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Derivatives of Pesticides with an α-Amino Acid Function: Synthesis and Effect on Threonine Uptake

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Abstract: The synthesis of several derivatives of a pesticide and an α-amino acid are described. The process involves three steps, the last one (deprotection of the α-amino acid group) being the most critical. The resulting products preserved their α-amino acid group and were globally neutral. They were tested for their effect on the transport of a neutral amino acid, threonine, and two sugars, sucrose and glucose. Two derivatives markedly and specifically inhibited threonine uptake by leaf tissues of *Vicia faba* L. and phloem loading. Preliminary experiments suggested that the lysine-2,4-D derivative is a competitive inhibitor of threonine uptake. The apparent K_i (0·5 mM) was much lower than the apparent K_m (3 mM) of the natural substrate.

1 INTRODUCTION

Most systemic pesticides move within the apoplast (vessels, cell walls). However, some herbicides are translocated using a symplastic pathway, via the intracellular medium and phloem. These xenobiotics enter the cytoplasm by diffusion across the lipidic bilayer of the plasma membrane. Most are weak acids which accumulate in the phloem by the 'acid-trap' mechanism.[1–5] Some compounds, which are neutral products, are nevertheless phloem-mobile because of their intermediate permeability coefficient.[1,4,6,7]

Recently, it has been shown that two herbicides enter into the cells by plasma membrane carriers: glyphosate, which is transported by the phosphate carrier[8,9] and paraquat, transported by a polyamine carrier.[10,11] In spite of its theoretical and practical interest, the xenobiotic/plasma membrane carrier recognition process is a little-explored field. It would be interesting to synthesize xenobiotics capable of inhibiting the membrane transport of a natural substrate, thus disturbing the growth of organisms by nutrient deficiency, or to develop xenobiotics which are transported by a carrier, thus enhancing their transport to their target site via conducting tissues, especially phloem.

With the exception of a number of herbicides, phloem-mobile pesticides are uncommon. From this point of view, amino acids and small peptides, carriers of plasma membrane, have a strategic interest. In higher plants, depending on the plant material, all amino acids are transported into the cytoplasm by two to four carriers[12–22] whose specificity depends, among other things, on the charge of the amino acid carried. The same carrier is able to recognize and to transport amino acids of various sizes. As the α-amino acid function is essential for this recognition,[23] it would be interesting to test the effect on amino acid transport of pesticides with an α-amino acid function. As such compounds do not exist, we have developed methods for their synthesis and have studied their effects on the uptake of a neutral α-amino acid, threonine. In sugar-beet leaves, this amino acid is translocated by the two carriers involved in neutral amino acid translocation.[18]

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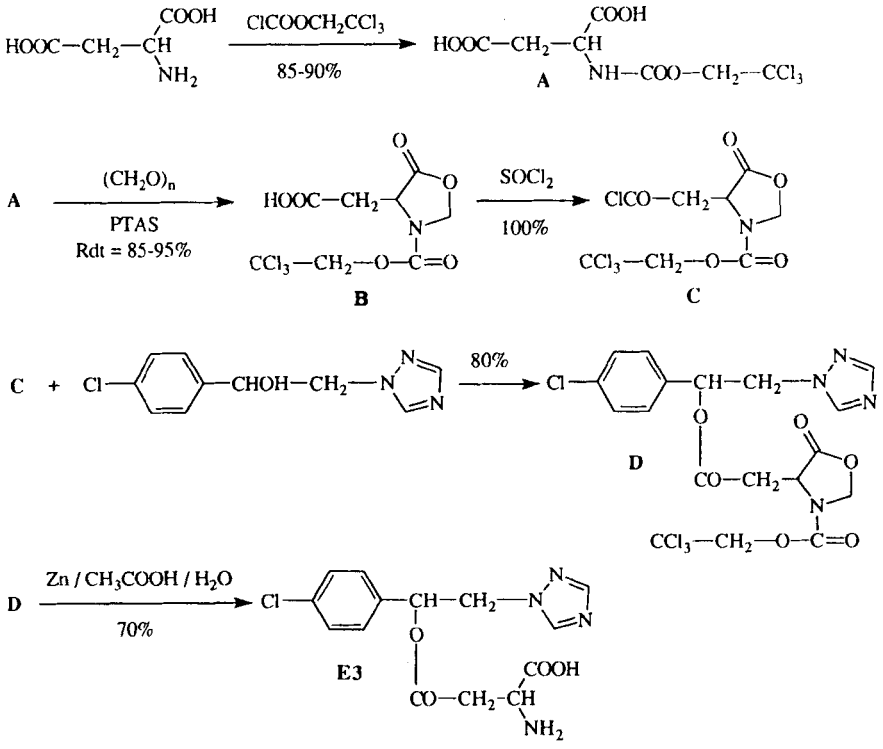


