009), and two members of the nitrate/peptide transporter family (GTR1 and GTR2) are involved in the phloem loading of these defence compounds (Nour-Eldin *et al.*, 2012). Small and hydrophilic glucosides such as salicin (M_r=286.28; log P=−1.48) and arbutin (M_r=272.25; log P=−1.14) are translocated by AtSUC2 expressed in *Xenopus laevis* oocytes (Chandran *et al.*, 2003). By contrast, the presence of phlorizin in the phloem sap has not been reported until now. This glucoside of phloretin is a non-transported competitive inhibitor of Na^+–glucose cotransporters in animal cells (Toggenburger *et al.*, 1982; Hummel *et al.*, 2011). In plants, phlorizin is recognized by hexose and Suc carrier systems but more efficiently inhibits Suc phloem loading than hexose uptake in broad bean leaves (Lemoine and Delrot, 1987). Due to the glycosyl hydroxyls (Hitz *et al.*, 1986; Delrot *et al.*, 1991), D-GFC may be recognized by Suc carriers but the size (M_r=551.38) and the amphiphilic structure (Fig. 1) of the compound are completely inappropriate for translocation considering the molecular structural requirements suitable for Suc carrier activity (Hitz *et al.*, 1986; Delrot *et al.*, 1991; Chandran *et al.*, 2003). Therefore, we hypothesized that D-GFC would affect sugar translocation systems. We use *Ricinus* as our model to test this hypothesis because it can load in the phloem not only endogenous Suc but also exogenous hexoses as mentioned above.

The purpose of this work was initially to compare the effect of D-GFC and PCMBS on Suc, 3-*O*-methylglucose

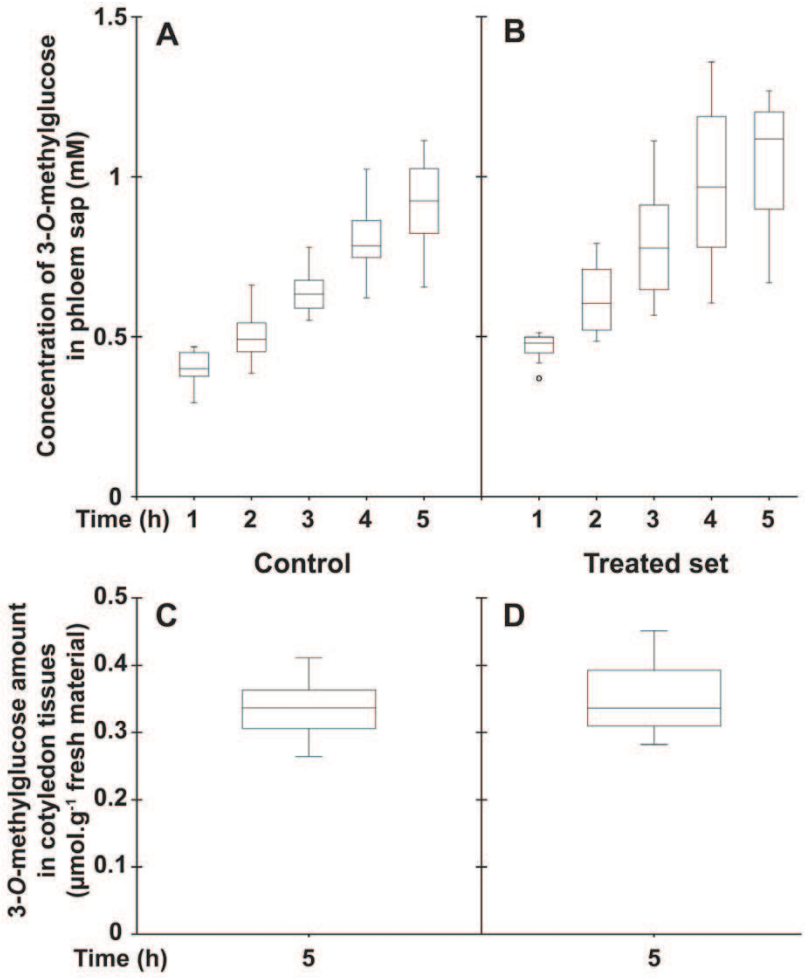

Fig. 4. Effect of D-GFC on 3-*O*-MeG uptake in the *Ricinus* model. Cotyledons were preincubated in a standard solution buffered at pH 5.0 for 30 min and then incubated in the same solution without (control; A, C) or with (treated set; B, D) 0.5 mM D-GFC. Thirty minutes later, 3-*O*-[^{3}H]MeG and unlabelled 3-*O*-MeG were added to the solution to get 0.5 mM final concentration (specific activity: 0.30 mCi mmol^{-1}; 10 ml per plant). After 30 min, the hypocotyl was severed at the hook region and then the sap was collected every hour for 5 h and then analysed (A, B). At the end of experiment, the amount of 3-*O*-MeG (and metabolites) in cotyledon tissues was determined by liquid scintillation counting (C, D). The Mann–Whitney test was used to assess statistically significant differences between the two sets at the 5% probability level. (A, B) *t*=1h, *P*=0.009; *t*=2h, *P*=0.023; *t*=3h, *P*=0.052; *t*=4h, *P*=0.143; *t*=5h, *P*=0.075. (C, D) no statistically significant difference was noted. For box plots, *n*=10.

(3-*O*-MeG) and glutamine (Gln) uptake and phloem transport in *Ricinus* seedlings. The results allowed a quantitative study of the contribution of the two pathways involved in phloem loading after endosperm removal and led us to extend the investigation to other biological models.

Materials and methods

Plant material

Castor bean seeds (*Ricinus communis* L. cv Sanguineus) were grown as previously described (Delétage-Grandon *et al.*, 2001). After 6 days of growth in vermiculite, seedlings of average size were selected for the experiments.

Broad bean (*Vicia faba* cv Aguadulce) plants were grown on vermiculite and watered daily with a nutrient solution as already described (Lemoine *et al.*, 1984). The experiments were performed on plants possessing five mature bifoliate leaves.

Saccharomyces cerevisiae strain RS453 cells were grown and transformed as described in Henry *et al.* (2011).

Chemicals

We have previously described the detailed synthesis of the D-glucose–fenpiclonil conjugate (D-GFC; Fig. 1) (Wu *et al.*, 2016). This conjugate was obtained using click chemistry, by coupling fenpiclonil, a fungicide from the phenylpyrrole family, to D-glucose via a spacer group including a 1,2,3-triazole ring.

PCMBS was purchased from Toronto Research Chemicals Inc., 3-*O*-[^{3}H]MeG (60 Ci mmol^{-1}) was purchased from Isobio and [^{3}H]Gln (50.1 Ci mmol^{-1}) and [U-^{14}C]Suc (435 mCi mmol^{-1}) were purchased from PerkinElmer SAS.

Uptake and exudation experiments with Ricinus *seedlings*

The cotyledons were preincubated in the standard solution buffed at pH 5.0 (Rocher *et al.*, 2006). Then cotyledons were incubated in the same solution without or with D-GFC at 0.5 mM concentration for 30 min (10 ml per plant). Then, a mixture of unlabelled Suc and [^{14}C]Suc, unlabelled 3-*O*-MeG and 3-*O*-[^{3}H]MeG or unlabelled Gln and [^{3}H]Gln was supplied to the solution to obtain a 0.5 mM final concentration and a specific activity of 0.04 mCi mmol^{-1}, 0.30 mCi mmol^{-1} or 0.30 mCi mmol^{-1}, respectively. Thirty minutes later, the hypocotyl was cut in the hook region for

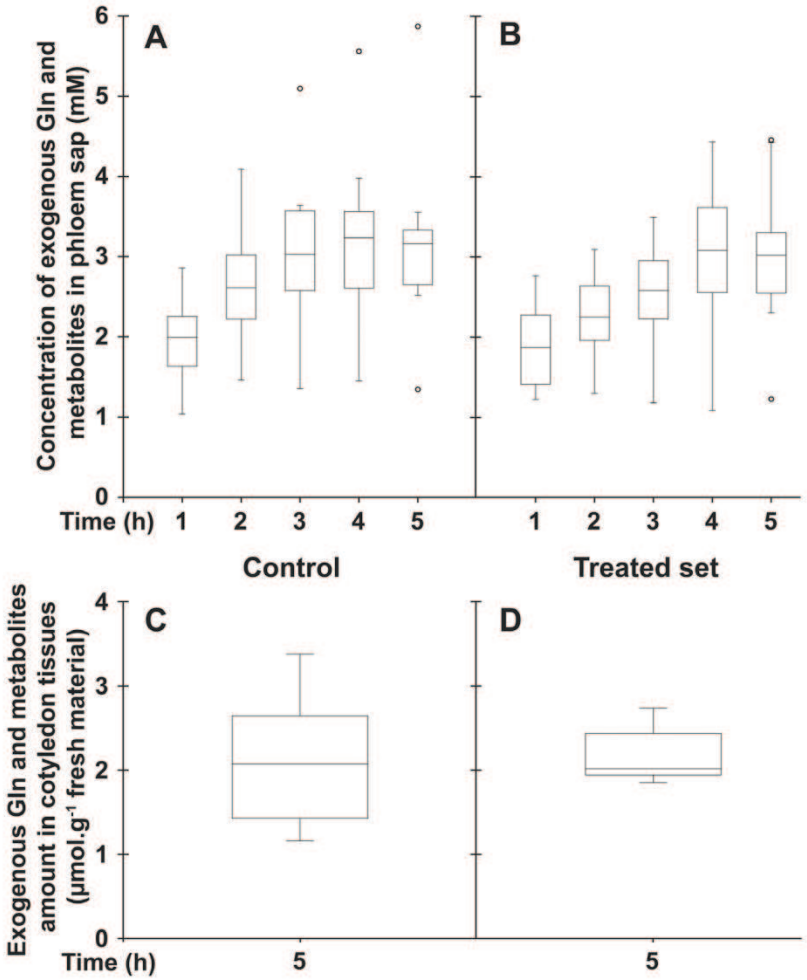

Fig. 5. Effect of D-GFC on Gln uptake in the *Ricinus* model. Cotyledons were preincubated in a standard buffered solution at pH 5.0 for 30 min and then incubated in the same solution without (control; A, C) or with (treated set; B, D) 0.5 mM D-GFC. Thirty minutes later, [^{3}H]Gln and unlabelled Gln were added to the solution to get 0.5 mM final concentration (specific activity: 0.30 mCi mmol^{-1}; 10 ml per plant). After 30 min, the hypocotyl was severed at the hook region and then the sap was collected every hour for 5 h and then analysed (A, B). At the end of experiment, the amount of exogenous Gln and metabolites (in Gln equivalent) in cotyledon tissues was determined by liquid scintillation counting (C, D). The Mann–Whitney test was used to assess statistically significant differences between the two sets at the 5% probability level: for each time (A, B) and between the two sets (C, D), no statistically significant difference was noted. For box plots, n=10.

phloem exudation of the exogenous solutes. The phloem sap was collected from the upper part of the hypocotyl for 5 h and was stored at −20 °C until analysis. Phloem sap was added to 4 ml scintillation cocktail (EcoLite, ICN Biomedicals). Radioactivity was measured with a liquid scintillation analyser (Tri-Carb 2910TR, PerkinElmer).

At the end of the exudation period, the cotyledons were rinsed with the buffer solution (3 × 2 min), wiped off with filter paper and weighed. Then, the cotyledons were digested overnight at 60 °C in a mixture of perchloric acid (65%; 0.56 ml), hydrogen peroxide (33%; 0 27 ml), and Triton X-100 (1 g l^{-1}; 0.17 ml). Radioactivity measurements were conducted on each seedling separately.

Analysis of endogenous sucrose, glucose, and fructose in Ricinus *phloem sap*

The *Ricinus* cotyledons were incubated in buffer solution (from pH 5.0 to 8.0) containing 0.25 mM $MgCl_2$ and 0.5 mM $CaCl_2$. The buffer used was 20 mM MES (pH 5.0 and 6.0) or 20 mM HEPES (pH 7.0 and 8.0) (Rocher *et al.*, 2006). The phloem sap was collected from the upper part of the *Ricinus* seedlings according to the methods already described (Kallarackal *et al.*, 1989). After removing the endosperm, the cotyledons of intact seedlings were preincubated for 30 min in the buffer solution. Then the cotyledons were incubated in the same buffer solution with or without 0.5 mM D-GFC. Thirty minutes later, the hypocotyl was cut in the hook region and the collected phloem sap was stored at −20 °C until analysis.

The endogenous sugars glucose, fructose, and Suc in the phloem sap were determined enzymatically using previously described methods (Orlich and Komor, 1992). A Suc/D-fructose/D-glucose assay kit (K-SUFRG; Megazyme, Ltd, Bray, Ireland) was used following the manufacturer's instructions. To measure the Suc concentration, the phloem sap was diluted 100-fold with pure water. All measurements were performed by using 1/4 of the amounts of the reagents recommended by the manufacturer.

pH transients in the incubation solution of Ricinus *seedlings*

The measurement of pH transients in the medium using *Ricinus* cotyledons was similar to that described previously (Komor *et al.*, 1977; Hutchings, 1978; Robinson and Beevers, 1981*b*). The cotyledon still

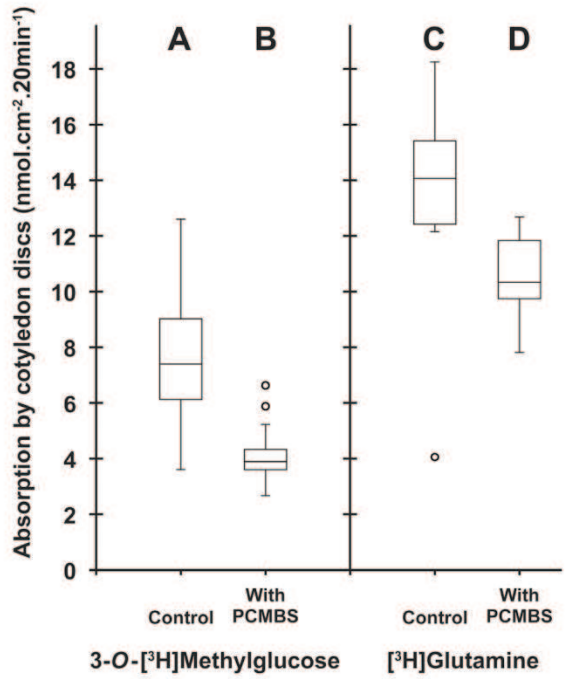

Fig. 6. Effect of PCMBS on 3-*O*-MeG uptake or on Gln uptake by *Ricinus* cotyledon discs. Cotyledons were incubated in a standard buffered solution at pH 5.0 for 10 min, then in the same solution without (controls; A, C) or with (treated sets; B, D) 0.5 mM PCMBS for 10 min and then, for both sets, in the same solution containing 3-*O*-[^{3}H]MeG and unlabelled 3-*O*-MeG (specific activity: 0.30 mCi mmol^{-1}; 20 ml per 15 discs) (A, B) or [^{3}H]Gln and unlabelled Gln (specific activity: 0.30 mCi mmol^{-1}; 20 ml per 12 discs) (C, D) at a 0.5 mM concentration for 20 min. Under these short-time experimental conditions, PCMBS did not affect the transmembrane proton gradient in intact cotyledons (Rocher *et al.*, 2009). Furthermore, it was confirmed that 0.5 mM D-GFC exhibited no statistically significant effect on Gln uptake. The Mann–Whitney test was used to assess statistically significant differences between controls and treated sets at the 5% probability level. For 3-*O*-MeG, P=0.00014; for Gln, P=0.0014. For box plots, n=15 (3-*O*-MeG) or n=12 (Gln).

attached to the seedling was incubated in a solution (10 ml) containing 0.25 mM $MgCl_2$ and 0.5 mM $CaCl_2$. The solution was stirred continuously and the pH of the solution was monitored every 30 s with a microelectrode and a pH meter (S220 SevenCompact pH/Ion Meter, Mettler Toledo). The pH of the solution bathing the cotyledons decreased to pH 4.6–4.9 after 30 min then stabilized between 4.8 and 5.0. Small aliquots of concentrated solutions of D-GFC or PCMBS (1 mM final concentration) were added at 30 min and small aliquots of concentrated solutions of Suc (20 mM final concentration) were added at 60 min. The pH was monitored continuously over 3 h.

Uptake experiments with leaf discs of broad bean

The leaf discs of broad bean were prepared as previously described (Lemoine and Delrot, 1987). After stripping off the lower epidermis, leaf discs (1.13 cm^2) were preincubated for 30 min in a buffer solution containing 0.25 mM $MgCl_2$, 0.5 mM $CaCl_2$, 250 mM mannitol, and 20 mM MES (pH 5.0). The discs were then incubated in the same buffer solution with or without 0.5 mM inhibitor (D-GFC or PCMBS) in the presence of a mixture of unlabelled and ^{14}C-labelled Suc (final concentration: 0.5 mM; specific activity: 0.20 mCi mmol^{-1}) or a mixture of unlabelled and ^{3}H-labelled 3-*O*-MeG (final concentration: 0.5 mM; specific activity: 0.30 mCi mmol^{-1}) for 30 min (20 ml per 15 discs). At the end of incubation the discs were rinsed in a solution similar to the preincubation medium (3 × 2 min). Each disc was then digested overnight at 60 °C in a mixture of perchloric acid (65%; 112 µl), hydrogen peroxide (33%; 54 µl), and Triton X-100 (1 g l^{-1}; 34 µl). Radioactivity was measured by the liquid scintillation analyser mentioned above. The measurements were made on each disc separately.

Uptake experiments in yeast

The *AtSUC2* coding region in the plasmid pDONR207 coding region was a generous gift from Dr F. Vilaine (Insitut Jean Pierre Bourgin, Versailles, France). The coding region was cloned by recombination into plasmid pDR-R1-R2-HIS3 (Cagnac *et al.*, 2007) derived from pDR 192. The plasmid containing *AtSUC2* and the empty plasmid were inserted into *Saccharomyces cerevisiae* RS453 and Suc uptake experiments were run as described in Henry *et al.* (2011). Briefly, yeast cells were grown to early logarithmic phase in YNB medium supplemented with 2% glucose. Cells were washed and resuspended with 50 mM MES buffer (pH 4.5) to reach a final OD_{600nm} value of 0.5. Aliquots (100 µl) of cell suspension were added to 100 µl of a solution containing 50 mM MES (pH 4.5) and a mixture of unlabelled and ^{14}C-labelled Suc (concentration: 1 mM; specific activity: 0.50 mCi mmol^{-1}) at 28 °C for 5 min. The final sucrose concentration in the medium was therefore 0.5 mM.

The reactions were stopped by adding 8 ml of cold water and immediate filtration on glass microfibre filters (25 mm, Fisher Bioblock, Illkirch, France). This step was repeated once. Radioactivity incorporated into cells collected on filters was evaluated using a liquid scintillation counter.

Results and discussion

Effect of the D-glucose–fenpiclonil conjugate on the uptake and phloem transport of exogenous Suc, 3-O-MeG, and Gln in Ricinus *seedlings in comparison with PCMBS*

The two nutrients and the sugar analogue were selected because of their high concentrations in the phloem sap and/or their poor metabolism. Suc is slowly metabolized in *Ricinus* cotyledon tissues (Kriedemann and Beevers, 1967; Komor *et al.*, 1977) and represents 98–99% of the total sugar in the phloem sap (Kallarackal and Komor, 1989). The mobile analogue 3-*O*-MeG is slowly phosphorylated in plant tissues (Cortès *et al.*, 2003). Chromatography analyses suggest the absence of metabolic transformation of 3-*O*-MeG in the *Ricinus* phloem exudate under short-time experiments (2 h) (Kallarackal and Komor, 1989). Gln is the major amino acid found in the *Ricinus* phloem sap, comprising 30–40% of the total amino acids (Robinson and Beevers, 1981*a*; Schobert and Komor, 1989) and dramatically accumulates in the phloem sap from the apoplast between the endosperm and the cotyledons (endogenous Gln) or from an incubation solution (exogenous Gln) (Schobert and Komor, 1989). The cotyledon tissues acidified the non-buffered solution to pH values of 4.8–5.0 as mentioned below. Therefore the experiments were conducted using incubation solutions buffered at pH 5.0.

