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

Vectorization of agrochemicals *via* amino acid carriers in the *Ricinus* system

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ABSTRACT

BACKGROUND: Excessive agrochemical use causes significant threats to environmental safety and human health. Reducing pesticide use without reducing yield is necessary for sustainable agriculture. Therefore we developed a vectorization 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 synthesized 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 (pHs 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 recognized by a pH-dependent transporter system and translocated at distance in the phloem. They exhibited a broader ability to 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 improvement of cultural practices, biological control of pathogens and pests, and development of new strategies conferring genetic resistance.¹⁻³ But, meanwhile, the discovery of optimal efficacy and low-use-rate pesticides to reduce the impact on human health and environment have 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 which not only can keep the dose at the lowest possible level, but also can improve the biological activity of active ingredient. For example, profungicides, which are likely to concentrate in plant phloem tissues, can be applied to control vascular diseases⁶ where the most existent fungicides are unable to access. One of attractive prodrug approach is to conjugate a drug with endogenous substrates, so that the resulting molecules can be recognized and transported by peptide, amino acid or glucose transporters.⁷⁻¹⁵

Carrier-mediated processes have been proved to be a promising approach to enhance the uptake and vectorisation of pesticides by utilizing 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 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 organ where the current commercialized fungicides are unable to access after foliar application.²¹

Fenpiclonil, a non-phloem mobile fungicide was selected in a 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 on phloem-mobile profungicides, a fenpiclonil-glucose and a fenpiclonil-glutamic acid conjugates were synthesized via click chemistry to compare the ability of amino acid and sugar carrier systems for profungicide uptake and phloem mobility.²⁴ The results of systemicity test indicated that the *L*-amino acid moiety was dramatically more favourable to phloem mobility than the *D*-amino acid and *D*-glucose moieties. 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 optimal characterization of chemical structures for recognition and translocation of amino acid conjugate 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 exhibiting 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. The 1,2,3-triazole, as a peptide bond isostere, is often described to improve 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 lead to peptidomimetics with retained receptor affinity and improved tumor-targeting capabilities.²⁷ Using click chemistry, this linker presents the advantage to be synthesized easily and quickly with 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 the spacer arm (i.e. the linkage between fenpiclonil and the α -aminoacid 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 a special attention for apoplast pH values measured in mature leaf tissues of various plant species.

2 MATERIAL AND METHODS

2.1 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. ¹H and ¹³C NMR spectra were performed in DMSO-*d*₆ using a Bruker AVANCE 400 MHz spectrometer. DEPT 135 and ¹H-¹³C 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 : 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 $27^{\circ}\text{C} \pm 1^{\circ}\text{C}$ prior to sowing in vermiculite watered with tap water. Seedlings were grown in a humid atmosphere ($80\% \pm 5\%$) at $27^{\circ}\text{C} \pm 1^{\circ}\text{C}$. 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, E).³² 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).

2.4 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]).

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 compound **11R₁** 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 CuAAC ("Copper(I)-catalyzed Azide-Alkyne Cycloaddition") 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 synthesized 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.

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

We first 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 trifluoromethanesulfonyl 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 trifluoromethanesulfonic anhydride with sodium azide in a mixture water-dichloromethane.⁴²⁻⁴⁴ Thus, the trifluoromethanesulfonyl azide **4** allowed to convert protected α -aminoacid compounds **5** or **6** respectively into the desired azides **7** and **8** in a one-pot reaction⁴⁵ in presence of potassium carbonate and copper (II) sulfate 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 mixture dichloromethane/water (42 mL, 2/1) trifluoromethanesulfonic anhydride (3.33 mL, 20.0 mmol, 2 equiv) was added 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 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) sulfate pentahydrate (25.0 mg, 0.10 mmol, 0.01 equiv) in a mixture methanol/water (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 fempiclonil **10** by click chemistry (Fig. 2; compounds **11R₂**, **11R₃**)

For this step, we prepared a key intermediate of fempiclonil 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 **11R₂** and **11R₃**.^{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 mixture *tert*-butanol-water. In these conditions the copper-catalyzed reaction allowed the synthesis of the 1,4-disubstituted regioisomers specifically.⁴⁷ The compounds **11R₂** and **11R₃** were obtained in moderate yields, 40% and 44% respectively. In a general approach, this pathway offers the possibility of a wide diversity for further structure-activity relationship study.