Fig. 1. General reaction scheme.

2 EXPERIMENTAL

2.1 Materials

[^{3}H]Threonine (15 Ci mmol^{-1}), [^{3}H]sucrose (14·4 Ci mmol^{-1}), [^{3}H]glucose (2·35 Ci mmol), [^{14}C]threonine (233 mCi mmol^{-1}) and [^{14}C]sucrose (10 mCi mmol^{-1}) were supplied by Amersham International.

2.2 Synthetic methods

2.2.1 General

In order to synthesize the amino acid–pesticide conjugate, it was necessary to choose an α-amino acid with an additional functional group (β- or γ-carboxyl group of aspartic and glutamic acids or amino group of lysine) and a pesticide which was able to react with that group. The synthesis of these compounds involved three steps:

—selective blocking of the α-amino acid group,
—conjugation of the carboxyl or amino group with the pesticide,
—selective deprotection of the α-amino acid group.

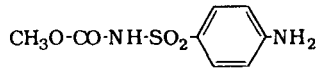
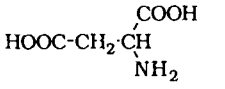
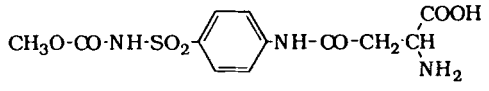
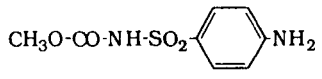
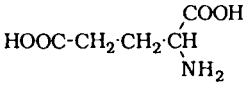
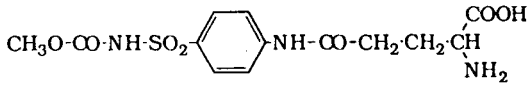
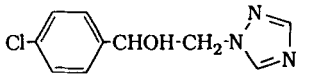
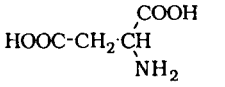
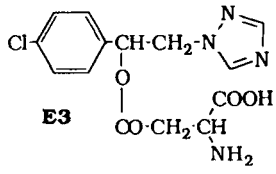
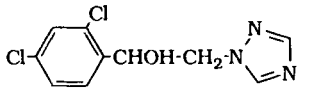
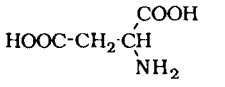
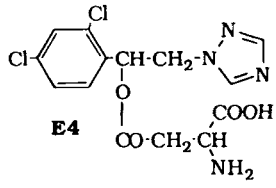
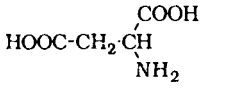
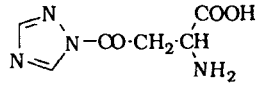
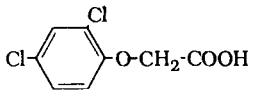
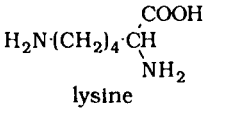
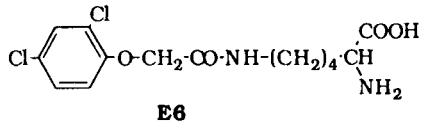
This can be illustrated by the typical example shown in Fig. 1. The resulting amino acid–pesticide conjugates are white solids insoluble in organic solvents, slightly soluble in water and freely soluble in hydrochloric acid (6 M). NMR spectra were recorded in deuterium chloride/deuterium oxide using sodium 3-(trimethylsilyl)(2,2,3,3-$^2H_4$)propionate as internal standard on a JEOL EX 90 spectrometer at 89·5 MHz (^{1}H) and 22·5 MHz (^{13}C). For all compounds, NMR spectra were in agreement with the expected structures, which are shown in Table 1.