Contrary to species of the Cucurbitaceae (Zhang *et al.*, 2012; Zimmermann *et al.*, 2013), after *Ricinus* hypocotyls were cut under our experimental conditions, an immediate and strong efflux of a mixture of phloem and xylem sap did not occur from the apical side of the cut. The concentration of Suc, which was approximately 300 mM in intact seedlings,

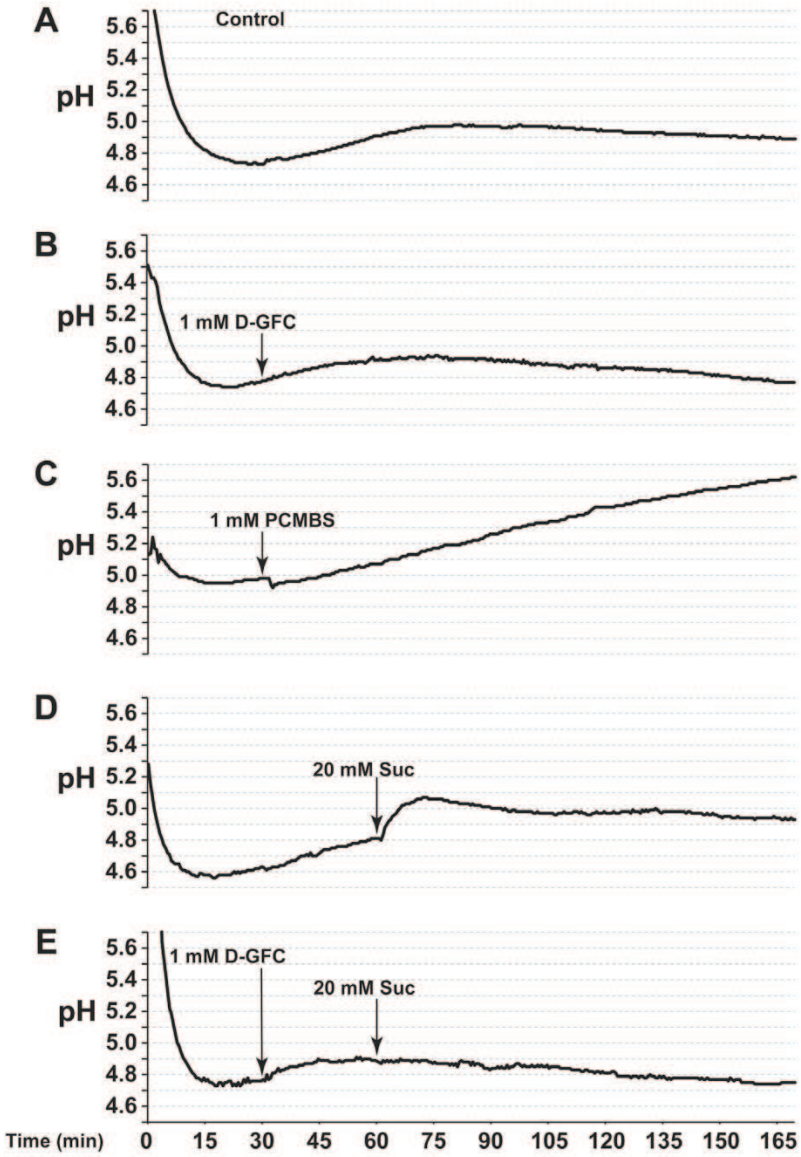

Fig. 7. pH time course variation of the *Ricinus* incubation medium after different treatments. The cotyledons attached to the seedlings were incubated in a solution (10 ml) containing 0.25 mM $MgCl_2$ and 0.5 mM $CaCl_2$ at 27 ± 1 °C. The pH of the solution bathing the cotyledons decreased to values of 4.6–4.9 after 30 min and then stabilized between 4.8 and 5.0. Small aliquots of concentrated solutions of D-GFC, PCMBS or Suc at the same pH were added after reaching a steady state. (A) Control; (B) D-GFC (1 mM final concentration) was added at time 30 min; (C) PCMBS (1 mM final concentration) was added at time 30 min; (D) Suc (20 mM final concentration) was added at 60 min; (E) D-GFC (1 mM final concentration) was added at time 30 min then Suc (20 mM final concentration) was added at 60 min. Each experiment was repeated three times with similar results.

decreased to approximately 100 mM 2 h after endosperm removal (Kallarackal and Komor, 1989) and to approximately 160 mM in our plant material. The concentrations of glucose and fructose were very low, approximately 2 and 0.7 mM, respectively. The concentrations of these three sugars remained stable for at least 5 h as detailed below. The pH of the sap remained almost unchanged (7.9–7.6) (Fig. 2A). After addition of 1 M $CaCl_2$, which induced a biphasic occlusion of sieve tubes (Furch *et al.*, 2010), no fluid in measurable quantities was released from the apical cut until the end of the experiment. The result was the same in the presence of D-GFC (Fig. 2B). All the data suggest that the phloem sap is very poorly contaminated by the apoplasmic fluid despite long incubation times in solution and indicate that phloem sap could be collected for at least 5 h after the first hour of preincubation, i.e. 6 h after endosperm removal.

Considering that a relatively small amino acid conjugate (L-Lys–2,4D; M_r=349.21) at 2.5 mM concentration was, in acidic conditions, an efficient inhibitor of uptake and phloem loading of various amino acids at a 1 mM concentration (Chollet *et al.*, 1997), we speculated that the large amphiphilic conjugate D-GFC could be used at low concentrations to affect the active uptake mediated by sugar carriers under their protonated form. Therefore, to compare the effects of D-GFC on uptake and phloem transport of the two sugars and the amino acid, the conjugate was used at 0.5 or 1 mM and exogenous Suc, 3-*O*-MeG, and Gln were used at 0.5 mM concentration in the incubation medium. Under

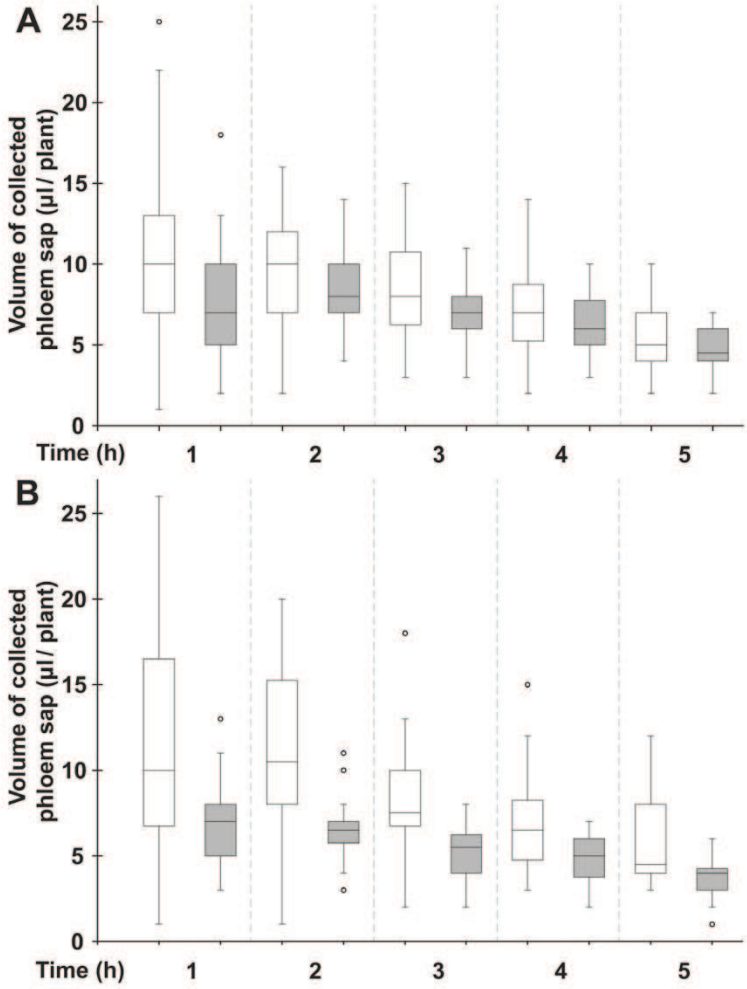

Fig. 8. Effect of D-GFC on the volume of phloem sap collected. (A) Effect of 0.5 mM D-GFC on the volume of phloem sap collected during the experiments already described in Figs 3, 4 and 5. (B) Effect of 1 mM D-GFC on the volume of phloem sap collected. Controls: white boxes; treated sets: grey boxes. The Mann–Whitney test was used to assess statistically significant differences between the control and treated set at the 5% probability level: (A) Except for t=1 h where P=0.04, there were no significant differences between the control and treated set for all times; (B) P<0.05 for all times. For box plots, n=30 (A) or n=20 (B).

these experimental conditions, D-GFC acted as a potent inhibitor of Suc uptake, especially Suc phloem transport. In a control set, exogenous Suc concentration in the phloem sap increased over time to reach a concentration factor (CF; concentration in the phloem sap/concentration in the incubation solution) of approximately 25 at the end of the experiment (Fig. 3A). In the treated set, the concentration of exogenous Suc in sieve tubes decreased by approximately 70% regardless of the time of sap collection (Fig. 3B). The amount of exogenous Suc and metabolites (in Suc equivalent) in cotyledon tissues collected at the end of the experiment (5 h) was reduced by 55% in the treated set (Fig. 3C, D). A slightly higher inhibition (65%; 0.96 and 0.34 µmol exogenous Suc g^{-1} fresh weight of *Ricinus* cotyledons in control and treated sets, respectively) occurred in a short-time experiment (1 h). As discussed below, Suc uptake by *Ricinus* cotyledons is also reduced by PCMBS (Orlich *et al.*, 1998). By contrast, D-GFC did not negatively affect the concentration of 3-*O*-MeG in the phloem sap, regardless of the time of phloem collection (Fig. 4A, B). This concentration increased over time to reach a CF of approximately 2 at the end of the experiment in both sets and was weakly but nevertheless significantly higher (Mann–Whitney test) in the treated set than in the control at times 1, 2, and 3 h. The amounts of 3-*O*-MeG and metabolites in cotyledon tissues were the same in both sets at 5 h (Fig. 4C, D). Similarly, the conjugate did not induce changes in the time course of accumulation of exogenous Gln and metabolites (in Gln equivalent) in the phloem sap. In both sets, their concentration plateaued during the last 2–3 h of the experiments to reach a CF of approximately 6 (Fig. 5A, B). In addition, the amounts of exogenous Gln and metabolites in cotyledon tissues were the same in control and treated sets (Fig. 5C, D). By contrast, 0.5 mM PCMBS reduced the uptake of 3-*O*-MeG in cotyledon tissues by 47% under short-time experiments (Fig. 6A, B). These experimental conditions were necessary because this thiol reagent progressively affects transmembrane proton gradients in *Ricinus* as mentioned below. At the same concentration, PCMBS moderately

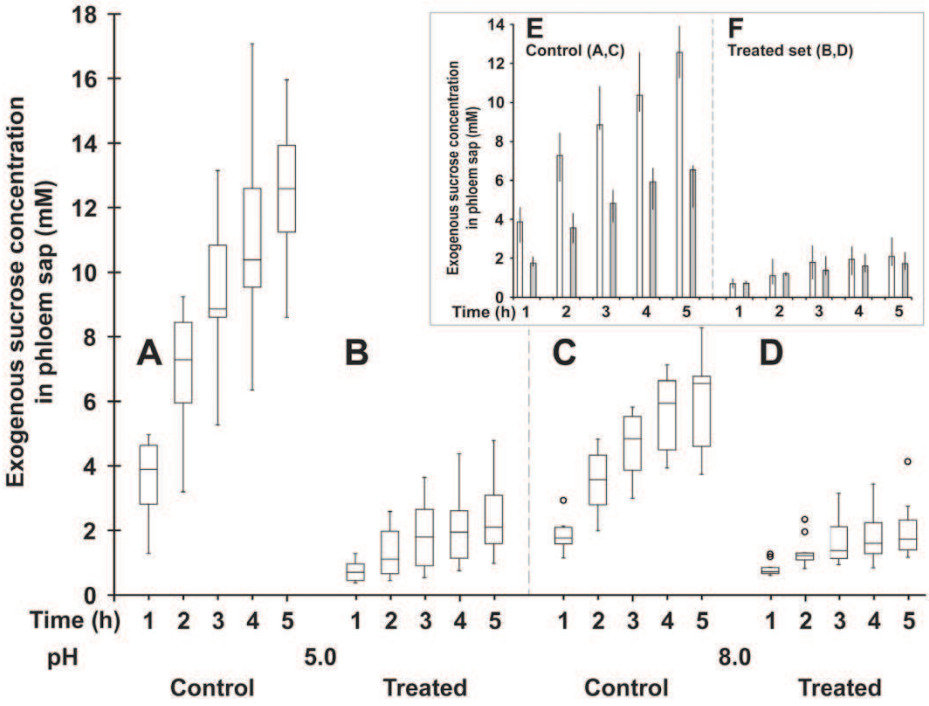

Fig. 9. Time course of exogenous Suc and metabolites (in Suc equivalent) concentration in phloem sap of *Ricinus* in the absence (A, C) or presence (B, D) of 1 mM D-GFC. Cotyledons were preincubated in a standard solution buffered at pH 5.0 (A, B) or pH 8.0 (C, D) for 30 min and then incubated in the same solution containing 0 mM (A, C) or 1 mM (B, D) D-GFC. Thirty minutes later, [^{14}C]Suc and unlabelled Suc were added to the solution to get 0.5 mM final concentration (specific activity: 0.04 mCi mmol^{-1}; 10 ml per plant). After 30 min, the hypocotyl was severed at the hook region and then the sap was collected every hour for 5 h. At pH 5.0, the experiments with D-GFC at 0.5 (Fig. 3) and 1 mM concentration (this figure) were conducted together. Therefore, the control was the same for both figures. For a given pH and for each time point, the Mann–Whitney test was used to assess statistically significant differences between the control and the treated sets (A *vs* B and C *vs* D). In all cases, $P<0.001$. For box plots, $n=10$. Taking into consideration separately control sets (A, C) or treated sets (B, D), the same test was used to assess statistically significant differences for each time between pH 5.0 and pH 8.0 (see inset (E, F); median±interquartile range; pH 5.0 white columns, pH 8.0 grey columns). For control sets (E), $P<0.005$ for all times; for treated sets (F), there were no statistically significant differences between pH 5.0 and 8.0 for all times.

Table 1. *Effect of 1 mM D-GFC on 0.5 mM Suc uptake (µmol g^{-1} fresh material) by* Ricinus *cotyledon tissues at pH values of 5.0 and 8.0*

Cotyledons were preincubated in a standard solution buffered at pH 5.0 or pH 8.0 for 30 min and then incubated in the same solution containing 0 mM (Control) or 1 mM (Treated set) D-GFC (10 ml per plant). Thirty minutes later, [^{14}C]Suc and unlabelled Suc were added to the solution to get 0.5 mM final concentration (specific activity: 0.04 mCi mmol^{-1}). After 30 min, the hypocotyl was severed at the hook region and then the sap was collected every hour for 5 h (see Fig. 9). At the end of experiment, the amount of exogenous Suc and metabolites (in Suc equivalent) in cotyledon tissues was determined by liquid scintillation counting. The data are means±95% CI, $n=10$.

pH 5.0			pH 8.0		
Control	**Treated set**	**Inhibition (%)**	**Control**	**Treated set**	**Inhibition (%)**
1.98 ± 0.23	0.59 ± 0.08	−70	0.97 ± 0.10	0.79 ± 0.05	−19

affected (approximately 25% inhibition) Gln uptake by tissues (Fig. 6C, D) but the inhibition was higher (≥50%) using plasma membrane vesicles from *Ricinus* cotyledons (Williams *et al.*, 1992).

Considering these results, complementary experiments were performed to get additional data on D-GFC specificity and some insights on concentration and pH dependence, as well as post-treatment duration of Suc transport inhibition induced by the chemical. Although 0.5 mM D-GFC led to a dramatic inhibition of Suc phloem loading in *Ricinus*, the chemical was used at 1 mM in the following experiments. Under this experimental condition, D-GFC did not affect the time course of incubation medium acidification by cotyledon tissues (Fig. 7A, B) contrary to PCMBS (Fig. 7C). Therefore this result provided evidence that D-GFC did not alter the activity of the plasma membrane H$^+$-ATPase via toxic effects on some cell functions, even for long-term experiments (several hours). In addition, 1 mM PCMBS quickly reduced the volume of phloem sap by 75% (Orlich *et al.*, 1998; Fig. 4B), while D-GFC had a less dramatic effect (from 32 to 42% inhibition) at the same concentration and a weak effect at 0.5 mM (Fig. 8A, B).