*Experimental procedure for the synthesis of derivatives **11R₂** and **11R₃** (Fig. 3)*

To a solution of compound **10** (3.47 g, 10.0 mmol, 1 equiv) in *tert*-butanol (28 mL), the solution of compound **7** or compound **8** previously prepared 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 ethyl acetate. The organic layer was extracted, washed with saturated ammonium chloride solution and brine, dried over MgSO₄, 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 **11R₂** as a pink powder (2.4 g, 40% yield) or compound **11R₃** as a beige powder (2.7 g, 44% yield).

2-(*Tert*-butoxycarbonylamino)-4-(4-{[2-(3-cyano-4-(2,3-dichlorophenyl)-1*H*-pyrrol-1-yl)propanamido]methyl}-1*H*-1,2,3-triazol-1-yl)butanoic acid (**11R₂**)

R_f = 0.60 (ethyl acetate/methanol: 5/5); Mp = 163-164 °C. ¹H NMR (400 MHz, DMSO-*d*₆): δ 13.01 (s, 1H, OH), 8.83 (t, 1H, ³*J* = 5.2 Hz, NH), 7.98 (s, 1H, H_d), 7.88 (d, 1H, ⁴*J* = 2.2 Hz,

H₂), 7.68 (dd, 1H, ³J = 7.3 Hz, ⁴J = 2.3 Hz, H₈), 7.49-7.43 (m, 2H, H₆ and H₇), 7.30 (d, 1H, ⁴J = 2.2 Hz, H₅), 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). ¹³C 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 (C₇ and C), 129.71 (C₈), 129.03 (C₂), 128.21 (C₆), 123.01 (C_d), 122.17 (C₄), 122.08 (C₅), 116.05 (CN), 91.98 (C₃), 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, CH₃CN): *m/z* calculated for C₂₆H₂₉Cl₂N₇O₅ [M + Na]⁺ 612.1505, *m/z* found 612.1501.

2-(*Tert*-butoxycarbonylamino)-6-(4-{[2-(3-cyano-4-(2,3-dichlorophenyl)-1*H*-pyrrol-1-yl)propanamido]methyl}-1*H*-1,2,3-triazol-1-yl)hexanoic acid (**11R₃**)

R_f = 0.13 (ethyl acetate/methanol: 9/1); M_p = 142-143 °C. ¹H 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, H₂), 7.68 (dd, 1H, ³J = 7.1 Hz, ⁴J = 2.5 Hz, H₈), 7.48-7.44 (m, 2H, H₆ and H₇), 7.30 (d, 1H, ⁴J = 2.2 Hz, H₅), 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). ¹³C 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 (C₇ and C), 129.72 (C₈), 129.01 (C₂), 128.22 (C₆), 122.81 (C_d), 122.18 (C₄), 122.08 (C₅), 116.05 (CN), 92.00 (C₃), 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, CH₃CN): *m/z* calculated for C₂₈H₃₃Cl₂N₇O₅ [M + Na]⁺ 640.1818, *m/z* found 640.1812.

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

We next investigated the preparation of another compound without the 1,2,3-triazole ring as 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 like commercial drugs.³⁸ The carboxylic acid moiety was first activated by an appropriate coupling reagent to form the *O*-acylisourea, a process allowing to convert the hydroxyl group of the acid into a good leaving group. The *O*-acylisourea intermediate was obtained from compound **9** previously described²³ using the 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 by-product 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 MgSO₄, 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)-1*H*-pyrrol-1-yl)propanamido]hexanoic acid (**12**)

R_f = 0.16 (ethyl acetate/methanol: 9/1); Mp = 107-108 °C. ¹H NMR (400 MHz, DMSO-*d*₆): δ 12.52 (s, 1H, OH), 8.28 (t, 1H, ³*J* = 5.5 Hz, NH), 7.85 (d, 1H, ⁴*J* = 2.2 Hz, H₂), 7.68 (dd, 1H, ³*J* = 7.2 Hz, ⁴*J* = 2.3 Hz, H₈), 7.49-7.43 (m, 2H, H₆ and H₇), 7.28 (d, 1H, ⁴*J* = 2.2 Hz, H₅), 7.05 (s, 1H, NH), 4.90 (q, 1H, ³*J* = 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). ¹³C 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 (C₇), 130.24 (C), 129.71 (C₈), 128.95 (C₂), 128.22 (C₆), 122.14 (C₅), 122.01 (C₄), 116.05 (CN), 91.92 (C₃), 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, CH₃CN): *m/z* calculated for C₂₅H₃₀Cl₂N₄O₅ [M + Na]⁺ 559.1491, *m/z* found 559.1492.