2.2.2 Aspartic and glutamic acid derivatives

Product **E3** is a typical example. The mixed masking of aspartic acid was carried out according to an already described method[24] and only the reaction with the pesticide and the deprotection of α-amino acid function are described here. A solution of oxazolidinone **B** (0·01 mol; 3·2 g) and thionyl chloride (0·05 mol; 3·7 ml) in carbon tetrachloride (2 ml) was refluxed until no more gas evolved. After removal of the solvent and excess thionyl chloride, the chloride **C** was treated with dichloromethane (2 × 15 ml) and this solution was evaporated to remove traces of thionyl chloride and hydrogen chloride. The acid chloride **C** was then dissolved in dichloromethane (50 ml). Under a nitrogen stream, 1-(4-chlorophenyl)-2-(1*H*-1,2,4-triazol-1-yl)ethanol (0·0095 mol; 2·13 g) was added gradually, alternately with pyridine (0·0095 mol; 0·8 ml). The mixture was heated at 40°C for 3 h and after cooling it was diluted with dichloromethane (20 ml) then washed with water (15 ml), sodium hydrogen carbonate saturated solution (15 ml) and again with water (2 × 15 ml). After drying the organic phase over magnesium sulfate, solvents were removed under reduced pressure. The product **D** appeared as a brown oil (4·47 g; 85%).

To compound **D** (0·0019 mol; 1 g) in solution in acetic acid (6 ml) and water (12 ml), powdered zinc was added (1 g). After 5 min, zinc was filtered off and the aqueous phase was washed with dichloromethane (2 × 10 ml).

TABLE 1
Characteristics of Amino Acid–Pesticide Derivatives.

 asulam	 aspartic acid	 **E1**	77%
 asulam	 glutamic acid	 **E2**	70%
 1-[4-chlorophenyl]-2-[(1H)-1,2,4-triazol-1-yl] ethanol	 aspartic acid	 **E3**	70%
 1-[2,4-dichlorophenyl]-2-[(1H)-1,2,4-triazol-1-yl] ethanol	 aspartic acid	 **E4**	65%
 1H-1,2,4-triazole	 aspartic acid	 **E5**	70%
 2,4-dichlorophenoxyacetic acid	 lysine	 **E6**	65%

Removal of water and acetic acid left **E3** as white crystals which were treated with methanol (25 ml) and then dried under vacuum (0·47 g; 70%; mp > 220°C).

2.2.3 Lysine derivatives

Product **E6** is a typical example.

In a 1-litre round-bottom flask, was placed lysine (0·05 mol; 7·3 g), copper(II)carbonate/copper(II)hydroxide (0·07 mol; 15·5 g) and water (600 ml). After refluxing for 2 h and filtering, the reaction mixture was concentrated to 100 ml. Concurrently with this operation, the acid chloride of 2,4-D was synthesized in a similar manner to that described in Section 2.2.2. The condensation between the copper complex of lysine and the acid chloride of 2,4-D was carried out in the following way: copper complex (0·025 mol/100 ml water) and sodium hydrogen carbonate solution (1 M; 50 ml) were placed in a 500-ml three-necked flask equipped in a classical manner. The mixture was cooled to 5°C and two solutions X and Y with the following compositions were then added alternately in small portions:

X: acid chloride of 2,4-D (0·054 mol) dioxane (30 ml)
Y: potassium hydroxide (0·065 mol; 3·65 g) water (16 ml)

The mixture was then kept for 15 min at 5°C and 4 h at room temperature. After filtration on a sintered glass funnel, crystals of the product (**D6**) were washed with water (100 ml), absolute ethanol (100 ml) and diethyl ether (100 ml) then dried under vacuum over phosphorus pentoxide (6·3 g; 35%).