Increasing conjugate concentration from 0.5 to 1 mM in the incubation medium buffered at pH 5.0 led to an

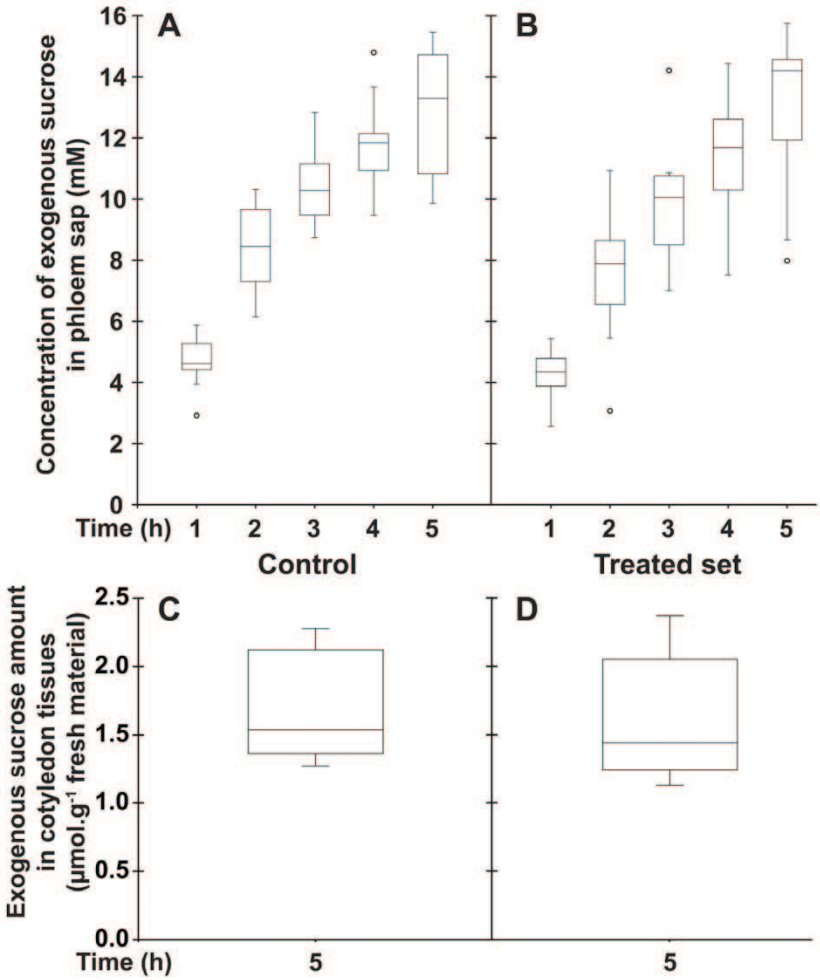

Fig. 10. Reversibility of the inhibitory effect of D-GFC on Suc uptake in the *Ricinus* model. Cotyledons were incubated in a standard buffered solution at pH 5.0 for 30 min and then in the same solution without (control; A, C) or with (treated set; B, D) 0.5 mM D-GFC for 60 min. Cotyledons were then washed 3 × 2 min with the standard buffer solution and were incubated (control and treated set) in the standard solution containing 0.5 mM [^{14}C]Suc and unlabelled Suc (final concentration, specific activity: 0.04 mCi mmol^{-1}; 10 ml per plant). After 30 min, the hypocotyl was severed at the hook region and the sap was collected every hour for 5 h and then analysed (A, B). At the end of experiment, the amount of [^{14}C]Suc (and labelled metabolites) in cotyledon tissues was determined by liquid scintillation counting (C, D). The Mann–Whitney test was used to assess statistically significant differences between the two sets at the 5% probability level. (A, B) For each time, no significant differences were noted; (C, D) no significant difference was noted. For box plots, *n*=10.

accentuated decrease of exogenous Suc concentration in the phloem sap (80–85% *vs* 70%) regardless of the time of sap collection (compare Fig. 3A, B and Fig. 9A, B). The same was observed for the amounts of exogenous Suc and metabolites in cotyledon tissues collected at the end of experiments (approximately 70% *vs* 55%) (compare Fig. 3C, D and Table 1). The uptake and phloem transport of exogenous Suc were either markedly dependent (control sets) or independent (treated sets) of the incubation medium pH. In control sets, the concentration of exogenous Suc in the sap and its amount in cotyledon tissues at 5 h were both reduced by approximately 50% from pH 5.0 to 8.0 (Fig. 9A, C, E; Table 1). By contrast, in the treated set, the low exogenous Suc contents in these two compartments were unaffected (Fig. 9B, D, F) or were weakly affected (Table 1) by the pH change, suggesting that 1 mM D-GFC reduced to nothing, or almost nothing, the pH-dependent component of the active Suc transport, i.e. the symport H^+–Suc. This well-known mechanism described several decades ago (Komor, 1977; Komor *et al.*, 1977; Hutchings, 1978; Malek and Baker, 1978; Delrot and Bonnemain, 1979, 1981) was notably supported by an alkalinization of the incubation medium due to concomitant influxes of protons and Suc in cotyledon tissues. At 1 mM, D-GFC completely abolished the transient alkalinization induced by these influxes after the addition of 20 mM Suc (final concentration) in the incubation solution (Fig. 7D, E). The inhibition of the active component of Suc transport was thoroughly removed after a short washing (3 × 2 min) of cotyledon tissues in the standard medium. Besides previous incubation of tissues for 1 h in a solution containing 1 mM conjugate (treated set) before washing, the time course enrichment of exogenous Suc in the phloem sap (Fig. 10A, B) and Suc uptake by cotyledon tissues (Fig. 10C, D) were similar in the control and treated sets.

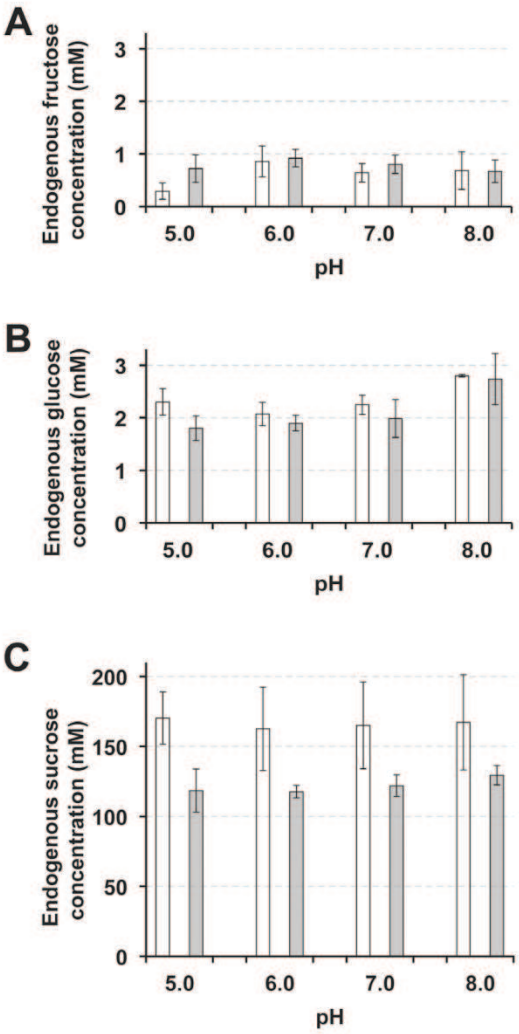

Fig. 11. Fru (A), Glc (B), and Suc (C) concentrations in the phloem sap of *Ricinus* at different pH values and in the absence (white columns) or presence (grey columns) of D-GFC. Cotyledons were preincubated in a standard buffered solution at pH 5.0, 6.0, 7.0, or 8.0 for 30 min and then incubated in the same solution containing 0.5 mM D-GFC. After 30 min, the hypocotyl was severed at the hook region and then the sap was collected during the fifth hour after the removal of endosperm from cotyledons and then analysed. Mean of three sets of seven plants each ±95% CI.

While PCMBS forms covalent bonds with protein cysteine residues, D-GFC acts as a reversible inhibitor like the natural glucoside phlorizin (Bush, 1993). The thiol reagent and D-GFC exhibit similarities and differences. The most astonishing similarity is that the conjugate (described here) and PCMBS (Orlich *et al.*, 1998) inhibit Suc transport in *Ricinus* by 80–90% at the same concentration (1 mM). The most striking difference is the specificity of D-GFC, which does not affect Gln or 3-*O*-MeG transport in cotyledon tissues, contrary to PCMBS. In addition, the xenobiotic glucoside does not change the time course acidification of the incubation medium by cotyledon tissues while the sulfhydryl reagent alters this process (Williams and Hall, 1987; Williams *et al.*, 1990; our data). It has been well known for decades, first by using plant tissues and then plasma membrane vesicles or complemented yeast mutants, that PCMBS inhibits not only Suc carriers (Delrot *et al.*, 1980; Bush, 1993; Lemoine, 2000; Barth *et al.*, 2003; Sauer, 2007) and oligopeptide carriers (Jamai *et al.*, 1994; Rentsch *et al.*, 1995), but also amino acid transporter systems to various degrees (from 5 to 75%) (Servaites *et al.*, 1979; Despeghel and Delrot, 1983; M'Batchi and Delrot, 1984; Montamat *et al.*, 1999). In that respect, some data emerged from *Ricinus* studies on the effect of PCMBS on nutrient uptake and the activity of the PM H^+-ATPase, especially during the 1980s and 1990s (Lorenc-Plucinska and Ziegler, 1987; Williams *et al.*, 1992, 1996; Weig and Komor, 1996; Marvier *et al.*, 1998). The effect of PCMBS on hexose transport is variable (from 0 to 70% inhibition) according to the plant material studied (Maynard and Lucas, 1982; Felker and Goodwin, 1988; Renault *et al.*, 1992). It can also react with many PM intrinsic proteins involved in the phloem transport of acidic organic compounds, such as salicylic acid (Rocher *et al.*, 2009) and auxin (Tamas and Davies, 2016), as well as mineral nutrient uptake, notably K^+ (Wilkinson and Ohki, 1991; Smart *et al.*, 1996). Therefore, it is not surprising that PCMBS reduces phloem sap exudation in *Ricinus* much more (Orlich *et al.*, 1998) than D-GFC (Fig. 8).

Effect of D-glucose–fenpiclonil conjugate on phloem loading of endogenous sugars in Ricinus *seedlings*

Because of its high specificity, at least in the heterotrophic tissues studied (Figs 3, 4, 5 and 7), D-GFC should be a suitable tool for long-term studies (at least several hours) on endogenous sugar transport and compartmentation. Therefore, we used it to investigate the pattern of sugar phloem loading during the fifth hour after the removal of endosperm from cotyledons, i.e. when Suc in the phloem sap derives uniquely from starch breakdown (Kallarackal *et al.*, 1989; Orlich *et al.*, 1998). In control sets, the concentrations of fructose (which exhibited large variations due to detection limits) and glucose in the sap (Fig. 11A, B) were similar to those previously reported (Kallarackal and Komor, 1989). Glucose amounts did not change markedly in response to the different pH values of the incubation medium (from pH 5.0 to 8.0). Similarly, the Suc concentration (whose values oscillated around 160 mM from the third to the sixth hour after endosperm removal in our plant material) remained stable regardless of the pH values of the incubation medium (Fig. 11C). The conjugate at 0.5 mM did not affect total hexose concentration from pH 5.0 to pH 8.0. In contrast, it reduced the concentration of Suc in the phloem sap from 30.4% (pH 5.0) to 22.6% (pH 8.0) (Fig. 11C). The data can be analysed in relation to the pattern of solute phloem loading in *Ricinus*.

Three different pathways have been considered for Suc transport from the endosperm (or incubation solution) to the companion cell–sieve element complex: a direct apoplasmic route, a symplasmic route and an indirect apoplasmic route (Orlich and Komor, 1992; Orlich *et al.*, 1998). This phloem loading pattern has been supported by structural, physiological, and molecular data. On the one hand, the high expression of the plasma membrane H^+-ATPase (Williams and Gregory, 2004)

Table 2. *Effect of D-GFC, PCMBS and phlorizin on the uptake of Suc and 3-O-MeG by broad bean leaf discs*

In the present study, leaf discs were preincubated in a standard buffer solution (pH 5.0) for 30 min. After preincubation, the discs were transferred to the same medium solution without (control) or with 0.5 mM D-GFC or 0.5 mM PCMBS in the presence of 0.5 mM labelled Suc (specific activity: 0.20 mCi $mmol^{-1}$; 20 ml per 15 discs) or 3-*O*-MeG (specific activity: 0.30 mCi $mmol^{-1}$; 20 ml per 12 discs) for 30 min. Radioactivity measurements were made on each disc separately. The medians were used to calculate the inhibition percentages relative to control discs.

Inhibitor	Substrate	Inhibition (%)	Reference
0.5 mM D-GFC	0.5 mM Suc	85.0	Present study
(pH 5.0)	0.5 mM 3-*O*-MeG	17.2	Present study
0.5 mM PCMBS	0.5 mM Suc	71.4	Present study
(pH 5.0)	0.5 mM 3-*O*-MeG	19.1	Present study
0.5 mM PCMBS	1 mM Suc	73.7	M'Batchi and Delrot (1984)
(pH 6.0)	1 mM 3-*O*-MeG	23.6	M'Batchi and Delrot (1984)
5 mM phlorizin	1 mM Suc	53	Lemoine and Delrot (1987)
(pH 5.0)	1 mM 3-*O*-MeG	23	Lemoine and Delrot (1987)

Table 3. *Effect of D-GFC on the uptake of Suc into transgenic* Saccharomyces cerevisiae *cells*

Uptake of Suc into *Saccharomyces cerevisiae* cells transformed with AtSUC2 or an empty vector in the presence of 0 mM (control), 0.25 mM, 0.5 mM, and 1mM D-GFC for 5 min. The [^{14}C]Suc concentration was 0.5 mM in all experiments at pH 4.5 (specific activity: 0.50 mCi $mmol^{-1}$). Data were expressed as the mean±95% CI (n=4). The experiment was repeated twice with similar results using 0.5 mM D-GFC

D-GFC concentration	Suc uptake (nmol min^{-1} mg $cells^{-1}$)			Inibition[b] (%)
	Empty vector	*AtSUC2*	Active uptake[a]	
Control (0 mM)	0.19 ± 0.08	0.91 ± 0.03	0.71	–
0.25 mM	0.17 ± 0.03	0.55 ± 0.05	0.37	47.9%
0.5 mM	0.21 ± 0.09	0.34 ± 0.04	0.13	81.5%
1 mM	0.13 ± 0.02	0.28 ± 0.01	0.15	79.1%

[a] The active uptake of Suc was calculated from the difference between the two *S. cerevisiae* cells (empty vector and *AtSUC2*).
[b] The D-GFC-induced inhibition was expressed as the percentage of active uptake/control uptake.

and a Suc carrier (Bick *et al.*, 1998) in the phloem, and the relative paucity of plasmodesmata between mesophyll and bundle sheath (Orlich *et al.*, 1998) are in agreement with a direct apoplasmic component of phloem loading of Suc exported from the endosperm. On the other hand, the lower epidermis cells are modified into transfer cells (Bick *et al.*, 1998) that possess (i) the proton pumping machinery and the Suc carrier equipment necessary for efficient sugar uptake from the endosperm (Bick *et al.*, 1998; Williams and Gregory, 2004) and (ii) high symplasmic connections with the mesophyll allowing cell to cell transport via plasmodesmata, at least to the proximity of the bundle sheath. In addition, a transient phloem loading of Suc occurs in the presence of PCMBS (Orlich *et al.*, 1998). These data support the involvement of a symplasmic component. Finally, time course analyses of labelled and non-labelled Suc in three compartments (mesophyll symplasm, cell-wall space and phloem exudate) indicate that an indirect apoplastic phloem loading occurs (Orlich and Komor, 1992).

Under our experimental conditions, the direct apoplasmic phloem loading and the primary symplasmic route from epidermal cells cannot operate because of the removal of the endosperm for several hours (Orlich and Komor, 1992). The involvement of a supplementary symplasmic route from storage compartments is supported by the lack of effect that pH values (from 5.0 to 8.0) of the incubation medium have on the endogenous Suc concentration in the phloem sap. In addition, a contribution of the indirect apoplasmic route is demonstrated by the slightly pH-dependent inhibition of endogenous Suc loading by the conjugate (Fig. 11C). Considering that the inhibition of Suc phloem loading by D-GFC is not optimal at 0.5 mM (compare Figs 3B and 9B), the contributions of the indirect apoplasmic route and the symplasmic route should constitute approximately one-third and two-thirds of phloem loading, respectively, under our experimental conditions (pH 5.0, fifth hour after the removal of endosperm). Therefore our data (i) support previous work (Orlich and Komor, 1992; Orlich *et al.*, 1998) and (ii) allow a quantitative approach to the relative contribution of these two latter ways.

Effect of D-glucose–fenpiclonil conjugate on Suc transport in two other biological models

Experiments were conducted on *Vicia faba* leaf tissues and *Saccharomyces cerevisiae* cells transformed with *AtSUC2*, which encodes the sucrose transporter involved in sucrose

phloem loading in Arabidopsis (Stadler and Sauer, 1996). *Vicia faba* is typically an apoplasmic phloem loader. The maturation of leaves from importing to exporting stages is characterized by a marked reduction of symplasmic connexions between the phloem and the mesophyll and, within the phloem itself, by an additional symplasmic isolation of the companion cell–sieve element complex (Bourquin *et al.*, 1990). Furthermore, in mature leaves, the companion cells are modified into transfer cells exhibiting a high expression and polarized addressing of the PM H^+-ATPase (Bouché-Pillon *et al.*, 1994). The effects of the conjugate on Suc and 3-*O*-MeG uptake by leaf discs were compared with data previously published concerning PCMBS (M'Batchi and Delrot, 1984) and phlorizin (Lemoine and Delrot, 1987) (Table 2). The dramatic inhibition of Suc uptake induced by 0.5 mM D-GFC in *Ricinus* was observed again using Fabaceae leaf discs. By comparison, 5 mM phlorizin is a relatively poor inhibitor. The effect of D-GFC in Fabaceae was not as specific as in *Ricinus* because of its slight effect on 3-*O*-MeG uptake. Nevertheless, considering the Suc/3-*O*-MeG inhibition ratio, its specificity is better than that of PCMBS and clearly of phlorizin (Table 2).

Transgenic *Saccharomyces cerevisiae* cells transformed with *AtSUC2* constituted an elegant model to study the effect of D-GFC on Suc uptake by the H^+–Suc symporter involved in Suc loading in Arabidopsis (Stadler and Sauer, 1996; Gottwald *et al.*, 2000). At a concentration as low as 0.25 mM, the active component of the Suc uptake was inhibited by approximately 50% in 5 min. The maximum inhibition plateaued (approximately 80%) at 0.5 mM (Table 3).

These data indicate that the potent inhibitory effect of D-GFC on sucrose carriers is not limited to cotyledons of seedlings with endosperm, i.e. heterotrophic tissues that function as the small intestinal wall (Robinson and Beevers, 1981*a*). The effect of D-GFC is similar to that of PCMBS in apoplasmic loaders and much higher than the natural glucoside phlorizin (Lemoine and Delrot, 1987; Bush, 1993).

Conclusion

Using different biological models (heterotrophic cotyledon tissues, mature exporting leaves, and transgenic *Saccharomyces cerevisiae* cells) from different plant families (Euphorbiaceae, Fabaceae, and *AtSUC2* from Brassicaceae), our data show that D-GFC is an inhibitor of Suc carriers as potent as PCMBS in acidic conditions. This xenobiotic glucoside reversibly blocks the H^+–Suc symporters involved in Suc exchanges at strategic sites of the plant, namely at the triploid endosperm–cotyledon tissues interface and phloem loading in cotyledons, as well as phloem loading in mature leaves of apoplasmic loaders. While PCMBS forms covalent bonds with sulfhydryl groups of many PM intrinsic proteins and therefore affects uptake and phloem transport of many solutes in addition to Suc, D-GFC can exhibit much more selectivity, especially in *Ricinus* seedlings and possibly in other seedlings with endosperm. Unlike PCMBS, this new tool in phloem biology allows long-term phloem exudation and therefore investigation, with quantitative analysis, of the pathways involved in phloem loading of endogenous Suc in *Ricinus*, as evidenced in the present work. The use of D-GFC can be extended to the study of sugar exchange between vascular tissue apoplasm and symplasm in response to abiotic stresses.

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References

Barth I, Meyer S, Sauer N. 2003. PmSUC3: characterization of a SUT2/SUC3-type sucrose transporter from *Plantago major*. The Plant Cell **15,** 1375–1385.

Bick JA, Neelam A, Smith E, Nelson SJ, Hall JL, Williams LE. 1998. Expression analysis of a sucrose carrier in the germinating seedling of *Ricinus communis*. Plant Molecular Biology **38,** 425–435.

Bouché-Pillon S, Fleurat-Lessard P, Fromont JC, Serrano R, Bonnemain JL. 1994. Immunolocalization of the plasma membrane H^+-ATPase in minor veins of *Vicia faba* in relation to phloem loading. Plant Physiology **105,** 691–697.

Bourquin S, Bonnemain JL, Delrot S. 1990. Inhibition of loading of ^{14}C assimilates by *p*-chloromercuribenzenesulfonic acid: localization of the apoplastic pathway in *Vicia faba*. Plant Physiology **92,** 97–102.

Bush DR. 1993. Inhibitors of the proton-sucrose symport. Archives of Biochemistry and Biophysics **307,** 355–360.

Cagnac O, Leterrier M, Yeager M, Blumwald E. 2007. Identification and characterization of Vnx1p, a novel type of vacuolar monovalent cation/H^+ antiporter of *Saccharomyces cerevisiae*. The Journal of Biological Chemistry **282,** 24284–24293.

Chandran D, Reinders A, Ward JM. 2003. Substrate specificity of the *Arabidopsis thaliana* sucrose transporter AtSUC2. The Journal of Biological Chemistry **278,** 44320–44325.

Chen L, Ortiz-Lopez A, Jung A, Bush DR. 2001. ANT1, an aromatic and neutral amino acid transporter in Arabidopsis. Plant Physiology **125,** 1813–1820.

Chollet JF, Delétage C, Faucher M, Miginiac L, Bonnemain JL. 1997. Synthesis and structure-activity relationships of some pesticides with an α-amino acid function. Biochimica et Biophysica Acta **1336,** 331–341.