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

The last step consisted of the deprotection of the α -aminoacid function of the compounds **11R₂**, **11R₃** and **12**.⁵¹ The reaction was performed 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 respectively to the final compounds **L-14**, **L-15** and **L-16** with 98% yield.

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

The compound **11R₂** (1.77 g, 3.0 mmol) or **11R₃** (1.86 g, 3.0 mmol) or **12** (1.61 g, 3.0 mmol) was diluted in a mixture anhydrous dichloromethane – trifluoroacetic acid (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 co-evaporated with ethyl acetate, 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)-1*H*-pyrrol-1-yl)propanamido]methyl}-1*H*-1,2,3-triazol-1-yl)butanoic acid (**L-14**)

R_f = 0.12 (ethyl acetate/methanol: 5/5); M_p = 174-175 °C. ¹H NMR (400 MHz, DMSO-*d*₆): δ 8.87 (t, 1H, ³*J* = 5.3 Hz, NH), 8.46 (s, 3H, NH₂ and OH), 8.03 (s, 1H, H_d), 7.87 (d, 1H, ⁴*J* = 2.2 Hz, H₂), 7.68 (dd, 1H, ³*J* = 7.4 Hz, ⁴*J* = 2.2 Hz, H₈), 7.49-7.42 (m, 2H, H₆ and H₇), 7.30 (d, 1H, ⁴*J* = 2.2 Hz, H₅), 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). ¹³C 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 (C₇ and C), 129.73 (C₈), 129.02 (C₂), 128.23 (C₆), 123.25 (C_d), 122.16 (C₄), 122.09 (C₅), 116.05 (CN), 91.99 (C₃), 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, CH₃CN): *m/z* calculated for C₂₁H₂₁Cl₂N₇O₃ [M + Na]⁺ 512.0981, *m/z* found 512.0984.

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

R_f = 0.14 (ethyl acetate/methanol: 5/5); M_p = 129-130 °C. ¹H NMR (400 MHz, DMSO-*d*₆): δ 8.86 (t, 1H, ³*J* = 5.5 Hz, NH), 8.28 (s, 3H, NH₂ and OH), 7.97 (s, 1H, H_d), 7.87 (d, 1H, ⁴*J* = 2.3 Hz, H₂), 7.68 (dd, 1H, ³*J* = 7.4 Hz, ⁴*J* = 2.2 Hz, H₈), 7.49-7.42 (m, 2H, H₆ and H₇), 7.30 (d, 1H, ⁴*J* = 2.3 Hz, H₅), 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). ¹³C 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 (C₇ and C), 130.55 (C₈), 129.81 (C₂), 129.03 (C₆), 123.53 (C_d), 122.94 (C₄), 122.84 (C₅), 116.77 (CN), 92.01 (C₃), 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, CH₃CN): *m/z* calculated for C₂₃H₂₅Cl₂N₇O₃ [M + Na]⁺ 518.1474, *m/z* found 518.1483.

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

R_f = 0.14 (ethyl acetate/methanol: 5/5); M_p = 112-113 °C. ¹H NMR (400 MHz, DMSO-*d*₆): δ 8.35 (t, 1H, ³*J* = 5.5 Hz, NH), 8.27 (s, 3H, NH₂ and OH), 7.85 (d, 1H, ⁴*J* = 2.2 Hz, H₂), 7.68

(dd, 1H, $^3J = 7.4$ Hz, $^4J = 2.2$ Hz, H₈), 7.49-7.43 (m, 2H, H₆ and H₇), 7.28 (d, 1H, $^4J = 2.2$ Hz, H₅), 4.90 (q, 1H, $^3J = 7.0$ 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, $^3J = 7.0$ 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 (C₇ and C), 129.74 (C₈), 128.94 (C₂), 128.25 (C₆), 122.15 (C₄), 122.03 (C₅), 116.06 (CN), 91.94 (C₃), 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, CH₃CN): m/z calculated for C₂₀H₂₂Cl₂N₄O₃ [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 determinant 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 improve xenobiotic uptake can be achieved through optimizing the physicochemical properties to increase passive membrane penetration.⁵³ Chemical descriptors and physicochemical properties of our conjugates were calculated and listed in Table 2. Lipinski's rule of five (Ro5) and Veber's rule which have been proven as effective approaches first for drug bioavailability^{54, 55} and then for agrochemicals uptake,^{56, 57} were used to predict plant membrane permeability of **L-13**, **L-14**, **L-15** and **L-16**. It should be noted that passive diffusion models 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 the 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 the Veber's rule, Table 2) exhibited the same pattern but showed the best systemicity (Fig. 4). At time 6 h, its concentration in the phloem sap (about