The deprotection of the α-amino acid function was carried out in the following way: to a boiling aqueous solution of ethylenediamine tetraacetic acid disodium salt (0·017 mol; 6·5 g; 175 ml water), was added **D6** (0·0087 mol; 6·3 g). After boiling for 30 min and subsequent cooling to 4°C, **E6** was obtained as white crystals which were isolated by filtration, washed with absolute alcohol (50 ml) and diethyl ether (50 ml) and finally dried under vacuum (1·95 g; 65%; mp > 260°C).

2.3 Biological testing

2.3.1 Plant material

Broad bean plants (*Vicia faba* L. cv. Aguadulce) were grown on vermiculite under a controlled environment: 16 h light (14 W m^{-2}, Sylvania tubes F65 W gro-lux) at 20(±1)°C and 8 h dark at 16(±1)°C; relative humidity

was 60(±5)%. The plants were watered with Hoagland's solution. The experiments were performed on plants possessing four mature bifoliate leaves.

Leaf discs (1·13 cm^2 surface) were obtained with a cork borer from the third and the fourth mature leaves, after the lower epidermis had been stripped off to facilitate exchanges between the tissues and the incubation medium.[25]

2.3.2 Uptake experiments

All the uptake experiments were performed in the light at pH 4·0. This pH value of the incubation medium was chosen for two reasons: first, uptake of neutral amino acids by *V. faba* leaf tissues was optimal in those conditions,[26] second, an acid medium favoured solubilization of the products. After removal of the lower epidermis, the discs were floated on a buffered solution: citric acid (10 mM); disodium phosphate (20 mM), containing mannitol (250 mM, as an osmoticum), calcium chloride (0·5 mM) and magnesium chloride (0·25 mM). This apoplast washing lasted for 30 min. The solutions, contained in Petri dishes, were continuously stirred on a reciprocal shaker (60 shakes per min). After this preincubation, different sets of discs were incubated in the same buffered solution with different concentrations of [^{3}H]threonine, [^{3}H]sucrose or [^{3}H]glucose (111 kBq in 25 ml) and for various times. The treated sets contained one of the amino acid–pesticide derivatives at different concentrations or the pesticide itself.

The amino acid–pesticide derivatives were solubilized in hydrochloric acid (6 M; 1% of final volume of incubation medium); the medium was readjusted to pH 4 with sodium hydroxide. Experiments reported elsewhere[27] showed that the uptake of organic nutrients is dependent on the osmotic pressure of the incubation medium. Therefore, in the control, the same quantities of hydrochloric acid and sodium hydroxide were added. The osmotic pressure of the medium (280 mM) was therefore identical in control and treated sets. 2,4-D and triazolyl alcohol (4-chloro derivative) were solubilized in 95° ethanol (0·2% of final volume of incubation medium). All the derivatives were stable under the experimental conditions: this was confirmed by NMR spectroscopy and HPLC.

At the end of incubation, the tissues were rinsed (2 × 3 min) in a similar medium to the preincubation solution. Discs were harvested and digested in a mixture of perchloric acid (65%; 20 µl), hydrogen peroxide (33%; 40 µl) and 'Triton' X-100 (1 g $litre^{-1}$; 50 µl). The radioactivity was counted by liquid scintillation spectrometry (Packard Tricarb 1900 TR).

Some discs were incubated with ^{14}C-nutrients (threonine or sucrose, 185 kBq in 25 ml). After 60 min incubation, the discs were rinsed, dry-ice frozen, lyophilized and autoradiographed.