Cortès S, Gromova M, Evrard A, Roby C, Heyraud A, Rolin DB, Raymond P, Brouquisse RM. 2003. In plants, 3-*O*-methylglucose is phosphorylated by hexokinase but not perceived as a sugar. Plant Physiology **131,** 824–837.

Delétage-Grandon C, Chollet JF, Faucher M, Rocher F, Komor E, Bonnemain JL. 2001. Carrier-mediated uptake and phloem systemy of a 350-dalton chlorinated xenobiotic with an alpha-amino acid function. Plant Physiology **125,** 1620–1632.

Delrot S, Bonnemain JL. 1979. H^+-Rb^+ exchange and H^+-sugar cotransport in leaf tissues of *Vicia faba* L. Comptes Rendus des Séances de l'Académie des Sciences. Série D, Sciences Naturelles **288,** 71–76.

Delrot S, Bonnemain JL. 1981. Involvement of protons as a substrate for the sucrose carrier during phloem loading in *Vicia faba* leaves. Plant Physiology **67,** 560–564.

Delrot S, Despeghel JP, Bonnemain JL. 1980. Phloem loading in *Vicia faba* leaves: effect of N-ethylmaleimide and parachloromercuribenzenesulfonic acid on H^+ extrusion, K^+ and sucrose uptake. Planta **149,** 144–148.

Delrot S, Roques N, Descotes G, Mentech J. 1991. Recognition of some deoxy-derivatives of sucrose by the sucrose transporter of

the plant plasma membrane. Plant Physiology and Biochemistry **29,** 25–29.

Despeghel JP, Delrot S. 1983. Energetics of amino acid uptake by *Vicia faba* leaf tissues. Plant Physiology **71,** 1–6.

Dufaud A, Chollet JF, Rudelle J, Miginiac L, Bonnemain JL. 1994. Derivatives of pesticides with an α-amino acid function: Synthesis and effect on threonine uptake. Pesticide Science **41,** 297–304.

Felker FC, Goodwin JC. 1988. Sugar uptake by maize endosperm suspension cultures. Plant Physiology **88,** 1235–1239.

Furch AC, Zimmermann MR, Will T, Hafke JB, van Bel AJ. 2010. Remote-controlled stop of phloem mass flow by biphasic occlusion in *Cucurbita maxima*. Journal of Experimental Botany **61,** 3697–3708.

Gerendas J, Schurr U. 1999. Physicochemical aspects of ion relations and pH regulation in plants—A quantitative approach. Journal of Experimental Botany **50,** 1101–1114.

Giaquinta R. 1976. Evidence for phloem loading from the apoplast: chemical modification of membrane sulfhydryl groups. Plant Physiology **57,** 872–875.

Giaquinta RT. 1983. Phloem loading of sucrose. Annual Review of Plant Physiology and Plant Molecular Biology **34,** 347–387.

Gottwald JR, Krysan PJ, Young JC, Evert RF, Sussman MR. 2000. Genetic evidence for the in planta role of phloem-specific plasma membrane sucrose transporters. Proceedings of the National Academy of Sciences, USA **97,** 13979–13984.

Henry C, Rabot A, Laloi M, Mortreau E, Sigogne M, Leduc N, Lemoine R, Sakr S, Vian A, Pelleschi-Travier S. 2011. Regulation of RhSUC2, a sucrose transporter, is correlated with the light control of bud burst in *Rosa* sp. Plant, Cell & Environment **34,** 1776–1789.

Hitz WD, Card PJ, Ripp KG. 1986. Substrate recognition by a sucrose transporting protein. The Journal of Biological Chemistry **261,** 11986–11991.

Hummel CS, Lu C, Loo DD, Hirayama BA, Voss AA, Wright EM. 2011. Glucose transport by human renal Na^+/D-glucose cotransporters SGLT1 and SGLT2. American Journal of Physiology Cell Physiology **300,** C14–C21.

Hutchings VM. 1978. Sucrose and proton cotransport in *Ricinus* cotyledons: I. H^+ influx associated with sucrose uptake. Planta **138,** 229–235.

Jamai A, Chollet JF, Delrot S. 1994. Proton-peptide co-transport in broad bean leaf tissues. Plant Physiology **106,** 1023–1031.

Kallarackal J, Bauer SN, Nowak H, Hajirezaei MR, Komor E. 2012. Diurnal changes in assimilate concentrations and fluxes in the phloem of castor bean (*Ricinus communis* L.) and tansy (*Tanacetum vulgare* L.). Planta **236,** 209–223.

Kallarackal J, Komor E. 1989. Transport of hexoses by the phloem of *Ricinus communis* L. seedlings. Planta **177,** 336–341.

Kallarackal J, Orlich G, Schobert C, Komor E. 1989. Sucrose transport into the phloem of *Ricinus communis* L. seedlings as measured by the analysis of sieve-tube sap. Planta **177,** 327–335.

Komor E. 1977. Sucrose uptake by cotyledons of *Ricinus communis* L.: characteristics, mechanism, and regulation. Planta **137,** 119–131.

Komor E, Rotter M, Tanner W. 1977. Proton cotransport system in a higher plant—sucrose transport in *Ricinus communis*. Plant Science Letters **9,** 153–162.

Komor E, Rotter M, Waldhauser J, Martin E, Cho BH. 1980. Sucrose proton symport for phloem loading in the *Ricinus* seedling. Berichte Der Deutschen Botanischen Gesellschaft **93,** 211–219.

Kriedemann P, Beevers H. 1967. Sugar uptake and translocation in the castor bean seedling II. Sugar transformations during uptake. Plant Physiology **42,** 174–180.

Lemoine R. 2000. Sucrose transporters in plants: update on function and structure. Biochimica et Biophysica Acta **1465,** 246–262.

Lemoine R, Delrot S. 1987. Recognition of phlorizin by the carriers of sucrose and hexose in broad bean leaves. Physiologia Plantarum **69,** 639–644.

Lemoine R, Delrot S, Auger E. 1984. Development of pH sensitivity of sucrose uptake during aging of *Vicia faba* leaf disks. Physiologia Plantarum **61,** 571–576.

Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. 2012. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Advanced Drug Delivery Reviews **64,** 4–17.

Liu DD, Chao WM, Turgeon R. 2012. Transport of sucrose, not hexose, in the phloem. Journal of Experimental Botany **63,** 4315–4320.

Lorenc-Plucinska G, Ziegler H. 1987. Effect of sulfite and sulfhydryl reagents on the phloem loading in cotyledons of *Ricinus communis* L. Journal of Plant Physiology **128,** 417–424.

Malek T, Baker DA. 1978. Effect of fusicoccin on proton co-transport of sugars in phloem loading of *Ricinus communis* L. Plant Science Letters **11,** 233–239.

Marhadour S, Wu H, Yang W, Marivingt-Mounir C, Bonnemain JL, Chollet JF. 2017. Vectorisation of agrochemicals via amino acid carriers: influence of the spacer arm structure on the phloem mobility of phenylpyrrole conjugates in the *Ricinus* system. Pest Management Science **73,** 1972–1982.

Marvier AC, Neelam A, Bick JA, Hall JL, Williams LE. 1998. Cloning of a cDNA coding for an amino acid carrier from *Ricinus communis* (RcAAP1) by functional complementation in yeast: kinetic analysis, inhibitor sensitivity and substrate specificity. Biochimica et Biophysica Acta **1373,** 321–331.

Maynard JW, Lucas WJ. 1982. Sucrose and glucose uptake into *Beta vulgaris* leaf tissues: a case for general (apoplastic) retrieval systems. Plant Physiology **70,** 1436–1443.

M'Batchi B, Delrot S. 1984. Parachloromercuribenzenesulfonic acid: a potential tool for differential labeling of the sucrose transporter. Plant Physiology **75,** 154–160.

Metzler A, Köckenberger W, Vonkienlin M, Komor E, Haase A. 1994. Quantitative measurement of sucrose distribution in *Ricinus communis* seedlings by chemical shift microscopy. Journal of Magnetic Resonance Series B **105,** 249–252.

Montamat F, Maurousset L, Tegeder M, Frommer W, Delrot S. 1999. Cloning and expression of amino acid transporters from broad bean. Plant Molecular Biology **41,** 259–268.

Nour-Eldin HH, Andersen TG, Burow M, Madsen SR, Jørgensen ME, Olsen CE, Dreyer I, Hedrich R, Geiger D, Halkier BA. 2012. NRT/PTR transporters are essential for translocation of glucosinolate defence compounds to seeds. Nature **488,** 531–534.

Orlich G, Hofbruckl M, Schulz A. 1998. A symplasmic flow of sucrose contributes to phloem loading in *Ricinus* cotyledons. Planta **206,** 108–116.

Orlich G, Komor E. 1992. Phloem loading in *Ricinus* cotyledons: sucrose pathways via the mesophyll and the apoplasm. Planta **187,** 460–474.

Renault S, Bonnemain JL, Faye L, Gaudillere JP. 1992. Physiological aspects of sugar exchange between the gametophyte and the sporophyte of *Polytrichum formosum*. Plant Physiology **100,** 1815–1822.

Rentsch D, Laloi M, Rouhara I, Schmelzer E, Delrot S, Frommer WB. 1995. NTR1 encodes a high affinity oligopeptide transporter in *Arabidopsis*. FEBS Letters **370,** 264–268.

Robinson SP, Beevers H. 1981*a*. Amino acid transport in germinating castor bean seedlings. Plant Physiology **68,** 560–566.

Robinson SP, Beevers H. 1981*b*. Evidence for amino-acid: proton cotransport in *Ricinus* cotyledons. Planta **152,** 527–533.

Rocher F, Chollet JF, Jousse C, Bonnemain JL. 2006. Salicylic acid, an ambimobile molecule exhibiting a high ability to accumulate in the phloem. Plant Physiology **141,** 1684–1693.

Rocher F, Chollet JF, Legros S, Jousse C, Lemoine R, Faucher M, Bush DR, Bonnemain JL. 2009. Salicylic acid transport in *Ricinus communis* involves a pH-dependent carrier system in addition to diffusion. Plant Physiology **150,** 2081–2091.

Sauer N. 2007. Molecular physiology of higher plant sucrose transporters. FEBS Letters **581,** 2309–2317.

Schobert C, Komor E. 1989. The differential transport of amino acids into the phloem of *Ricinus communis* L. seedlings as shown by the analysis of sieve-tube sap. Planta **177,** 342–349.

Servaites JC, Schrader LE, Jung DM. 1979. Energy-dependent loading of amino acids and sucrose into the phloem of soybean. Plant Physiology **64,** 546–550.

Smart CJ, Garvin DF, Prince JP, Lucas WJ, Kochian LV. 1996. The molecular basis of potassium nutrition in plants. Plant and Soil **187,** 81–89.

Stadler R, Sauer N. 1996. The *Arabidopsis thaliana AtSUC2* gene is specifically expressed in companion cells. Botanica Acta **109,** 299–306.

Tamas IA, Davies PJ. 2016. Dynamics and control of phloem loading of indole-3-acetic acid in seedling cotyledons of *Ricinus communis*. Journal of Experimental Botany **67,** 4755–4765.

Toggenburger G, Kessler M, Semenza G. 1982. Phlorizin as a probe of the small-intestinal Na^+,D-glucose cotransporter. A model. Biochimica et Biophysica Acta **688,** 557–571.

Turgeon R, Ayre BG. 2005. Pathways and mechanisms of phloem loading. In: Holbrook NM, Zwieniecki MA, eds. Vascular transport in plants. Burlington: Academic Press, 45–67.

Turgeon R, Medville R. 2004. Phloem loading. A reevaluation of the relationship between plasmodesmatal frequencies and loading strategies. Plant Physiology **136,** 3795–3803.

Turgeon R, Wolf S. 2009. Phloem transport: cellular pathways and molecular trafficking. Annual Review of Plant Biology **60,** 207–221.

van Bel AJ, Gamalei YV, Ammerlaan A, Bik LP. 1992. Dissimilar phloem loading in leaves with symplasmic or apoplasmic minor-vein configurations. Planta **186,** 518–525.

Veber DF, Johnson SR, Cheng HY, Smith BR, Ward KW, Kopple KD. 2002. Molecular properties that influence the oral bioavailability of drug candidates. Journal of Medicinal Chemistry **45,** 2615–2623.

Verscht J, Kalusche B, Köhler J, Köckenberger W, Metzler A, Haase A, Komor E. 1998. The kinetics of sucrose concentration in the phloem of individual vascular bundles of the *Ricinus communis* seedling measured by nuclear magnetic resonance microimaging. Planta **205,** 132–139.

Vreugdenhil D, Koot-Gronsveld EAM. 1989. Measurements of pH, sucrose and potassium ions in the phloem sap of castor bean *Ricinus communis* plants. Physiologia Plantarum **77,** 385–388.

Weig A, Komor E. 1996. An active sucrose carrier (Scr1) that is predominantly expressed in the seedling of *Ricinus communis* L. Journal of Plant Physiology **147,** 685–690.

Wilkinson RE, Ohki K. 1991. Effect of PCMBS and EPTC on mineral nutrient uptake and translocation in wheat. Journal of Plant Nutrition **14,** 93–108.

Williams LE, Bick JA, Neelam A, Weston KN, Hall JL. 1996. Biochemical and molecular characterization of sucrose and amino acid carriers in *Ricinus communis*. Journal of Experimental Botany **47 Spec No,** 1211–1216.

Williams LE, Gregory A. 2004. Changes in the expression pattern of the plasma membrane H^+-ATPase in developing *Ricinus communis* cotyledons undergoing the sink/source transition. Planta **218,** 562–568.

Williams L, Hall JL. 1987. ATPase and proton pumping activities in cotyledons and other phloem-containing tissues of *Ricinus communis*. Journal of Experimental Botany **38,** 185–202.

Williams LE, Nelson SJ, Hall JL. 1990. Characterization of solute transport in plasma membrane vesicles isolated from cotyledons of *Ricinus communis* L.: II. Evidence for a proton-coupled mechanism for sucrose and amino acid uptake. Planta **182,** 540–545.

Williams LE, Nelson SJ, Hall JL. 1992. Characterization of solute/proton cotransport in plasma membrane vesicles from *Ricinus* cotyledons, and a comparison with other tissues. Planta **186,** 541–550.

Wu H, Marhadour S, Lei Z-W, Yang W, Marivingt-Mounir C, Bonnemain J-L, Chollet J-F. 2016. Vectorization of agrochemicals: amino acid carriers are more efficient than sugar carriers to translocate phenylpyrrole conjugates in the *Ricinus* system. Environmental Science and Pollution Research International doi: 10.1007/s11356-11016-18107-x.

Wu HX, Yang W, Zhang ZX, Huang T, Yao GK, Xu HH. 2012. Uptake and phloem transport of glucose-fipronil conjugate in *Ricinus communis* involve a carrier-mediated mechanism. Journal of Agricultural and Food Chemistry **60,** 6088–6094.

Xie Y, Zhao JL, Wang CW, Yu AX, Liu N, Chen L, Lin F, Xu HH. 2016. Glycinergic-fipronil uptake is mediated by an amino acid carrier system and induces the expression of amino acid transporter genes in *Ricinus communis* seedlings. Journal of Agricultural and Food Chemistry **64,** 3810–3818.

Yang W, Wu HX, Xu HH, Hu AL, Lu ML. 2011. Synthesis of glucose-fipronil conjugate and its phloem mobility. Journal of Agricultural and Food Chemistry **59,** 12534–12542.

Yuan JG, Wu HX, Lu ML, Song GP, Xu HH. 2013. Synthesis of a series of monosaccharide-fipronil conjugates and their phloem mobility. Journal of Agricultural and Food Chemistry **61,** 4236–4241.

Zhang C, Yu X, Ayre BG, Turgeon R. 2012. The origin and composition of cucurbit "phloem" exudate. Plant Physiology **158,** 1873–1882.

Zimmermann MR, Hafke JB, van Bel AJ, Furch AC. 2013. Interaction of xylem and phloem during exudation and wound occlusion in *Cucurbita maxima*. Plant, Cell & Environment **36,** 237–247.

Chapter 5. Improving the amino acid conjugate strategy

- Journal Article «Vectorization of agrochemicals via amino acid carriers: influence of the spacer arm structure on the phloem mobility of phenylpyrrole conjugates in the *Ricinus* system», *Pest Management Science*. 2017, 73(9): 1972-1982, DOI 10.1002/ps.4575.

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Vectorisation of agrochemicals via amino acid carriers: influence of the spacer arm structure on the phloem mobility of phenylpyrrole conjugates in the *Ricinus* system

Sophie Marhadour,[a†] Hanxiang Wu,[a,c†] Wen Yang,[b] Cécile Marivingt-Mounir,[a] Jean-Louis Bonnemain[c] and Jean-François Chollet[a*]

Abstract

BACKGROUND: Excessive agrochemical use poses significant threats to environmental safety and human health. Reducing pesticide use without reducing yield is necessary for sustainable agriculture. Therefore, we developed a vectorisation strategy to enhance agrochemical delivery through plant amino acid carriers.

RESULTS: In addition to a fenpiclonil conjugate recently described, three new amino acid conjugates were synthesised by coupling fenpiclonil to an L-α-amino acid. Phloem mobility of these conjugates, which exhibit different structures of the spacer arm introduced between fenpiclonil and the α-amino acid function, was studied using the *Ricinus* model. Conjugate L-14, which contains a triazole ring with the shortest amino acid chain, showed the best phloem systemicity among the four conjugates. By contrast, removing the triazole ring in the spacer arm did not improve systemicity. L-14 exhibited phloem systemicity at all reported pH values (pH values from 5.0 to 6.5) of the foliar apoplast, while acidic derivatives of fenpiclonil were translocated only at pH values near 5.0.

CONCLUSION: The conjugates were recognised by a pH-dependent transporter system and translocated at distance in the phloem. They exhibited a broader phloem systemicity than fenpiclonil acidic derivatives within the pH value range of the foliar apoplast.

Keywords: phenylpyrroles; fungicide; conjugate systemicity; phloem transport; sustainable agriculture; amino acid carrier

1 INTRODUCTION

To protect crops from damage by weeds, pathogens and animal pests, agrochemicals remain an important part of current agricultural systems to secure food production, in addition to the improvement in cultural practices, biological control of pathogens and pests and the development of new strategies conferring genetic resistance.[1–3] However, the search for optimum efficacy and for low-use-rate pesticides to reduce the impact on human health and environment has become urgent and necessary. Prodrug approaches in pharmaceutical research have been developed to enhance bioavailability through targeted drug delivery and accumulation,[4] which have set a good example for agrochemical design.[5] Ensuring pesticide effective delivery to sites of action presents a promising strategy that not only can keep the dose at the lowest possible level but also can improve the biological activity of the active ingredient. For example, profungicides, which are likely to concentrate in plant phloem tissues, can be applied to control vascular diseases[6] that most existing fungicides are unable to access. One attractive prodrug approach is to conjugate a drug with endogenous substrates, so that the resulting molecules can be recognised and transported by peptide, amino acid or glucose transporters.[7–15]

Carrier-mediated processes have been shown to be a promising approach to enhancing the uptake and vectorisation of pesticides by utilising plant sugar or amino acid transporters as well.[16,17] Both glucose and amino acid pesticide conjugates were reported to involve carrier-mediated uptake and displayed

* *Correspondence to: J-F Chollet, Institut de Chimie des Milieux et des Matériaux de Poitiers, Université de Poitiers, Poitiers, France. E-mail: jean.franc.chollet@univ-poitiers.fr*

† *These authors contributed equally to the paper*

a *Institut de Chimie des Milieux et des Matériaux de Poitiers, Université de Poitiers, Poitiers, France*

b *Guizhou Tea Reasearch Institute, Guizhou Academy of Agricultural Science, Guiyang, Guizhou, China*

c *Laboratoire Écologie et Biologie des Interactions, Université de Poitiers, Poitiers, France*

phloem mobility.[18–20] This propesticide strategy could be applied to develop phloem mobile insecticides, which are needed to control root or vascular pests.[17,19] Similarly, profungicides designed to move at long distance in the phloem tissue could be applied to control vascular or root diseases of herbaceous cultures, i.e. tissues or organs that the current commercialised fungicides are unable to access after foliar application.[21]

Fenpiclonil, a non-phloem mobile fungicide, was selected in the recent past as a model compound because of the numerous possibilities of adding substituents, notably carboxylic groups,[22,23] at various sites in the molecule. In a recent study of phloem-mobile profungicides, fenpiclonil-glucose and fenpiclonil-glutamic acid conjugates were synthesised via click chemistry in order to compare the capacity of amino acid and sugar carrier systems for profungicide uptake and phloem mobility.[24] The results of systemicity tests indicated that the L-amino acid promoiety was dramatically more favourable to phloem mobility than the D-amino acid and D-glucose promoieties. However, the structure–activity relationship between conjugates and amino acid carrier systems has not yet been extended to other parameters such as the structure of the L-amino acid promoiety and the possible effect of the triazole spacer. The optimum characterisation of chemical structures for recognition and translocation of amino acid conjugates remains unknown.