30 μ M) was 2.5 times higher than these of conjugate **L-13** and a glycinergic-fipronil conjugate recently synthesized.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 influence positively phloem mobility (Fig. 4). In other words, 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 to predict 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 indicate 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 unit exist between the vein apoplast and the mesophyll apoplast. For instance, in *Helianthus annuus* 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 varies from pH 5.0 in light period to pH 5.4 in dark period. This oscillation is possibly due to changes in the activity of the plasmamembrane H⁺-ATPase⁶⁴ 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 carboxylic acid group in previous works.^{22, 23} The concentration of these acidic derivatives in the phloem sap was closely correlated to the percentage of their undissociated form in the external incubation solution at various biological pH values and dropped 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 by about 3-4 times at pHs 7.0 and 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 it is

the case for neutral amino acid uptake by leaf tissues.⁷² The different phloem mobility behaviors of fenpiclonil acidic derivatives and compound **L-14** indicate that 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 length of 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 recognizing 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 physico-chemical properties, it is possible to clearly 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 conjugation reaction.⁷³ Introduction of a triazole ring in the spacer of our conjugates did not display 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 of studying the distribution pattern of conjugates within the whole plant as indicated before,²⁴ it will be interesting to focus the investigations on degradable spacer group in order to release the active ingredient. For example, pH-sensitive linkage can be used based on the pH difference between the leaf apoplast and symplast.⁷⁴

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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 tested 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* (28).

- First step (only when spacer includes 1,2,3-triazole ring): obtention of azido derivatives N_3-R_x from protected amino acids

- Second step: coupling azido derivatives N_3-R_x and a propargyl derivative of fenpiclonil by click chemistry (when spacer includes 1,2,3-triazole ring) or protected amino acid derivative **6** with the carboxylic acid derivative of fenpiclonil **9**

- Third step: deprotection of the amino acid moiety

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

Figure 3. Fenciclonil derivatives numbering for ^1H and ^{13}C assignments

Figure 4. Time course of amino acid conjugates **L-13** (A), **L-15** (B), **L-16** (C) and **L-14** (D) 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 compounds **L-13**, **L-15**, **L-16** or **L-14** at 100 μ M 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.

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, 2006) 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 reach a plateau, ie after 5 and 6 hours (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 = 11 (pH 8.2) (B).

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.

Table 1. Chromatographic data for tested products

Mobile Phase (gradient)			Delivery (ml.min ⁻¹)	Detection UV (nm)	Compound	Retention Time (min)
Time (min)	Water + TFA 0.1%	CH ₃ CN				
t = 0	70	30	0.8	218	Fenpiclonil	24.6
					9	22.9
t = 30	40	60			L-13	8.0
t = 35	70	30			L-14	8.8
					L-15	9.7
t = 37	70	30			L-16	9.2

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 ($MW \leq 500$ Da ; $HBD \leq 5$; $HBA \leq 10$; $\log P \leq 5.0$) and to Veber rule ($FRB \leq 10$; $PSA \leq 140 \text{ \AA}^2$). At biological pHs (from 5.0 to 8.0), compound **9** is predicted to be almost under its undissociated form ($pK_a = 3.58 \pm 0.40$) and compounds **L-13** – **L-16** are predicted to be under their zwitterionic form.
MW : molecular weight ; *HBD* : number of hydrogen bond donors ; *HBA* : number of hydrogen bond acceptors ; *FRB* : free rotatable bonds ; *PSA* : polar surface area.

No	Structure	MW	HBD	HBA	Log D (pH 5.0)	Log D (pH 8.0)	FRB	PSA ($\text{\AA}^2$)	Lipinski's rule of five violation (*)	Veber rules violation (**)
Fenpi clonil		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