3 RESULTS

The uptake of 1 mM [^{3}H]threonine was linear with time over 90 min (Figs 2 and 3).[26] Since the solubilities of the derivatives were low, the highest concentrations tested were 2 mM for **E3** and 5 mM for **E1**, **E5** and **E6**. The lysine–2,4-D derivative (**E6**) inhibited significantly the uptake of 1 mM [^{3}H]threonine: inhibition was 35% at 0·1 mM xenobiotic and about 90% at 5 mM. This inhibition was observed as soon as uptake began. The aspartic acid–triazolyl alcohol derivative (**E3**) also was an effective inhibitor, although less so than **E6**. The inhibition was about 55% with 2 mM xenobiotic and was detectable within the first minute (Fig. 3). The uptake of 1 mM [^{3}H]threonine was only slightly affected by 5 mM aspartic acid–asulam (**E1**) whereas 5 mM aspartic acid–1,2,4-triazole (**E5**) was without effect (Table 2).

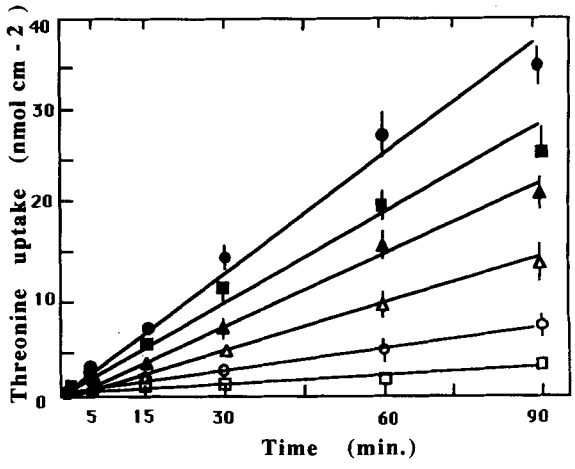


Fig. 2. Time course of the effect of various concentrations of **E6** on 1 mM threonine uptake. (●) control; (■) 0·1 mM **E6**; (▲) 0·25 mM **E6**; (△) 1 mM **E6**; (○) 2·5 mM **E6**; (□) 5 mM **E6**). Each point is the mean of 20 values. Bar = SE.

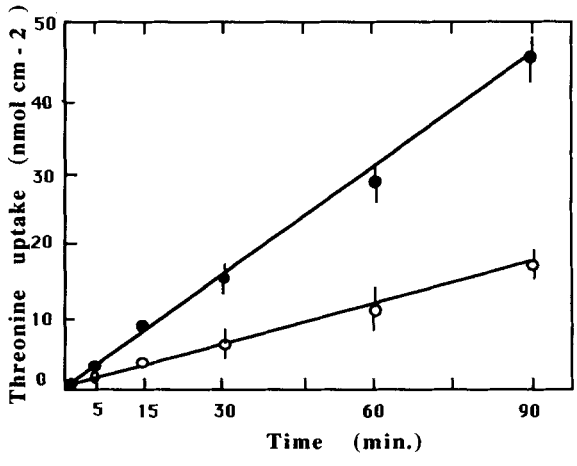


Fig. 3. Time course of the effect of (○) 2 mM **E3** on 1 mM threonine uptake; (●) control. Each point is the mean of 20 values. Bar = SE.

TABLE 2
Effect of Aminoacid–Pesticide Derivatives on Threonine Uptake by *Vicia faba* Leaf Tissues

	E1		**E3**		**E5**		**E6**		
Derivative (mM)	*0*	*5*	*0*	*2*	*0*	*5*	*0*	*2·5*	*5*
Uptake of 1 mM threonine[a] (nmol cm^{-2} h^{-1})	24·8(±1·7)	20·2(±1·6) (NS)[b]	28·4(±2·6)	12·5(±2) (S)[b]	29(±1·1)	29·2(±2) (NS)[b]	27·5(±2·5)	4(±0·6) (S)[b]	2·2 (S)[b]
(%)	100	81·5	100	44·1	100	99·3	100	14·5	8

[a] Each value is the mean of 50 values (±SE).
[b] NS = no significant effect, S = significant effect.