In the present work, we describe the synthesis and phloem systemicity of three new amino acid conjugates in which fenpiclonil is attached to L-α-amino acids with different chain lengths and with or without a triazole spacer. In order to avoid any interaction of the antifungal moiety with the active site of the carrier, we added a spacer linker between the amino acid function and the antifungal compound. The structure notably included a 1,2,3-triazole ring connected to the antifungal moiety via an amide bond. 1,2,3-Triazole, as a peptide bond isostere, is often described as improving the stability, absorption or aqueous solubility of different bioconjugation compounds.[25,26] In drug discovery, the replacement of amide bonds in the backbone of peptides by 1,4-disubstituted 1,2,3-triazole leads to peptidomimetics with retained receptor affinity and improved tumour-targeting capabilities.[27] Using click chemistry, this linker has the advantage of being synthesised easily and quickly in good yields.[28] Phloem systemicity studies were conducted using *Ricinus* seedling cotyledons, which function as the small intestine wall.[29,30] The aim of the second part of this paper is (1) to investigate the influence of different chain length and type of spacer arm (i.e. the linkage between fenpiclonil and the α-amino acid function) on phloem mobility and (2) to compare the phloem systemicity of the most mobile amino acid conjugate with the acidic derivatives of fenpiclonil at various external pH values, with special attention paid to apoplast pH values measured in mature leaf tissues of various plant species.

2 MATERIALS AND METHODS

2.1 Synthesis

Some reactions were carried out under nitrogen. All reactions were monitored by thin-layer chromatographic analysis using Merck silica gel 60F-254 thin-layer plates (Merck, Darmstadt, Germany). Column chromatography was carried out on silica gel Merck 60 (0.015–0.04 mm). Melting points were determined on an Electrothermal IA 9200 melting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were taken in DMSO-d_6 using a Bruker Avance 400 MHz spectrometer (Bruker, Billerica, MA). DEPT-135 and ^{1}H and ^{13}C experiments were used to confirm the NMR peak assignements. Chemical shifts are reported as δ values in parts per million (ppm) relative to tetramethylsilane as internal standard, and coupling constants (J) are given in hertz (Hz). The following abbreviations are used to describe peak patterns when appropriate: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). High-resolution mass spectra were obtained on a Bruker qTOF Maxis Impact spectrometer.

2.2 Plant material

Castor bean seeds (*Ricinus communis* L. cv. Sanguineus), obtained from Graines Girerd et Fils (Le Thor, France) were placed in wet cotton wool for 24 h at 27 ± 1°C prior to sowing in vermiculite watered with tap water. Seedlings were grown in a humid atmosphere (80 ± 5%) at 27 ± 1°C. As the brush border of the small intestine, cotyledon tissues exhibit a high ability to take up small nutrient molecules, but from an external compartment, namely the endosperm (Fig. 1A).[30,31]

2.3 Phloem sap collection and analysis

The sap collection method was similar to that already described (Figs 1B, C, D and E).[32] The phloem sap was analysed by high-performance liquid chromatography after dilution with UHQ-grade water (1 + 9 v/v). We employed reverse-phase chromatography using an Ascentis Express RP-amide C16 column (length 250 mm, internal diameter 4.6 mm, 5 µM) (Supelco, Bellefonte, PA) in accordance with the procedure set out in Table 1. Results were processed with PC 1000 software v.3.5 from Thermo Fisher Scientific (Courtaboeuf, France).

2.4 Chemicals

The compounds to be added to the incubation solutions were from Acros Organics (Noisy-le-Grand, France) – 4-morpholinoethanesulphonic acid (MES) and 2-[4-(2-hydroxyethyl)-1-piperazine]ethanesulphonic acid (HEPES).

2.5 Physicochemical properties

Physicochemical properties and descriptors were predicted using ACD/Labs Percepta 2015 release (Build 2726) software from Advanced Chemistry Development, Inc. (Toronto, Canada). The calculated properties (Table 2) were chosen according to their influence on passive membrane transport in plants.[33]

3 RESULTS AND DISCUSSION

3.1 Synthesis of amino acid conjugates

In a previous work, we described the three-step synthesis of compound **L-13** which associated fenpiclonil, a fungicide from the phenylpyrrole family, to L-glutamic acid via a spacer group including a 1,2,3-triazole ring. This conjugate was obtained by coupling the azido derivative **3** of protected glutamic acid with the propargyl derivative of fenpiclonil **10**. Then the deprotection of the α-amino acid function of the resulting coumpound **11R$_1$** gave the desired conjugate **L-13** (Fig. 2).[24] A similar synthetic route was adapted in this work to obtain the conjugates **L-14** and **L-15**. The procedure involves a copper(I)-catalysed azide-alkyne cycloaddition (CuAAC) permitting the introduction of a 1,2,3-triazole ring by click chemistry.[34–36] In order to study the influence of the triazole ring on phloem mobility, we synthesised compound **L-16** which does not have this heterocycle in its structure. In this case, the strategy was to form an amide bond using a coupling reagent.[37–40] The different processes are outlined in Fig. 2.

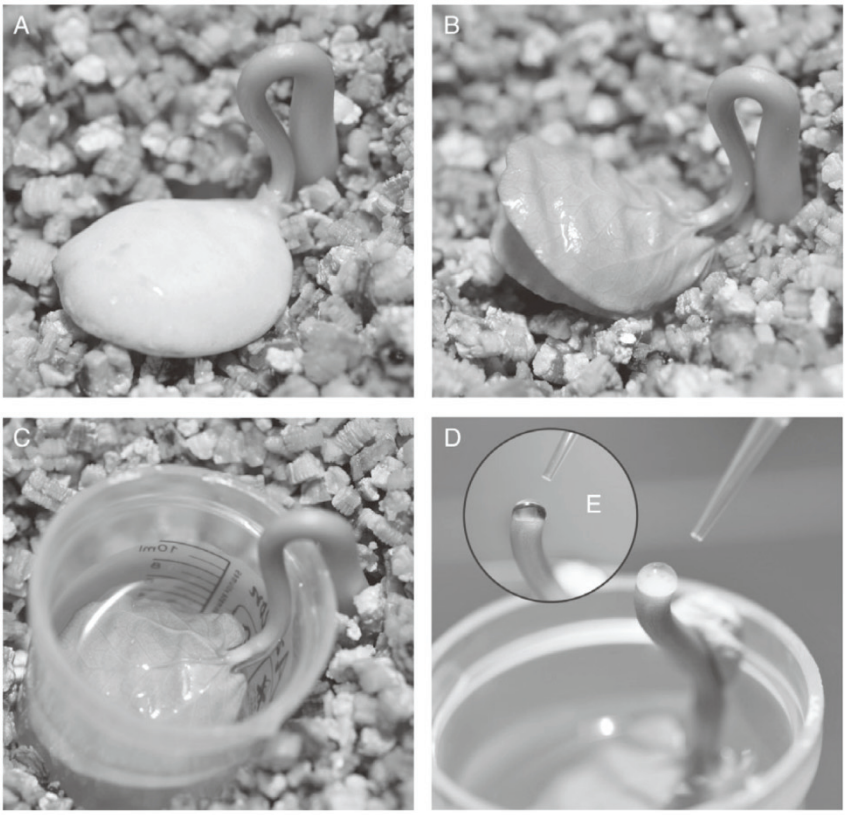

Figure 1. The *Ricinus* model. (A) The *Ricinus* seedlings 6 days after sowing. (B) The endosperm was carefully removed. (C) Cotyledons were immersed in a buffered solution containing the test product. (D)/(E) After cutting the hypocotyl, phloem sap accumulated on the cross-section and then was collected with a micropipette. See also Fig. 1 from Kallarackal *et al.*[29]

Table 1. Chromatographic data for tested products

Mobile phase (gradient)						
Time (min)	Water + TFA 0.1%	CH_3CN	Delivery (mL min^{-1})	Detection UV (nm)	Compound	Retention time (min)
$t = 0$	70	30	0.8	218	Fenpiclonil	24.6
$t = 30$	40	60			**9**	22.9
$t = 35$	70	30			**L-13**	8.0
$t = 37$	70	30			**L-14**	8.8
					L-15	9.7
					L-16	9.2

*3.1.1 Obtaining azido derivatives from protected amino acid (Fig. 2), compounds **7** and **8***

We started with the synthesis of azido derivatives N_3-R_x from commercially available protected amino acid. The conversion of primary amines to azides via diazo transfer occurred using trifluoromethanesulphonyl azide (TfN_3), an efficient organic-soluble reactive described by Cavender and Shiner.[41] TfN_3 is not available commercially and was prepared immediately prior to use. It was made by reacting trifluoromethanesulphonic anhydride with sodium azide in a water–dichloromethane mixture.[42–44] Thus, the trifluoromethanesulphonyl azide **4** made it possible to convert protected α-amino acid compounds **5** or **6** respectively into the desired azides **7** and **8** in a one-pot reaction[45] in the presence of potassium carbonate and copper(II) sulphate pentahydrate in a methanol–water medium.

*Experimental procedure for the synthesis of 4-azido-2-[(tert-butoxycarbonyl)amino]butanoic acid (**7**) and 6-azido-2-[(tert-butoxycarbonyl)amino]hexanoic acid (**8**).*

To a solution of sodium azide (6.52 g, 100.0 mmol, 10 equiv.) in a dichloromethane/water mixture (42 mL, 2:1) was added trifluoromethanesulphonic anhydride (3.33 mL, 20.0 mmol, 2 equiv.) at 0 °C. The reaction mixture was allowed to warm to room temperature and was stirred for 2 h. The water layer was extracted with dichloromethane, and the combined organic layers were washed with saturated sodium carbonate solution. The resulting solution of TfN_3 **4** in dichloromethane was added slowly to a solution of

Table 2. Structures, chemical descriptors and physicochemical properties of the studied compounds computed with ACD/Labs Percepta 2015 release (Build 2726) software. The interpretation of the computed properties to predict crossing a biological membrane is given according to Lipinski's rule of five (MW ≤ 500 Da; HBD ≤ 5; HBA ≤ 10; log P ≤ 5.0) and to Veber's rule (FRB ≤ 10; PSA ≤ 140 Å^2). At biological pHs (from 5.0 to 8.0), compound **9** is predicted to be almost in its undissociated form (pK_a = 3.58 ± 0.40), and compounds **L-13** to **L-16** are predicted to be in their zwitterionic form[a]

Number	Structure	MW	HBD	HBA	log D(pH 5.0)	log D(pH 8.0)	FRB	PSA(Å^2)	Lipinski's rule of five violation(*)	Veber's rule violation(**)
Fenpiclonil		237.08	1	2	3.92	3.92	2	39.58	0/4	0/2
9		309.15	1	4	2.05	−0.45	4	66.02	0/4	0/2
L-13		561.42 (*)	5	12 (*)	0.42	0.39	13 (**)	180.95 (**)	2/4	2/2
L-14		490.34	4	10	1.32	1.31	10	151.85 (**)	0/4	1/2
L-15		518.40 (*)	4	10	1.38	1.37	12 (**)	151.85 (**)	1/4	2/2
L-16		437.32	4	7	1.98	1.97	10	121.14	0/4	0/2

[a] MW: molecular weight; HBD: number of hydrogen bond donors; HBA: number of hydrogen bond acceptors; FRB: free rotatable bonds; PSA: polar surface area.

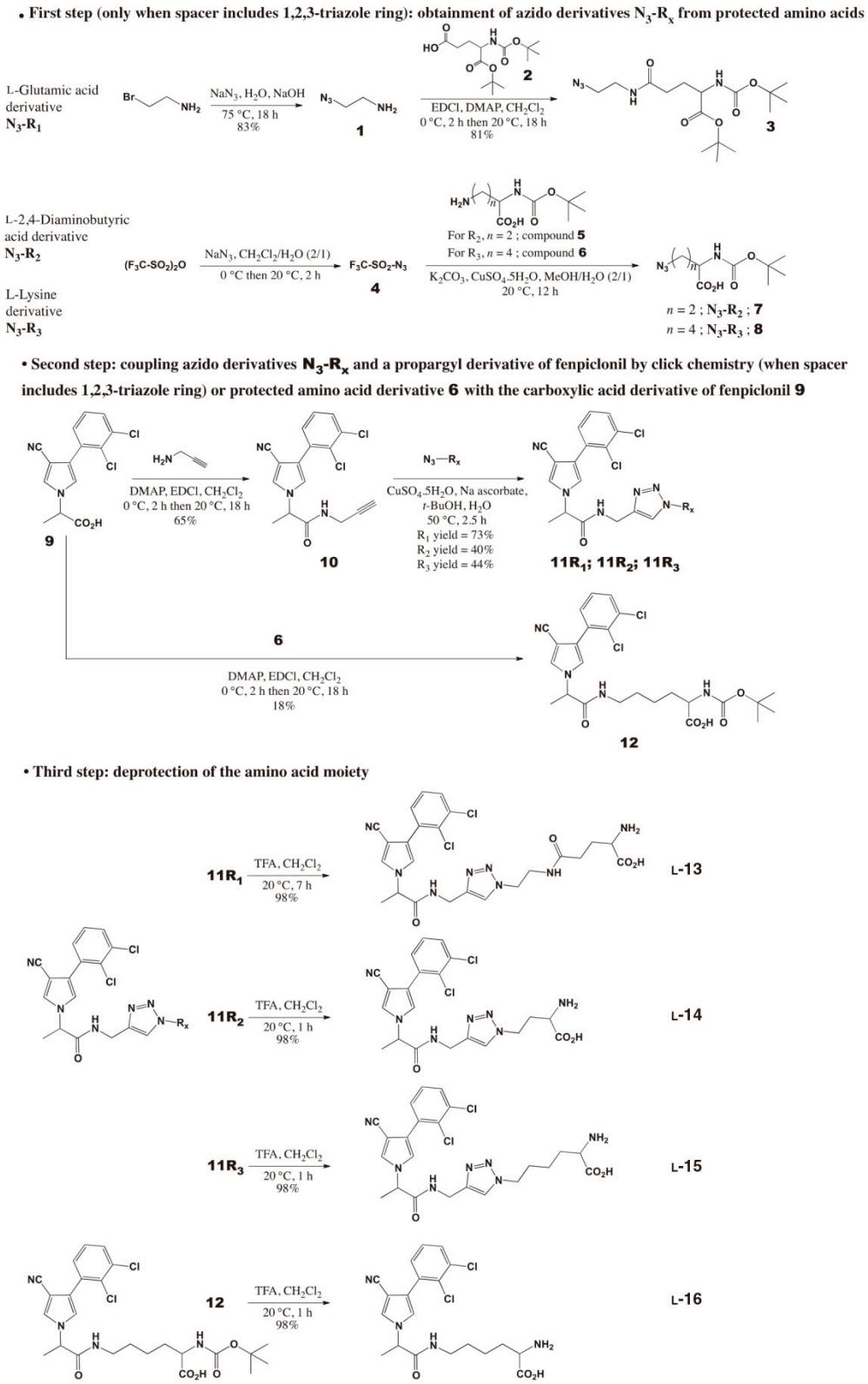

Figure 2. General reaction scheme showing the different steps of the synthesis of the amino acid-fungicide derivatives. DMAP: 4-dimethylaminopyridine; EDCI: 1-ethyl-3-(3- dimethylaminopropyl)carbodiimide hydrochloride; TFA: trifluoroacetic acid.

protected amino acid *N*-α-Boc-L-2,4-diaminobutyric acid **5** (2.18 g, 10.0 mmol, 1 equiv.) or *N*-α-Boc-L-lysine **6** (2.47 g, 10.0 mmol, 1 equiv.), potassium carbonate (2.08 g, 15.0 mmol, 1.5 equiv.) and copper(II) sulphate pentahydrate (25.0 mg, 0.10 mmol, 0.01 equiv.) in a methanol/water mixture (96 mL, 2:1). The reaction mixture was stirred at room temperature for 12 h, and the organic solvents were evaporated under reduced pressure. The aqueous layer was used directly for the next step.

3.1.2 *Coupling azido derivatives N_3-R_x and the propargyl derivative of fenpiclonil **10** by click chemistry (Fig. 2), compounds **11R$_2$** and **11R$_3$***

For this step, we prepared a key intermediate of fenpiclonil substituted with an alkyne function (compound **10**), as previously described.[24] Thus, we considered a 1,3-cycloaddition by click chemistry with an appropriate coupling partner, the azido derivatives **7** and **8**, giving the 1,2,3-triazole ring as a spacer group to obtain targeted compounds **11R$_2$** and **11R$_3$**.[34,46] This reaction was carried out using as catalyst the active Cu(I), generated from Cu(II) salts with sodium ascorbate as the reducing agent in a *tert*-butanol–water mixture. In these conditions, the copper-catalysed reaction allowed the synthesis of the 1,4-disubstituted regioisomers specifically.[47] The compounds **11R$_2$** and **11R$_3$** were obtained in moderate yields, 40% and 44% respectively. In a general approach, this pathway offers a wide diversity for further structure–activity relationship studies.

*Experimental procedure for the synthesis of derivatives **11R$_2$** and **11R$_3$** (Fig. 3).*

To a solution of compound **10** (3.47 g, 10.0 mmol, 1 equiv.) in *tert*-butanol (28 mL) was added the solution of compound **7** or compound **8** previously prepared. Then, a solution of copper(II) sulphate pentahydrate (500 mg, 2.0 mmol, 0.2 equiv.) and L-ascorbic acid sodium salt (794 mg, 4.0 mmol, 0.4 equiv.) in water (28 mL) was added to the reaction mixture. The resulting solution was heated at 50 °C for 2.5 h. After cooling to room temperature, the resulting mixture was diluted with ethyl acetate. The organic layer was extracted, washed with saturated ammonium chloride solution and brine, dried over $MgSO_4$, filtered and concentrated under vacuum. The crude product was purified by silica gel column chromatography using ethyl acetate/methanol (9:1) as eluent to obtain respectively compound **11R$_2$** as a pink powder (2.4 g, 40% yield) or compound **11R$_3$** as a beige powder (2.7 g, 44% yield).