Autoradiographs of control discs (Fig. 4A) showed that both mesophyll and veins were involved in [^{14}C]threonine uptake, although the veins were labelled the most, as found previously.[26] The inhibitory effect of 2·5 mM **E6** (Fig. 4B) and 2 mM **E3** (Fig. 4C) on the uptake of 1 mM threonine concerned both mesophyll and veins; thus, the phloem loading of threonine was strongly inhibited by these two products. By contrast, no effect of 2·5 mM **E6** or 2 mM **E3** products was found on the uptake of 1 mM [^{3}H]sucrose or 1 mM [^{3}H]glucose (Table 3). Autoradiographs confirmed that the phloem loading of [^{14}C]sugars was not modified (Figs 4D to 4F).

Triazolyl alcohol (the pesticide moiety of **E3**), tested at the same concentration as the **E3** derivative, did not change [^{3}H]threonine or [^{3}H]sugar uptake (Table 4). Influx of the three nutrients tested was not, or was only slightly, inhibited by 0·01 mM 2,4-D (pesticide moiety of **E6**) whereas with higher concentrations, uptake of both threonine and sugars decreased in the same proportion (Fig. 5).

The kinetic parameters for [^{3}H]threonine uptake in the presence of varying concentrations of **E6** derivative were studied. Lineweaver–Burk transformation of the results suggested that the **E6** derivative competitively inhibited threonine uptake (Fig. 6) with an apparent K_i of 0·5 mM (Fig. 7).

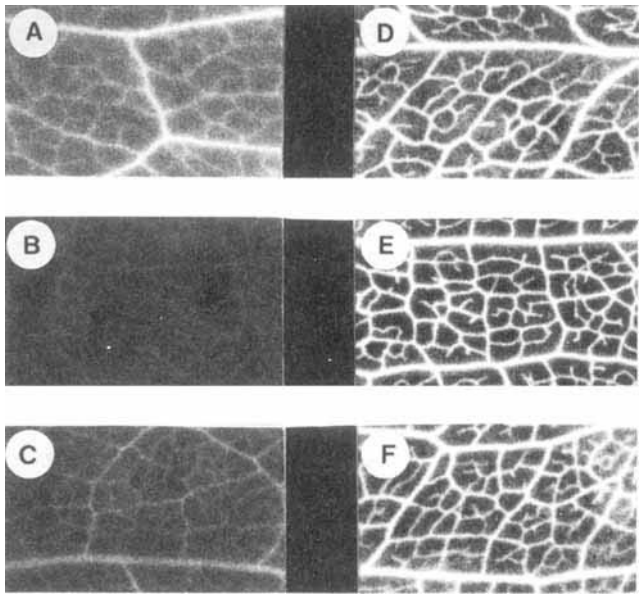


Fig. 4. Autoradiographs showing the effect of **E6** and **E3** associations on 1 mM [U-^{14}C]threonine uptake (A, B, C) or on 1 mM [U-^{14}C]sucrose uptake (D, E, F) by leaf tissues. A and D: control; B and E: 2·5 mM **E6**; C and F: 2 mM **E3**. The radioactivity appears in white. The exposure time was 3·5 days. The samples were exposed on the same film and the background is shown between A, B, C and D, E, F. Tissues are shown at 7× magnification.

TABLE 3
Effect of Amino Acid–Pesticide Derivatives on Sucrose or Glucose Uptake by *Vicia faba* Leaf Tissues

	E3		**E6**	
Derivative (mM)	*0*	*2*	*0*	*2·5*
Uptake of 1mM sucrose[a] (nmol cm^{-2} h^{-1})	11·85(±1)	10·9(±0·6) (NS)[b]	14·4(±0·6)	14·5(±0·7) (NS)[b]
%	100	92	100	99·9
Uptake of 1 mM glucose[a] (nmol cm^{-2} h^{-1})	7·18(±0·9)	6·2(±0·7) (NS)[b]	5·25(±0·5)	5·85(±0·3) (NS)[b]
%	100	96·1	100	111·4

[a] Each value is the mean of 50 values (±SE).
[b] NS = no significant effect.

TABLE 4
Effect of Triazolyl Alcohol on Uptake of Three Nutrients by *Vicia faba* Leaf Tissues

Triazolyl alcohol (mM)	*0*	*2*
Uptake of 1 mM threonine[a] (nmol $cm^{-2} h^{-1}$)	22·7(±0·6)	19·8(±2) (NS)[b]
(%)	100	87·2
Uptake of 1 mM sucrose[a] (nmol $cm^{-2} h^{-1}$)	10·5(±0·8)	10·8(±1·4) (NS)[b]
(%)	100	102·8
Uptake of 1 mM glucose[a] (nmol $cm^{-2} h^{-1}$)	7·5(±0·5)	6·7(±0·4) (NS)[b]
(%)	100	89·3

[a] Each value is the mean of 30 values (±SE).
[b] NS = no significant effect.

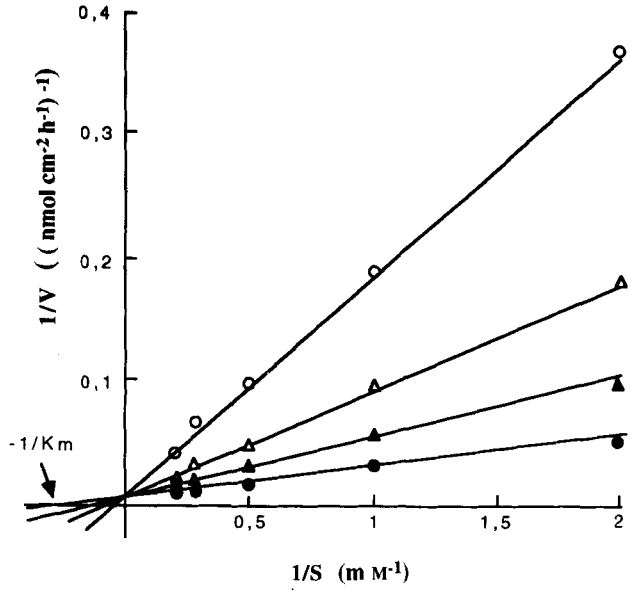


Fig. 6. Double reciprocal plot of the kinetics of [^{3}H]threonine transport in the presence of lysine-2,4-D association at (●) 0 mM; (▲) 0·25 mM; (△) 1 mM; and (○) 2·5 mM. Each point is the mean of 20 values.

4 DISCUSSION

In this work, we have synthesized a new type of molecule by the conjugation of an α-amino acid and a pesticide. Such synthesis is difficult because the bond between pesticide and α-amino acid must not be broken during selective deprotection of the α-amino acid group. The main problem is the choice of a suitable protecting group.[24]

One aim was to determine if plasma membrane amino acid carriers could recognize our derivatives. Both lysine–2,4-D (**E6**) and aspartic acid–triazolyl alcohol (**E3**) inhibited threonine uptake immediately and significantly. This uptake remained linear with time. This suggests that (i) the target of such products is probably on the plasma membrane and (ii) these products do not