*2-(tert-Butoxycarbonylamino)-4-[4-({2-[3-cyano-4-(2,3-dichlorophenyl)-1H-pyrrol-1-yl]propanamido}methyl)-1H-1,2,3-triazol-1-yl]butanoic acid (**11R$_2$**)*

$R_f = 0.60$ (ethyl acetate/methanol: 5:5), mp = 163–164 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 13.01 (s, 1H, OH), 8.83 (t, 1H, $^3J = 5.2$ Hz, NH), 7.98 (s, 1H, H_d), 7.88 (d, 1H, $^4J = 2.2$ Hz, H_2), 7.68 (dd, 1H, $^3J = 7.3$ Hz, $^4J = 2.3$ Hz, H_8), 7.49–7.43 (m, 2H, H_6 and H_7), 7.30 (d, 1H, $^4J = 2.2$ Hz, H_5), 7.19 (s, 1H, NH), 4.98 (q, 1H, $^3J = 7.0$ Hz, H_b), 4.43–4.32 (m, 4H, H_c and H_e), 3.86–3.82 (m, 1H, H_g), 2.26–2.08 (m, 2H, H_f), 1.65 (d, 3H, $^3J = 7.0$ Hz, H_a), 1.42 (s, 9H, H_h). ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.33 (C = O), 169.04 (C = O), 156.55 (C = O), 144.94 (C), 133.98 (C–Cl), 132.47 (C–Cl), 130.26 (C_7 and C), 129.71 (C_8), 129.03 (C_2), 128.21 (C_6), 123.01 (C_d), 122.17 (C_4), 122.08 (C_5), 116.05 (CN), 91.98 (C_3), 78.18 (C), 59.75 (C_g), 57.57 (C_b), 46.86 (C_e), 34.47 (C_c), 31.72 (C_f), 28.18 (3 C_h), 18.28 (C_a). HRMS (ESI, CH_3CN): *m/z* calcd for $C_{26}H_{29}Cl_2N_7O_5$ $[M + Na]^+$ 612.1505, *m/z* found 612.1501.

*2-(tert-Butoxycarbonylamino)-6-[4-({2-[3-cyano-4-(2,3-dichlorophenyl)-1H-pyrrol-1-yl]propanamido}methyl)-1H-1,2,3-triazol-1-yl]hexanoic acid (**11R$_3$**)*

$R_f = 0.13$ (ethyl acetate/methanol: 9:1), mp = 142–143 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.56 (s, 1H, OH), 8.87 (t, 1H, $^3J = 5.3$ Hz, NH), 7.97 (s, 1H, H_d), 7.88 (d, 1H, $^4J = 2.2$ Hz, H_2), 7.68 (dd, 1H, $^3J = 7.1$ Hz, $^4J = 2.5$ Hz, H_8), 7.48–7.44 (m, 2H, H_6 and H_7), 7.30 (d, 1H, $^4J = 2.2$ Hz, H_5), 6.64 (d, 1H, $^3J = 7.6$ Hz, NH), 4.99 (q, 1H, $^3J = 7.0$ Hz, H_b), 4.38–4.32 (m, 4H, H_c and H_e), 3.87–3.81 (m, 1H, H_i), 1.85–1.52 (m, 7H, H_a, H_f and H_h), 1.40–1.31 (m, 11H, H_g and H_j). ^{13}C NMR (100 MHz, DMSO-d_6): δ 174.15 (C = O), 169.03 (C = O), 155.52 (C = O), 143.93 (C), 133.98 (C–Cl), 132.50 (C–Cl), 130.25 (C_7 and C), 129.72 (C_8), 129.01 (C_2), 128.22 (C_6), 122.81 (C_d), 122.18 (C_4), 122.08 (C_5), 116.05 (CN), 92.00 (C_3), 77.92 (C), 57.57 (C_b), 53.46 (C_i), 49.15 (C_e), 34.52 (C_c), 30.34 (C_h), 29.46 (C_f), 28.21 (3 C_j), 22.63 (C_g), 18.23 (C_a). HRMS (ESI, CH_3CN): *m/z* calcd for $C_{28}H_{33}Cl_2N_7O_5$ $[M + Na]^+$ 640.1818, *m/z* found 640.1812.

3.1.3 *Coupling protected amino acid derivative with the carboxylic acid derivative of fenpiclonil **9** (Fig. 2), compound **12***

We next investigated the preparation of another compound without the 1,2,3-triazole ring as a spacer group in order to compare the interaction between these amino acid conjugates and their respective carriers. Amide bonds play a major role in biological systems but also in a wide range of molecules such as commercial drugs.[38] The carboxylic acid moiety was first activated by an appropriate coupling reagent to form the *O*-acylisourea, a process making it possible to convert the hydroxyl group of the acid into a good leaving group. The *O*-acylisourea intermediate was obtained from compound **9** previously described[23] using water-soluble 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI)[48,49] and 4-dimethylaminopyridine (DMAP) as catalyst.[50] Then, this intermediate compound reacted *in situ* with the *N*-α-Boc-L-lysine derivative **6** to give the desired compound **12** which was easily extracted by an organic solvent, the urea derivative byproduct being eliminated in the aqueous layer.

*Experimental procedure for the synthesis of compound **12** (Fig. 3)*

To a solution of compound **9** (3.10 g, 10.0 mmol, 1 equiv.) in anhydrous dichloromethane (30 mL) cooled to 0 °C was added *N*-α-Boc-L-lysine (7.41 g, 30.0 mmol, 3 equiv.), EDCI (5.96 g, 31.0 mmol, 3.1 equiv.) and 4-DMAP (123 mg, 1.00 mmol, 0.1 equiv.). The reaction mixture was purged with nitrogen through the septum and then stirred at 0 °C for 2 h. The mixture was allowed to reach room temperature and then stirred for 18 h. Water was added, and the organic layer was extracted twice with dichloromethane. The combined organic layers were washed with water, dried over $MgSO_4$, filtered and concentrated under vacuum. The crude product was purified by silica gel column chromatography using pentane/ethyl acetate (5:5) as eluent to obtain compound **12** as a pink powder (0.97 g, 18% yield).

*2-(tert-Butoxycarbonylamino)-6-{2-[3-cyano-4-(2,3-dichlorophenyl)-1H-pyrrol-1-yl]propanamido}hexanoic acid (**12**)*

$R_f = 0.16$ (ethyl acetate/methanol: 9:1), mp = 107–108 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.52 (s, 1H, OH), 8.28 (t, 1H, $^3J = 5.5$ Hz, NH), 7.85 (d, 1H, $^4J = 2.2$ Hz, H_2), 7.68 (dd, 1H, $^3J = 7.2$ Hz, $^4J = 2.3$ Hz, H_8), 7.49–7.43 (m, 2H, H_6 and H_7), 7.28 (d, 1H, $^4J = 2.2$ Hz, H_5), 7.05 (s, 1H, NH), 4.90 (q, 1H, $^3J = 7.0$ Hz, H_b), 3.88–3.83 (m, 1H, H_g), 3.16–3.01 (m, 2H, H_c), 1.74–1.56 (m, 5H, H_a and H_f), 1.50–1.27 (m, 13H, H_d, H_e and H_h). ^{13}C NMR (100 MHz, DMSO-d_6): δ 174.23 (C = O), 168.94 (C = O), 155.59 (C = O), 133.99 (C–Cl), 132.47 (C–Cl), 130.26 (C_7), 130.24 (C), 129.71 (C_8), 128.95 (C_2), 128.22 (C_6), 122.14 (C_5), 122.01 (C_4), 116.05 (CN), 91.92 (C_3), 77.94 (C), 57.74 (C_b), 53.36 (C_g), 38.89 (C_c), 30.69 (C_f), 28.38 (C_d), 28.19 (3 C_h), 22.96 (C_e), 18.33 (C_a). HRMS (ESI, CH_3CN): *m/z* calcd for $C_{25}H_{30}Cl_2N_4O_5$ $[M + Na]^+$ 559.1491, *m/z* found 559.1492.

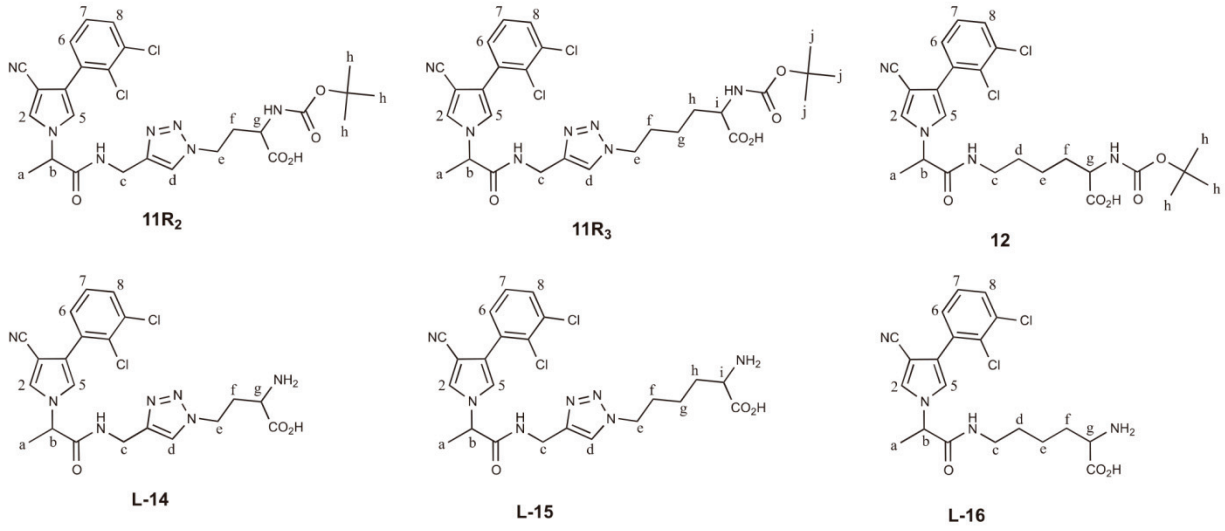

Figure 3. Fenpiclonil derivative numbering for ^{1}H and ^{13}C assignments.

*3.1.4 Deprotection of the amino acid moiety (Fig. 2), compounds **L-14**, **L-15** and **L-16***

The last step consisted of the deprotection of the α-amino acid function of the compounds **11R$_2$**, **11R$_3$** and **12**.[51] The reaction was performed with trifluoroacetic acid in dichloromethane for 1 h at room temperature, making it possible to remove the protecting *t*-butoxycarbonyl group of the amino function and leading respectively to the final compounds **L-14**, **L-15** and **L-16** in 98% yield.

*Experimental procedure for the synthesis of compounds **L-14, L-15** and **L-16** (Fig. 3)*

The compound **11R$_2$** (1.77 g, 3.0 mmol) or **11R$_3$** (1.86 g, 3.0 mmol) or **12** (1.61 g, 3.0 mmol) was diluted in an anhydrous dichloromethane–trifluoroacetic acid mixture (9.4 mL, 1:1). The reaction mixture was purged with nitrogen through the septum and then stirred at room temperature for 1 h before being evaporated. The residue was coevaporated with ethyl acetate and dried under vacuum to obtain respectively the compound **L-14** as a pink powder (1.4 g, 98% yield), **L-15** as a beige powder (1.5 g, 98% yield) or **L-16** as a pink powder (1.44 g, 98% yield).

*2-Amino-4-[4-({2-[3-cyano-4-(2,3-dichlorophenyl)-1H-pyrrol-1-yl]propanamido}methyl)-1H-1,2,3-triazol-1-yl]butanoic acid (**L-14**)*

$R_f = 0.12$ (ethyl acetate/methanol: 5:5), mp = 174–175 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.87 (t, 1H, $^{3}J = 5.3$ Hz, NH), 8.46 (s, 3H, NH_2 and OH), 8.03 (s, 1H, H_d), 7.87 (d, 1H, $^{4}J = 2.2$ Hz, H_2), 7.68 (dd, 1H, $^{3}J = 7.4$ Hz, $^{4}J = 2.2$ Hz, H_8), 7.49–7.42 (m, 2H, H_6 and H_7), 7.30 (d, 1H, $^{4}J = 2.2$ Hz, H_5), 4.98 (q, 1H, $^{3}J = 7.0$ Hz, H_b), 4.56 (t, 2H, $^{3}J = 7.5$ Hz, H_e), 4.39 (t, 2H, $^{3}J = 5.3$ Hz, H_c), 3.98–3.96 (m, 1H, H_g), 2.46–2.26 (m, 2H, H_f), 1.64 (d, 3H, $^{3}J = 7.0$ Hz, H_a). ^{13}C NMR (100 MHz, DMSO-d_6): δ 170.25 (C = O), 169.11 (C = O), 144.23 (C), 133.96 (C-Cl), 132.49 (C–Cl), 130.24 (C_7 and C), 129.73 (C_8), 129.02 (C_2), 128.23 (C_6), 123.25 (C_d), 122.16 (C_4), 122.09 (C_5), 116.05 (CN), 91.99 (C_3), 57.58 (C_b), 49.67 (C_g), 45.59 (C_e), 34.45 (C_c), 30.73 (C_f), 18.29 (C_a). HRMS (ESI, CH_3CN): *m/z* calcd for $C_{21}H_{21}Cl_2N_7O_3$ $[M + Na]^+$ 512.0981, *m/z* found 512.0984.

*2-Amino-6-[4-({2-[3-cyano-4-(2,3-dichlorophenyl)-1H-pyrrol-1-yl]propanamido}methyl)-1H-1,2,3-triazol-1-yl]hexanoic acid (**L-15**)*

$R_f = 0.14$ (ethyl acetate/methanol: 5:5), mp = 129–130 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.86 (t, 1H, $^{3}J = 5.5$ Hz, NH), 8.28 (s, 3H, NH_2 and OH), 7.97 (s, 1H, H_d), 7.87 (d, 1H, $^{4}J = 2.3$ Hz, H_2), 7.68 (dd, 1H, $^{3}J = 7.4$ Hz, $^{4}J = 2.2$ Hz, H_8), 7.49–7.42 (m, 2H, H_6 and H_7), 7.30 (d, 1H, $^{4}J = 2.3$ Hz, H_5), 4.99 (q, 1H, $^{3}J = 7.0$ Hz, H_b), 4.38–4.34 (m, 4H, H_c and H_e), 3.95–3.92 (m, 1H, H_i), 1.88–1.64 (m, 4H, H_f and H_h), 1.49 (d, 3H, $^{3}J = 7.0$ Hz, H_a), 1.34–1.24 (m, 2H, H_g). ^{13}C NMR (100 MHz, DMSO-d_6): δ 172.09 (C = O), 170.12 (C = O), 144.97 (C), 134.79 (C–Cl), 133.32 (C–Cl), 131.06 (C_7 and C), 130.55 (C_8), 129.81 (C_2), 129.03 (C_6), 123.53 (C_d), 122.94 (C_4), 122.84 (C_5), 116.77 (CN), 92.01 (C_3), 57.60 (C_b), 51.76 (C_i), 48.97 (C_e), 34.50 (C_c), 29.43 (C_h), 29.28 (C_f), 21.49 (C_g), 18.24 (C_a). HRMS (ESI, CH_3CN): *m/z* calcd for $C_{23}H_{25}Cl_2N_7O_3$ $[M + Na]^+$ 518.1474, *m/z* found 518.1483.

*2-Amino-6-{2-[3-cyano-4-(2,3-dichlorophenyl)-1H-pyrrol-1-yl]propanamido}hexanoic acid (**L-16**)*

$R_f = 0.14$ (ethyl acetate/methanol: 5:5), mp = 112–113 °C. ^{1}H NMR (400 MHz, DMSO-d_6): δ 8.35 (t, 1H, $^{3}J = 5.5$ Hz, NH), 8.27 (s, 3H, NH_2 and OH), 7.85 (d, 1H, $^{4}J = 2.2$ Hz, H_2), 7.68 (dd, 1H, $^{3}J = 7.4$ Hz, $^{4}J = 2.2$ Hz, H_8), 7.49–7.43 (m, 2H, H_6 and H_7), 7.28 (d, 1H, $^{4}J = 2.2$ Hz, H_5), 4.90 (q, 1H, $^{3}J = 7.0$ Hz, H_b), 3.92–3.91 (m, 1H, H_g), 3.17–3.05 (m, 2H, H_c), 1.85–1.71 (m, 2H, H_f), 1.63 (d, 3H, $^{3}J = 7.0$ Hz, H_a), 1.49–1.26 (m, 4H, H_d and H_e). ^{13}C NMR (100 MHz, DMSO-d_6): δ 171.09 (C = O), 169.02 (C = O), 133.98 (C–Cl), 132.49 (C–Cl), 130.26 (C_7 and C), 129.74 (C_8), 128.94 (C_2), 128.25 (C_6), 122.15 (C_4), 122.03 (C_5), 116.06 (CN), 91.94 (C_3), 57.74 (C_b), 51.85 (C_g), 38.89 (C_c), 29.62 (C_f), 28.29 (C_d), 21.70 (C_e), 18.34 (C_a). HRMS (ESI, CH_3CN): *m/z* calcd for $C_{20}H_{22}Cl_2N_4O_3$ $[M + Na]^+$ 459.0967, *m/z* found 459.0986.

3.2 Comparison of the phloem mobility of compounds L-13, L-14, L-15 and L-16 using the *Ricinus* model

The permeability of plant plasma membrane is one of the key determinants of pesticide phloem systemicity, because any pesticides that can be transported in the vascular system must cross the plasma membrane before entering the symplast.[52] The classical approach to improving xenobiotic uptake can be achieved by optimising the physicochemical properties to increase passive membrane penetration.[53] Chemical descriptors and physicochemical properties of our conjugates were calculated and are listed in Table 2. Lipinski's rule of five (Ro5) and Veber's rule, which have been proven as effective approaches firstly for drug bioavailability[54,55] and then for agrochemical uptake,[56,57] were used to predict the plant membrane permeability of **L-13, L-14, L-15** and **L-16**. It should be noted that passive diffusion models

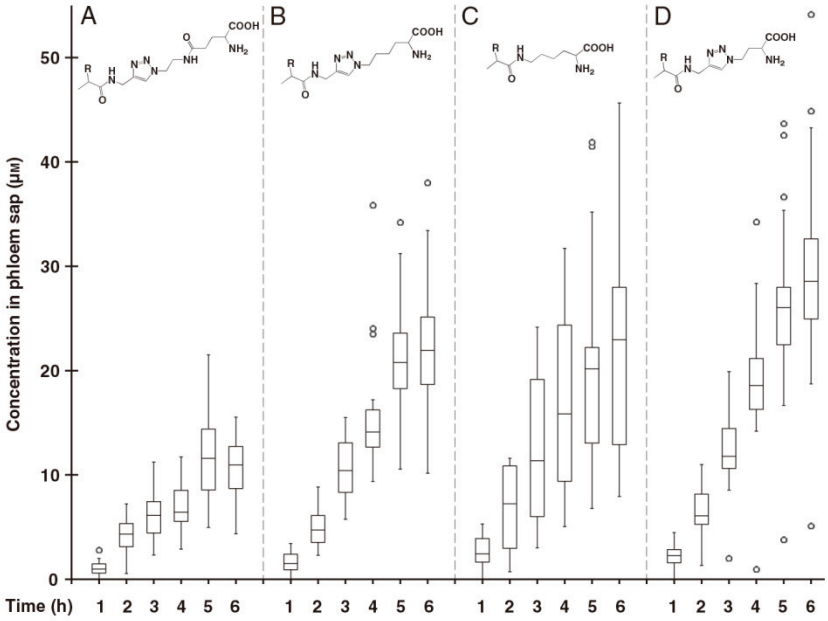

Figure 4. Time course of amino acid conjugate **L-13** (A), **L-15** (B), **L-16** (C) and **L-14** (D) concentrations in phloem sap of *Ricinus*. Cotyledons were incubated in a standard buffered solution at pH 5.0 (Rocher *et al.*[32]) for 30 min, then in the same solution containing compounds **L-13**, **L-15**, **L-16** or **L-14** at 100 µM concentration. After 30 min, the hypocotyl was severed in the hook region and then the sap was collected every hour for 6 h. For box plots, $n = 22$ (A), $n = 18$ (B), $n = 20$ (C and D). R = fenpiclonil.