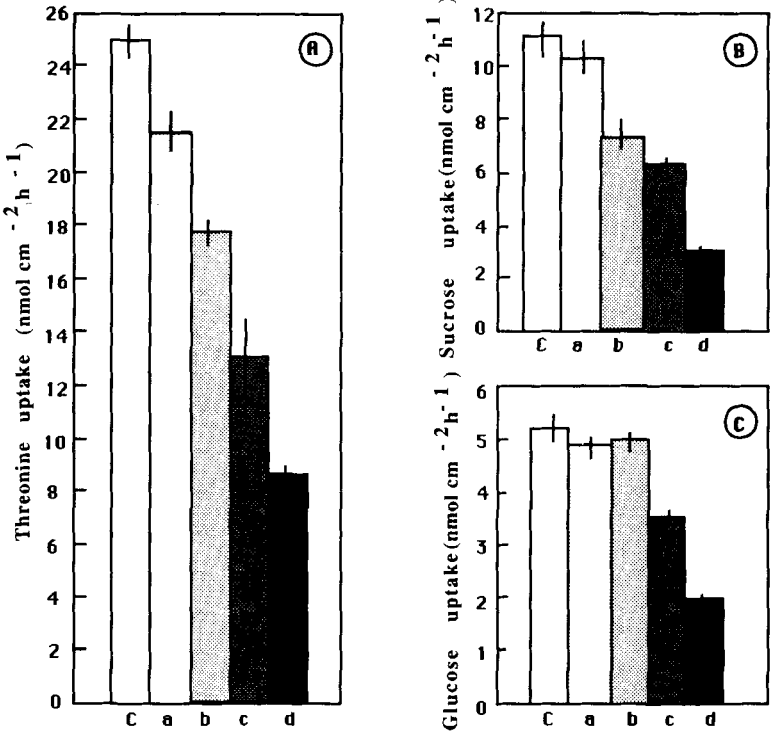


Fig. 5. Effect of various concentrations of 2,4-D on uptake of (A) 1 mM threonine, (B) 1 mM sucrose or (C) 1 mM glucose. C: control; a: 0·01 mM 2,4-D; b: 0·1 mM 2,4-D; c, 0·3 mM 2,4-D; d: 1 mM 2,4-D. Mean of 20 values ± SE by set.

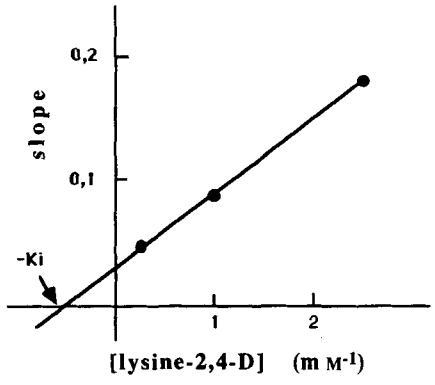


Fig. 7. Apparent affinity of the threonine carrier for the lysine–2,4-D derivative.

change the structural integrity of the plasma membrane. The inhibitory capacity of 5 mM **E6** was found to be stronger than that obtained with the uncoupler carbonyl cyanide *m*-chlorophenyl hydrazide (CCCP; 0·01 mM) or the thiol reagent *p*-chloromercuribenzene sulfonic acid (PCMBS; 5 mM) on the same material, i.e. leaf discs of *V. faba*.[27] As the present products do not inhibit the uptake of 1 mM sucrose or 1 mM glucose (Tables 3 and 4), their inhibitory effect is specific for threonine uptake (Table 2).

Moreover, the inhibition observed is due to the amino acid–pesticide derivative and not to the pesticide alone (Table 4 and Fig. 5). The non-selective effect of 2,4-D noted at high concentrations (Fig. 5) is not surprising, since auxinic herbicides are known to alter the permeability of plants' membranes to different ions and nutrients such as H^+, K^+ and amino acids.[28]

Preliminary results, limited to the influx of [^{3}H]threonine versus threonine concentration in presence or absence of lysine–2,4-D (**E6**) suggest that this derivative is a competitive inhibitor of threonine uptake. Moreover, the apparent affinity of the threonine carrier for **E6** (apparent $K_i = 0{\cdot}5$ mM) (Fig. 7) appears to be much higher than for threonine (apparent $K_m = 3$ mM) (Fig. 6). The structural similarity between amino acid–pesticide derivatives and threonine is weak except for the α-amino acid function. However, even if the α-amino acid group is essential for recognition between substrate and carrier,[29] it does not appear to be sufficient, as the **E5** derivative did not affect threonine uptake, at least under our experimental conditions.

In conclusion, it has been shown that it is possible to synthesize molecules which dramatically inhibit the uptake of amino acids and which might be able to induce an amino acid depletion in cells. The selective inhibition of threonine uptake suggests recognition between **E3**, **E6** and the natural substrate carrier, and this point is supported by the transport characteristics of [^{3}H]threonine in the presence of various concentrations of **E6**. This confirms that amino acids carriers do not have a high specificity for their substrates, in contrast to the sucrose carrier.[30–33] However, these data do not imply that these two derivatives are transported.

Future work will focus on improving our understanding of the interaction between xenobiotic conjugates and carriers and testing the ability of these products to be translocated within the plant.

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