are not applicable to active carrier-mediated molecules.[58] In addition, they must be supplemented by other models[53,59,60] to explain phloem systemicity or non-systemicity. For instance, fenpiclonil, which is predicted to diffuse through membranes (Table 2), is not phloem mobile, in agreement with Kleier's map.[23]

To determine the influence and contribution of the spacer arm structure on phloem mobility, the three new conjugates were assessed in the *Ricinus* model under the same experimental conditions. The cotyledons of *Ricinus* seedlings were incubated with tested conjugates at 100 µM concentration at pH 5.0, which is close to the pH value of the cotyledon tissue apoplast under our experimental conditions. Our previous study revealed that the concentration of conjugate **L-13** in phloem sap reached a plateau at about 12 µM after 5 h incubation.[24] In the present study, the concentration of conjugates **L-15** and **L-16** increased linearly up to 5–6 h, to reach about 20 µM under our experimental conditions (Fig. 4). The time-course concentration of conjugate **L-14** (which violates Veber's rule) (Table 2) exhibited the same pattern but showed the best systemicity (Fig. 4). At 6 h, its concentration in the phloem sap (about 30 µM) was 2.5 times higher than those of conjugate **L-13** and a glycinergic-fipronil conjugate recently synthesised.[19]

A comparison between conjugates **L-13**, **L-14** and **L-15**, which contain the same triazole spacer, indicated that reducing the chain of the L-amino acid increased phloem mobility, notably by removing the amido group (Fig. 4). In contrast, comparison between conjugate **L-15** (which violates both Lipinski's and Veber's rules) (Table 2) and conjugate **L-16** (which is predicted to diffuse easily through membranes) (Table 2) showed that removing the triazole ring did not have a positive influence on phloem mobility (Fig. 4). In other words, the addition of a triazole spacer did not affect the systemicity of our compounds. Finally, our data are in agreement with the paradigm that passive diffusion models are not suitable for predicting transport mediated by active carrier systems.[58]

3.3 Comparison of phloem mobility of conjugate L-14 and acidic derivatives of fenpiclonil in the physiological pH range of mature leaf apoplast

For about three decades, fluorescence methods have been used to measure the apoplast pH of the leaf tissues of a large number of plant species, gymnosperms included. The data indicated that it ranges from 5.0 to 6.6 according to species, cell types and environmental conditions.[61–67] pH gradients of 0.5–1.0 units exist between the vein apoplast and the mesophyll apoplast. For instance, in *Helianthus annus* the apoplastic pH values are about 5.6 and 6.2 in the vein area and the mesophyll respectively.[64] The most acidic pH values are found in *Vicia faba* and vary from pH 5.0 in the light period to pH 5.4 in the dark period. This oscillation is possibly due to changes in the activity of the plasma membrane H^+-ATPase,[64] which is highly expressed in the phloem tissue of this apoplastic loader.[68] There are two strategies in developing pesticides with an efficient phloem systemicity, initially the ion-trap mechanism[53,59,69] and recently the carrier-mediated process.[16] Using the ion-trap mechanism, fenpiclonil was modified by adding a carboxylic acid group in previous studies.[22,23] The concentration of these acidic derivatives in the phloem sap was closely correlated with the percentage of their undissociated form in the external incubation solution at various biological pH values and droped from pH 5.0 to pH 6.0. For instance, the concentration factor of compound **9** (Table 2) was 0.3, 0.04 and below 0.001 at pH 5.0, 5.5 and 6.0 respectively (Fig. 5B). By contrast, the concentration factor of conjugate **L-14** did not exhibit significant change from pH 5.0 to 6.0 (Fig. 5A). Then, it decreased about 3–4-fold at pH 7.0 and pH 8.0. This pH dependence was similar to active uptake of neutral amino acids, which are manipulated by proton-amino acid symporters.[70,71] There was a residual uptake at neutral and slightly alkaline pH values (Fig. 5A), as is the case for neutral amino acid uptake by leaf tissues.[72] The different phloem mobility behaviours of fenpiclonil acidic derivatives and compound **L-14** indicate that

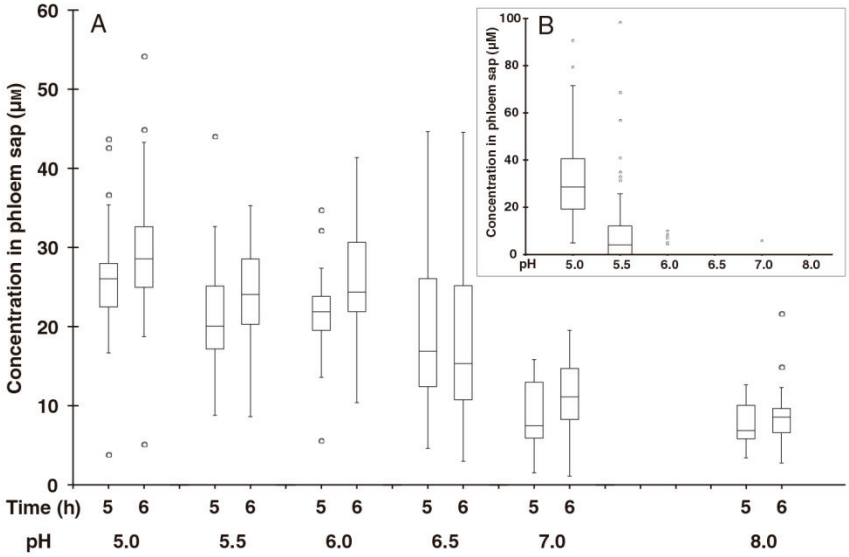

Figure 5. Concentration of amino acid conjugate **L-14** (A) or acidic derivative **9** (B) in phloem sap of *Ricinus* as a function of the pH of the incubation medium. Cotyledons were incubated in a standard buffered solution at pH 5.0, 5.5, 6.0, 6.5, 7 .0 or 8.0 (Rocher *et al.*[32]) for 30 min, then in the same solution containing compound **L-14** or **9** at 100 µM concentration. After 30 min, the hypocotyl was severed in the hook region and then the sap was collected when the concentration reached a plateau, i.e. after 5 and 6 h (A) or during the third and fourth hours of incubation (B). For box plots, $n = 20$ for all pHs (A), $n = 58$ (pH 5.0), $n = 88$ (pH 5.5), $n = 39$ (pH 6.0), $n = 22$ (pH 6.5), $n = 12$ (pH 7.0) and $n = 1$ 1 (pH 8.2) (B).

carrier-mediated processes can be more efficient for conferring systemicity to phenylpyrroles, taking into account the pH values of the leaf tissue apoplast mentioned above.

4 CONCLUSION

In this study, three L-amino acid conjugates of fenpiclonil exhibiting a free L-α-amino acid function were successfully prepared with different lengths of the spacer arm. The selected spacer structure incorporated a triazole ring for two conjugates. The phloem mobility of these conjugates was investigated using the *Ricinus* model. The results showed that the conjugates were manipulated by a pH-dependent transporter system recognising the L-α-amino acid function and translocated at distance in the phloem. Despite the steric hindrance due to these large chlorinated conjugates, i.e. xenobiotics much larger than natural substrates and exhibiting different physicochemical properties, it is possible clearly to improve their phloem mobility by reducing the length of the L-amino acid chain. While the phloem systemicity of acidic derivatives of fenpiclonil was limited to pH values near 5.0, the carrier-mediated conjugates can be transported in the whole range of foliar apoplast pH values (from 5.0 to 6.5) measured in angiosperms and gymnosperms (Fig. 6).

Click chemistry is a practical and reliable approach in the conjugation reaction.[73] Introduction of a triazole ring into the spacer of our conjugates did not have any negative effect on phloem mobility. In addition, no parent compound was detected in phloem sap after the treatment of all four conjugates, indicating that all selected spacer arms were non-degradable in the companion cell–sieve cell complex. However, as a successful prodrug strategy, the active ingredient should be released from the prodrug to exert its biological action. Therefore, in addition to studying the distribution pattern of conjugates within the whole plant as indicated before,[24] it will be interesting to focus investigations on a degradable spacer group in order to release the active ingredient.

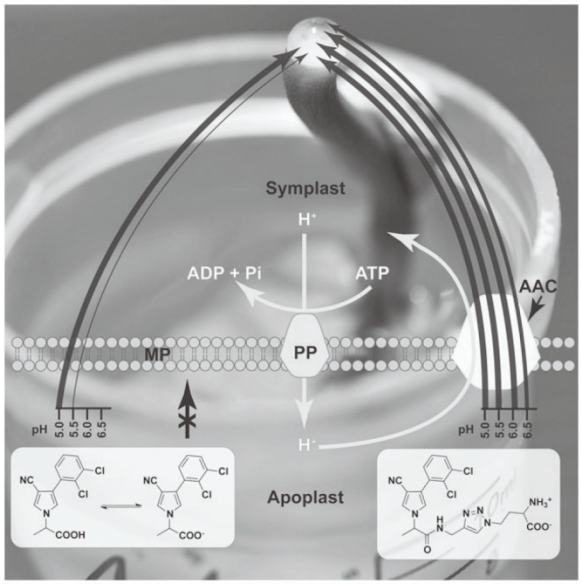

Figure 6. Phloem systemicity dependence of the acidic derivative (**9**) and the L-α-amino acid conjugate (**L-14**) of fenpiclonil at apoplastic pH values (from pH 5.0 to 6.5). The thickness of each arrow is related to the concentrations of both compounds found in the phloem sap. AAC: amino acid carrier; MP: plasma membrane; PP: proton pump.

For example, pH-sensitive linkage can be used, based on the pH difference between the leaf apoplast and symplast.[74]

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REFERENCES

1 Chandrasekaran J, Brumin M, Wolf D, Leibman D, Klap C, Pearlsman M *et al.*, Development of broad virus resistance in non-transgenic cucumber using CRISPR/Cas9 technology. *Mol Plant Pathol* **17**:1140–1153 (2016).
2 Dedryver CA, Le Ralec A and Fabre F, The conflicting relationships between aphids and men: a review of aphid damage and control strategies. *C R Biol* **333**:539–553 (2010).
3 Dogimont C, Bendahmane A, Chovelon V and Boissot N, Host plant resistance to aphids in cultivated crops: genetic and molecular bases, and interactions with aphid populations. *C R Biol* **333**:566–573 (2010).
4 Han HK and Amidon GL, Targeted prodrug design to optimize drug delivery. *AAPS PharmSci* **2**:E6 (2000).
5 Jeschke P, Propesticides and their use as agrochemicals. *Pest Manag Sci* **72**:210–225 (2016).
6 Smith PH, Chamberlain K, Sugars JM and Bromilow RH, Fungicidal activity of *N*-(2-cyano-2-methoximinoacetyl)amino acids and their derivatives. *Pestic Sci* **44**:219–224 (1995).
7 del Amo EM, Urtti A and Yliperttula M, Pharmacokinetic role of L-type amino acid transporters LAT1 and LAT2. *Eur J Pharm Sci* **35**:161–174 (2008).
8 Dobson PD and Kell DB, Carrier-mediated cellular uptake of pharmaceutical drugs: an exception or the rule? *Nat Rev Drug Discov* **7**:205–220 (2008).
9 Fan W, Wu Y, Li XK, Yao N, Li X, Yu YG *et al.*, Design, synthesis and biological evaluation of brain-specific glucosyl thiamine disulfide prodrugs of naproxen. *Eur J Med Chem* **46**:3651–3661 (2011).
10 Janzer M, Larbig G, Kubelbeck A, Wischnjow A, Haberkorn U and Mier W, Drug conjugation affects pharmacokinetics and specificity of kidney-targeted peptide carriers. *Bioconjugate Chem* **27**:2441–2449 (2016).
11 Peura L, Malmioja K, Laine K, Leppanen J, Gynther M, Isotalo A *et al.*, Large amino acid transporter 1 (LAT1) prodrugs of valproic acid: new prodrug design ideas for central nervous system delivery. *Mol Pharm* **8**:1857–1866 (2011).
12 Peura L, Malmioja K, Huttunen K, Leppanen J, Hamalainen M, Forsberg MM *et al.*, Design, synthesis and brain uptake of LAT1-targeted amino acid prodrugs of dopamine. *Pharm Res* **30**:2523–2537 (2013).
13 Sugano K, Kansy M, Artursson P, Avdeef A, Bendels S, Di L *et al.*, Coexistence of passive and carrier-mediated processes in drug transport. *Nat Rev Drug Discov* **9**:597–614 (2010).
14 Sugawara M, Huang W, Fei YJ, Leibach FH, Ganapathy V and Ganapathy ME, Transport of valganciclovir, a ganciclovir prodrug, via peptide transporters PEPT1 and PEPT2. *J Pharm Sci* **89**:781–789 (2000).
15 Zawilska JB, Wojcieszak J and Olejniczak AB, Prodrugs: a challenge for the drug development. *Pharmacol Rep* **65**:1–14 (2013).
16 Chollet JF, Deletage C, Faucher M, Miginiac L and Bonnemain JL, Synthesis and structure–activity relationships of some pesticides with an alpha-amino acid function. *Biochim Biophys Acta* **1336**:331–341 (1997).
17 Yang W, Wu HX, Xu HH, Hu AL and Lu ML, Synthesis of glucose-fipronil conjugate and its phloem mobility. *J Agric Food Chem* **59**:12 534–12 542 (2011).
18 Wu HX, Yang W, Zhang ZX, Huang T, Yao GK and Xu HH, Uptake and phloem transport of glucose-fipronil conjugate in *Ricinus communis* involve a carrier-mediated mechanism. *J Agric Food Chem* **60**:6088–6094 (2012).
19 Xie Y, Zhao JL, Wang CW, Yu AX, Liu N, Chen L *et al.*, Glycinergic-fipronil uptake is mediated by an amino acid carrier system and induces the expression of amino acid transporter genes in *Ricinus communis* seedlings. *J Agric Food Chem* **64**:3810–3818 (2016).
20 Deletage-Grandon C, Chollet JF, Faucher M, Rocher F, Komor E and Bonnemain JL, Carrier-mediated uptake and phloem systemy of a 350-Dalton chlorinated xenobiotic with an alpha-amino acid function. *Plant Physiol* **125**:1620–1632 (2001).
21 Tjamos EC, Problems and prospects in controlling Verticillium wilt, in *Vascular Wilt Diseases of Plants*, ed. by Tjamos EC. Springer, Berlin/Heidelberg, Germany, pp. 441–456 (1989).
22 Chollet JF, Rocher F, Jousse C, Deletage-Grandon C, Bashiardes G and Bonnemain JL, Synthesis and phloem mobility of acidic derivatives of the fungicide fenpiclonil. *Pest Manag Sci* **60**:1063–1072 (2004).
23 Chollet JF, Rocher F, Jousse C, Deletage-Grandon C, Bashiardes G and Bonnemain JL, Acidic derivatives of the fungicide fenpiclonil: effect of adding a methyl group to the *N*-substituted chain on systemicity and fungicidal activity. *Pest Manag Sci* **61**:377–382 (2005).
24 Wu H, Marhadour S, Lei Z-W, Yang W, Marivingt-Mounir C, Bonnemain J-L *et al.*, Vectorization of agrochemicals: amino acid carriers are more efficient than sugar carriers to translocate phenylpyrrole conjugates in the *Ricinus* system. *Environ Sci Pollut Res Int* https://doi.org/10.1007/s11356-016-8107-x (2016).
25 Brik A, Alexandratos J, Lin YC, Elder JH, Olson AJ, Wlodawer A *et al.*, 1,2,3-Triazole as a peptide surrogate in the rapid synthesis of HIV-1 protease inhibitors. *ChemBioChem* **6**:1167–1169 (2005).
26 Bock VD, Speijer D, Hiemstra H and van Maarseveen JH, 1,2,3-Triazoles as peptide bond isosteres: synthesis and biological evaluation of cyclotetrapeptide mimics. *Org Biomol Chem* **5**:971–975 (2007).
27 Valverde IE, Bauman A, Kluba CA, Vomstein S, Walter MA and Mindt TL, 1,2,3-Triazoles as amide bond mimics: triazole scan yields protease-resistant peptidomimetics for tumor targeting. *Angew Chem Int Ed* **52**:8957–8960 (2013).
28 Hein CD, Liu XM and Wang D, Click chemistry, a powerful tool for pharmaceutical sciences. *Pharm Res* **25**:2216–2230 (2008).
29 Kallarackal J, Orlich G, Schobert C and Komor E, Sucrose transport into the phloem of *Ricinus communis* L. seedlings as measured by the analysis of sieve-tube sap. *Planta* **177**:327–335 (1989).
30 Robinson SP and Beevers H, Amino acid transport in germinating castor bean seedlings. *Plant Physiol* **68**:560–566 (1981).
31 Komor E, Rotter M and Tanner W, Proton cotransport system in a higher plant – sucrose transport in *Ricinus communis*. *Plant Sci Lett* **9**:153–162 (1977).
32 Rocher F, Chollet JF, Jousse C and Bonnemain JL, Salicylic acid, an ambimobile molecule exhibiting a high ability to accumulate in the phloem. *Plant Physiol* **141**:1684–1693 (2006).
33 Rocher F, Roblin G and Chollet J-F, Modifications of the chemical structure of phenolics differentially affect physiological activities in pulvinar cells of *Mimosa pudica* L. II. Influence of various molecular properties in relation to membrane transport. *Environ Sci Pollut Res Int* **24**:6910–6922 (2017).
34 Kolb HC, Finn MG and Sharpless KB, Click chemistry: diverse chemical function from a few good reactions. *Angew Chem Int Ed* **40**:2004–2021 (2001).
35 Leyden R and Murphy PV, Glycotriazolophane synthesis via click chemistry. *Synlett* 1949–1950 (**2009**).
36 Meldal M and Tornoe CW, Cu-catalyzed azide-alkyne cycloaddition. *Chem Rev* **108**:2952–3015 (2008).
37 Sheehan JC, Boshart GL and Cruickshank PA, Convenient synthesis of water-soluble carbodiimides. *J Org Chem* **26**:2525–2528 (1961).
38 Valeur E and Bradley M, Amide bond formation: beyond the myth of coupling reagents. *Chem Soc Rev* **38**:606–631 (2009).
39 Sheehan JC and Hess GP, A new method of forming peptide bonds. *J Am Chem Soc* **77**:1067–1068 (1955).
40 Totaro KA, Liao XL, Bhattacharya K, Finneman JI, Sperry JB, Massa MA *et al.*, Systematic investigation of EDC/sNHS-mediated bioconjugation reactions for carboxylated peptide substrates. *Bioconjugate Chem* **27**:994–1004 (2016).
41 Cavender CJ and Shiner VJ, Trifluoromethanesulfonyl azide – its reaction with alkyl amines to form alkyl azides. *J Org Chem* **37**:3567–3569 (1972).
42 Das M, Bandyopadhyay D, Mishra D, Datir S, Dhak P, Jain S *et al.*, 'Clickable', trifunctional magnetite nanoparticles and their chemoselective biofunctionalization. *Bioconjugate Chem* **22**:1181–1193 (2011).
43 Khoee S and Abedini N, One-pot synthesis of amphiphilic nanogels from vinylated SPIONs/HEMA/PEG via a combination of click chemistry and surfactant-free emulsion photopolymerization: unveiling of the protein–nanoparticle interactions. *Polymer* **55**:5635–5647 (2014).
44 Titz A, Radic Z, Schwardt O and Ernst B, A safe and convenient method for the preparation of triflyl azide, and its use in diazo transfer reactions to primary amines. *Tetrahedron Lett* **47**:2383–2385 (2006).
45 Link AJ, Vink MKS and Tirrell DA, Presentation and detection of azide functionality in bacterial cell surface proteins. *J Am Chem Soc* **126**:10 598–10 602 (2004).

46 Huisgen R, Knorr R, Mobius L and Szeimies G, 1,3-Dipolare cycloadditionen. 23. Einige beobachtungen zur addition organischer azide an cc-dreifachbindungen. *Chem Ber Recl* **98**:4014–4021 (1965).

47 Rostovtsev VV, Green LG, Fokin VV and Sharpless KB, A stepwise Huisgen cycloaddition process: copper(I)-catalyzed regioselective 'ligation' of azides and terminal alkynes. *Angew Chem Int Ed* **41**:2596–2599 (2002).

48 Han SY and Kim YA, Recent development of peptide coupling reagents in organic synthesis. *Tetrahedron* **60**:2447–2467 (2004).

49 Nakajima N and Ikada Y, Mechanism of amide formation by carbodiimide for bioconjugation in aqueous-media. *Bioconjugate Chem* **6**:123–130 (1995).

50 Montalbetti C and Falque V, Amide bond formation and peptide coupling. *Tetrahedron* **61**:10 827–10 852 (2005).

51 Siebum AHG, Tsang RKF, van der Steen R, Raap J and Lugtenburg J, Synthesis of (ε-^{13}C,ε-^{15}N)-enriched L-lysine – establishing schemes for the preparation of all possible ^{13}C and ^{15}N isotopomers of L-lysine, L-ornithine, and L-proline. *Eur J Org Chem* 2004:4391–4396 (**2004**).

52 Sicbaldi F, Sacchi GA, Trevisan M and DelRe AAM, Root uptake and xylem translocation of pesticides from different chemical classes. *Pestic Sci* **50**:111–119 (1997).

53 Kleier DA and Hsu FC, Phloem mobility of xenobiotics. 7. The design of phloem systemic pesticides. *Weed Sci* **44**:749–756 (1996).

54 Lipinski CA, Lombardo F, Dominy BW and Feeney PJ, Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev* **64**:4–17 (2012).

55 Veber DF, Johnson SR, Cheng HY, Smith BR, Ward KW and Kopple KD, Molecular properties that influence the oral bioavailability of drug candidates. *J Med Chem* **45**:2615–2623 (2002).

56 Tice CM, Selecting the right compounds for screening: does Lipinski's rule of five for pharmaceuticals apply to agrochemicals? *Pest Manag Sci* **57**:316 (2001).

57 Avram S, Funar-Timofei S, Borota A, Chennamaneni SR, Manchala AK and Muresan S, Quantitative estimation of pesticide-likeness for agrochemical discovery. *J Cheminform* **6**:11 (2014).

58 Rocher F, Chollet JF, Legros S, Jousse C, Lemoine R, Faucher M *et al.*, Salicylic acid transport in *Ricinus communis* involves a pH-dependent carrier system in addition to diffusion. *Plant Physiol* **150**:2081–2091 (2009).

59 Bromilow RH, Chamberlain K and Evans AA, Physicochemical aspects of phloem translocation of herbicides. *Weed Sci* **38**:305–314 (1990).

60 Satchivi NM, Stoller EW, Wax LM and Briskin DP, A nonlinear, dynamic, simulation model for transport, and whole plant allocation of systemic xenobiotics following foliar application. IV: Physicochemical properties requirements for optimum absorption and translocation. *Pestic Biochem Physiol* **84**:83–97 (2006).

61 Husted S and Schjoerring JK, Apoplastic pH and ammonium concentration in leaves of *Brassica napus* L. *Plant Physiol* **109**:1453–1460 (1995).

62 López-Millán AF, Morales F, Abadia A and Abadia J, Effects of iron deficiency on the composition of the leaf apoplastic fluid and xylem sap in sugar beet. Implications for iron and carbon transport. *Plant Physiol* **124**:873–884 (2000).

63 Loubet B, Milford C, Hill PW, Tang YS, Cellier P and Sutton MA, Seasonal variability of apoplastic NH_4^+ and pH in an intensively managed grassland. *Plant Soil* **238**:97–110 (2002).

64 Mühling KH and Läuchli A, Light-induced pH and K+ changes in the apoplast of intact leaves. *Planta* **212**:9–15 (2000).

65 Mühling KH, Plieth C, Hansen UP and Sattelmacher B, Apoplastic pH of intact leaves of *Vicia faba* as influenced by light. *J Exp Bot* **46**:377–382 (1995).

66 Pfanz H and Dietz KJ, A fluorescence method for the determination of the apoplastic proton concentration in intact leaf tissues. *J Plant Physiol* **129**:41–48 (1987).

67 Pitann B, Schubert S and Mühling KH, Decline in leaf growth under salt stress is due to an inhibition of H + -pumping activity and increase in apoplastic pH of maize leaves. *J Plant Nutr Soil Sci* **172**:535–543 (2009).

68 Bouché-Pillon S, Fleurat-Lessard P, Fromont JC, Serrano R and Bonnemain JL, Immunolocalization of the plasma membrane H^+-ATPase in minor veins of *Vicia faba* in relation to phloem loading. *Plant Physiol* **105**:691–697 (1994).

69 Delrot S and Bonnemain J. Absorption et migration des herbicides, in *Les Herbicides, Mode d'action et Principes d'Utilisation*, ed. by Scala R. Éditions INRA, Paris, France, pp. 51–77 (1991).

70 Ortiz-Lopez A, Chang HC and Bush DR, Amino acid transporters in plants. *Biochim Biophys Acta* **1465**:275–280 (2000).

71 Tegeder M and Ward JM, Molecular evolution of plant AAP and LHT amino acid transporters. *Front Plant Sci* **3**:11 (2012).

72 Despeghel JP and Delrot S, Energetics of amino acid uptake by *Vicia faba* leaf tissues. *Plant Physiol* **71**:1–6 (1983).

73 Kolb HC and Sharpless KB, The growing impact of click chemistry on drug discovery. *Drug Discov Today* **8**:1128–1137 (2003).

74 Gillies ER, Goodwin AP and Frechet JMJ, Acetals as pH-sensitive linkages for drug delivery. *Bioconjugate Chem* **15**:1254–1263 (2004).

Chapter 6. Conclusions and perspectives

6.1 General conclusion

Prodrug strategy has been considered as an important concept in design of modern agrochemicals with optimal efficacy, environmental safety, user friendliness and economic variability[1]. Profungicides are promising to offer new opportunities in root and vascular disease control via plant phloem targeting of active ingredients[2,3]. Two propesticide approaches, ion-trap mechanism and carried-mediated transport, have been explored for developing phloem systemic pesticides in the last 20 years[3-7]. However profungicide design is still in an early stage of development, especially for carried-mediated processes.

Most of recent fungicides are substituted with halogens[8] and the molecular weight of 90% fungicides located between 220 and 440 Da[9]. Our objective was to determine the possibility of manipulating the systemicity of fungicides through conjugating them with an amino acid or a glucose. In order to keep the continuity of our academic research, fenpiclonil, which have been studied for a long time in our laboratory, was selected as the model compound in this thesis.

Therefore, we emphasized the carrier-mediated processes of profungicide. The transport mechanism of fenpiclonil conjugates was investigated. In general, the results presented in chapter 3 to 5 provided structure-activity information to optimize the design of carrier-mediated profungicides concerning the selection of promoiety and spacer arm. Key achievements and findings are as following:

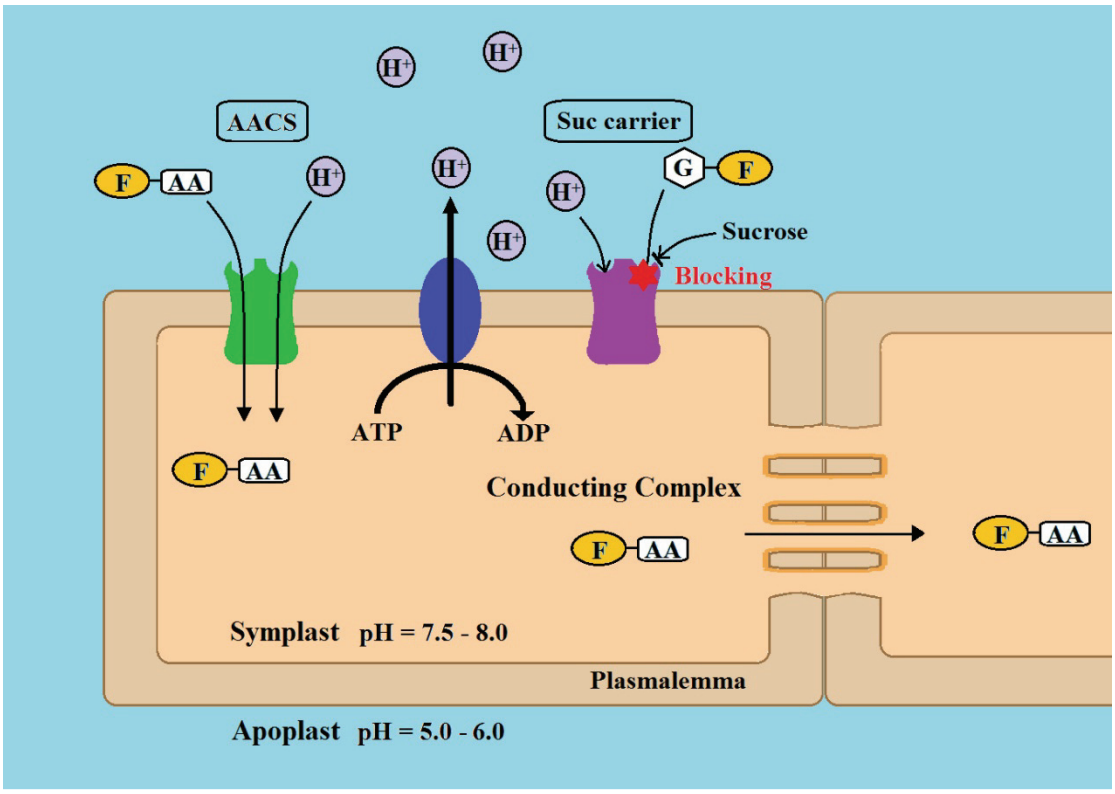

Figure 6-1. Phloem loading mechanism of L-amino acid fenpiclonil conjugates (AA-F) and possible mechanism of sucrose phloem loading inhibition by stearic hindrance due to the large size and structure of D-glucose fenpiclonil conjugate (G-F). For simplification, the amino acid carrier system (AACS) includes all the carriers recognizing the L-amino acid function[10], but the symporters which translocate F-AAs remain to be identified. Conducting complex: no distinction has been made between the sieve element and the companion cell.

1. The physicochemical properties of D-glucose fenpiclonil conjugate (D-GFC) and four L-amino acid fenpilonil conjugates violate the Lipinski's rule of five (Ro5) and Veber rules, which are two passive diffusion rules using to predict the passive membrane permeability of drug candidates and argochemicals. However, all conjugates were detected in phloem sap. The opposing results of prediction and systemicity tests in *Ricinus* model implied that carrier-mediated processes were involved in the phloem systemicity of there large chlorinated conjugates.
2. The phloem mobility of the D-glucose fenpiclonil conjugate and the first synthesized L-glutamic acid conjugate was compared. The L-amino acid promoiety

was dramatically more favourable to phloem mobility than the D-glucose pro-moiety in this study, indicating that amino acid carriers have a relatively higher capacity than sugar carriers to translocate phenylpyrrole conjugates.

3. The L-glutamic acid conjugate was five times more concentrated in phloem sap than its counterpart of D-glutamic acid conjugate and the systemicity of L-derivative is pH dependent and almost completely inhibited by the uncoupler of oxidative phosphorylation CCCP, suggesting that the phloem transport of the L-amino acid conjugate is governed by a stereospecific amino acid carrier system energized by the proton motive force (Fig 6-1).
4. Despite very low concentration of D-GFC in phloem sap, the behavior of this conjugate led us to conduct an investigation of the mechanism of its absorption. In a previous work[11], it has been shown that the systemicity of a glucose-fipronil conjugate possibly involved an active absorption. On the other hand, it is well known that the sucrose transporters are only capable to translocate their natural substrate and some small glucosides. Hence, it is possible that D-GFC is recognized by sugar transporters, but unable to translocate through the transporters.
5. Competitive inhibition tests showed that D-GFC dramatically inhibited the uptake and phloem transport of [^{14}C]-sucrose in *Ricinus*, while no effect on [^{3}H]3-O-methyl-D-glucose or [^{3}H]-glutamine uptake was observed, unlike PCMBS. At 0.5 mM concentration, it also affected (80 % inhibition) sucrose uptake by broad bean leaf tissues and by AtSUC2 expressed in yeast. These results demonstrated that D-GFC can act as a potent and selective inhibitor of sucrose carriers involved in phloem loading and therefore constitute a new tool in phloemology (Fig 6-1).
6. The structure-activity study of the spacer arm between fenpiclonil and the L-α-amino acid function suggested that reducing the length of the chain between the triazole ring and the L-α-amino acid function led clearly to an improvement

of the phloem systemicity in the *Ricinus* model. By contrast, removing the triazole ring in the structure of the spacer arm did not improve systemicity.

7. The L-amino acid conjugate **L-14** was detected in the phloem sap at all physiological pH range of the apoplast (5.0-6.5), while the phloem systemicity of acidic derivatives of fenpiclonil was limited to pH values near 5.0. The different phloem mobility behaviors of fenpiclonil acidic derivatives and L-amino acid conjugate indicated that carrier-mediated processes can be more promising for confering phloem systemicity to phenylpyrroles than the ion-trap mechanism.

6.2 Perspectives

Plant transporters which are capable to transport pesticides may result in the vectorization of agrochemicals as well as reduction of toxicity. There are several unfinished works which might be interesting to promote the development of carrier-mediated profungicide.

1. As amino acid conjugate strategy can improve the phloem systemicity of profungicides, it is also interesting to evaluate *in vitro* and *in vivo* fungicidal activity of amino acid conjugates against plant vascular pathogenic fungus, for example, grapevine trunk diseases and Fusarium wilt of banana.
2. For a successful prodrug strategy, the active ingredient should be released from the prodrug to exert its biological action. All selected spacer arms were non-degradable in this study, so it is interesting to explore a degradable spacer group in order to release the active ingredient. For example, pH-sensitive linkage can be used, based on the pH difference of the plant apoplast and symplast.
3. Phloem-mobile fluorescent probes have been proven to be an effective tool to study phloem loading and transport[12]. Thus we plan to synthesize some fluorescent fungicide conjugates to visualize the uptake and transport of profungicide in the *Ricinus* model.
4. Fenpiclonil, a contact fungicide from the phenylpyrrole family, was used as a model molecule in this study. As carrier-mediated processes were proven to be

effective, the same strategy should be extended to fungicides of different chemical groups and find another suitable active ingredient as parent compound. This strategy also could be extended to some defense molecules.

5. The development of molecular biology provides new opportunities to get more physiological and structural information for plant sucrose and amino acid transporters[13,14]. Identifying the transporters of conjugates might be useful to optimize the structure-activity relationship of carrier-mediated profungicide.

Finally, the prodrug strategy is one of the most promising approaches to enhance the efficacy and reduce the adverse effects of agrochemicals. Continuous efforts are needed to promote the discovery and development of successful propesticides.

References

1. Jeschke, P., *Propesticides and Their Use as Agrochemicals.* Pest Management Science, 2016. **72**(2): p. 210-225.
2. Smith, P. H., Chamberlain, K., Sugars, J. M., and Bromilow, R. H., *Fungicidal Activity of N-(2-Cyano-2-Methoximinoacetyl)Amino Acids and Their Derivatives.* Pesticide Science, 1995. **44**(3): p. 219-224.
3. Deletage-Grandon, C., Chollet, J. F., Faucher, M., Rocher, F., Komor, E., and Bonnemain, J. L., *Carrier-Mediated Uptake and Phloem Systemy of a 350-Dalton Chlorinated Xenobiotic with an Alpha-Amino Acid Function.* Plant physiology, 2001. **125**(4): p. 1620-1632.
4. Chollet, J. F., Rocher, F., Jousse, C., Deletage-Grandon, C., Bashiardes, G., and Bonnemain, J. L., *Synthesis and Phloem Mobility of Acidic Derivatives of the Fungicide Fenpiclonil.* Pest Management Science, 2004. **60**(11): p. 1063-1072.
5. Chollet, J. F., Rocher, F., Jousse, C., Deletage-Grandon, C., Bashiardes, G., and Bonnemain, J. L., *Acidic Derivatives of the Fungicide Fenpiclonil: Effect of Adding a Methyl Group to the N-Substituted Chain on Systemicity and Fungicidal Activity.* Pest Management Science, 2005. **61**(4): p. 377-382.
6. Yang, W., Wu, H. X., Xu, H. H., Hu, A. L., and Lu, M. L., *Synthesis of Glucose-Fipronil Conjugate and Its Phloem Mobility.* Journal of Agricultural and Food Chemistry, 2011. **59**(23): p. 12534-12542.
7. Yuan, J. G., Wu, H. X., Lu, M. L., Song, G. P., and Xu, H. H., *Synthesis of a Series of Monosaccharide-Fipronil Conjugates and Their Phloem Mobility.* Journal of Agricultural and Food Chemistry, 2013. **61**(18): p. 4236-4241.
8. Jeschke, P., *The Unique Role of Halogen Substituents in the Design of Modern Agrochemicals.* Pest Management Science, 2010. **66**(1): p. 10-27.
9. Hao, G. F., Dong, Q. J., and Yang, G. F., *A Comparative Study on the Constitutive Properties of Marketed Pesticides.* Molecular Informatics, 2011. **30**(6-7): p. 614-622.
10. Tegeder, M. and Ward, J. M., *Molecular Evolution of Plant Aap and Lht Amino Acid Transporters.* Frontiers in Plant Science, 2012. **3**.
11. Wu, H. X., Yang, W., Zhang, Z. X., Huang, T., Yao, G. K., and Xu, H. H., *Uptake and Phloem Transport of Glucose-Fipronil Conjugate in Ricinus Communis Involve a Carrier-Mediated Mechanism.* Journal of Agricultural and Food Chemistry, 2012. **60**(24): p. 6088-6094.
12. Knoblauch, M., Vendrell, M., de Leau, E., Paterlini, A., Knox, K., Ross-Elliot, T., Reinders, A., Brockman, S. A., Ward, J., and Oparka, K., *Multispectral Phloem-Mobile Probes: Properties and Applications.* Plant physiology, 2015. **167**(4): p. 1211-1220.
13. Rentsch, D., *Transporters for Amino Acids and Peptides in Plants.* Amino Acids, 2009. **37**(1): p. 47-47.
14. Kuhn, C. and Grof, C. P. L., *Sucrose Transporters of Higher Plants.* Current Opinion in Plant Biology, 2010. **13**(3): p. 287-298.

Publications

（1） **Hanxiang Wu**, Sophie Marhadour, Zhi-Wei Lei, Émilie Dugaro, Cécile Gaillard, Benoit Porcheron, Cécile Marivingt-Mounir, Rémi Lemoine, Jean-François Chollet, Jean-Louis Bonnemain*, Use of D-Glucose–fenpiclonil Conjugate as a Potent and Specific Inhibitor of Sucrose Carriers. *Journal of Experimental Botany*. 2017, 68(20): 5599-5613

（2） Sophie Marhadour, **Hanxiang Wu**, Wen Yang, Cécile Marivingt-Mounir, Jean-Louis Bonnemain, Jean-François Chollet*, Vectorisation of agrochemicals via amino acid carriers: influence of the spacer arm structure on the phloem mobility of phenylpyrrole conjugates in the *Ricinus* system. *Pest Management Science*. 2017, 73(9): 1972-1982

（3） **Hanxiang Wu**, Sophie Marhadour, Zhi-Wei Lei, Wen Yang, Cécile Marivingt-Mounir, Jean-Louis Bonnemain, Jean-François Chollet*, Vectorization of agrochemicals: amino acid carriers are more efficient than sugar carriers to translocate phenylpyrrole conjugates in the Ricinus system. *Environmental Science and Pollution Research*. 2016, (doi:10.1007/s11356-016-8107-x)