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Acidic derivatives of the fungicide fenpiclonil: effect of adding a methyl group to the *N*-substituted chain on both systemicity and fungicidal activity

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Jean-François Chollet ^{a 1}, Françoise Rocher ^{a 1}, Cyril Jousse ^{a b}, Céline Delétage-Grandon ^b, Georges Bashiardes ^a and Jean-Louis Bonnemain ^{b*}

10 ^a *Laboratoire «Synthèse et Réactivité des Substances Naturelles», Unité Mixte de Recherche CNRS 6514, 40, av. du Recteur Pineau, 86022 Poitiers cedex, France*

^b *Laboratoire «Transport des Assimilats», Unité Mixte de Recherche CNRS 6161, 40, av. du Recteur Pineau, 86022 Poitiers cedex, France*

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Abstract: A new acidic derivative of the fungicide fenpiclonil was synthesized containing a methyl group on the alpha-position of the carboxyl function of the *N*-carboxymethyl-3-cyano-4-(2,3-dichlorophenyl)pyrrole recently described. The phloem mobility of the new *N*-(1-carboxyethyl)-3-cyano-4-(2,3-dichlorophenyl)pyrrole was comparable to that of the former compound, but was higher at external pH 5.0. Unlike the derivatives previously synthesized, it was comparable in its fungicidal activity to fenpiclonil against the pathogenic fungus *Eutypa lata*.

20

Keywords: phenylpyrroles, fenpiclonil, acidic derivatives, systemicity, phloem mobility, antifungal activity, *Eutypa lata*

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* Correspondence to: Jean-Louis Bonnemain, Laboratoire «Transport des Assimilats», Unité Mixte de Recherche CNRS 6161, Université de Poitiers, 40, av. du Recteur Pineau, 86022 Poitiers cedex, France

E-mail: jl.bonnemain@voila.fr

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¹ These authors contributed equally to the paper

1 INTRODUCTION

The development of fungicides that can be translocated downward in the phloem to control root or vascular pathogens has long been needed.¹ Unlike several herbicides (especially auxins, glyphosate, sulfonylureas, imidazolinones) that are phloem mobile, the so-called “systemic” fungicides are mobile only within the apoplastic compartment.^{1,2} The phloem mobility of these moderate lipophilic xenobiotics is either undetectable or minute. Attempts to derivatize fungicides to make them phloem mobile have generally had the discouraging consequence of decreasing their biological activity.^{1,3} However, it must be mentioned that two derivatives of cymoxanil containing a free carboxyl group, a moiety which has been shown to confer phloem systemicity, exhibit high biological activity against *Peronospora parasitica*.⁴ This incompatibility between induced phloem mobility and biological activity also concerns, excepting at least one case², the insecticide-nematicide group.^{2,3}

As reported recently,⁵ the fungicide fenpiclonil was used as a model molecule because of the possibilities of adding a carboxyl group at various sites on the benzene or pyrrole rings. Using the mathematical model of Kleier,^{3,6} all these acidic analogues are predicted to be phloem-mobile. However, only the group of *N*-substituted derivatives exhibited some long-term fungicidal activity, particularly *N*-carboxymethyl-3-cyano-4-(2,3-dichlorophenyl)pyrrole (compound **2a**, Fig 1), ie the compound with the shortest *N*-chain. The phloem mobility of this xenobiotic was investigated. It was shown that its concentration in the sieve tube sap was correlated to the percentage of the undissociated form of the molecule (predicted using ACD LogD v. 7.0 software) in the apoplastic incubation solution.

According to the predictions using the ACD LogD software, the second group of *N*-substituted derivatives (**11a**, Fig 1) also exhibits a good profile for phloem systemicity (see Figure 4 in reference 5), taking into account the high percentage of its undissociated form at apoplastic pH values (pH 5.0 to 6.0). However the fungicidal activity of this group is less than that of compound **2a**. Another strategy to modulate lipophilicity and dissociation could be to insert a lipophilic substituent on the carbon adjacent to the acidic function. The present paper describes the synthesis and biological properties of *N*-(1-carboxyethyl)-3-cyano-4-(2,3-dichlorophenyl)pyrrole.

2 EXPERIMENTAL

2.1 Synthesis

This proceeds in two stages: obtaining an ester derivative and hydrolysis of this to obtain the carboxylic acid derivative.

5 The synthesis of compound **2a** (compound **9** of reference 5) has been previously described.⁵

2.1.1 Synthesis of 3-cyano-4-(2,3-dichlorophenyl)-N-(1-methoxycarbonyl)pyrrole (Fig 2, **1b**)

Fenpiclonil (2.37 g, 10 mmol) dissolved in anhydrous degassed tetrahydrofuran (THF) (30 ml) was lithiated by a dropwise addition of a solution of n-butyllithium in hexane (6.6 ml of 1.6 M solution, 10.05 mmol). The mixture was stirred for 3 h at 0 °C. Methyl 2-bromopropionate (2.5 g, 15 mmol) in degassed THF (20 ml) was added dropwise and the mixture was stirred for 1 h at 0 °C then 15 h at 60 °C. After cooling to 0 °C, cold water (50 ml) was carefully added dropwise. The aqueous phase was extracted with dichloromethane (3 x 25 ml). The combined dichloromethane layers were washed with water (20 ml), dried over magnesium sulfate and evaporated to provide **1b** as an oil. 10
15 The product was then used without further purification.

1b. Yield: 90 %; **NMR** ¹H (CDCl₃) δ (ppm): 1.75 (d, 3H, CH₃, J 7.3 Hz), 3.76 (s, 3H, OCH₃), 4.83 (q, 1H, CH, J 7.3 Hz), 7.00 (d, 1H, H₅, J 2.3 Hz), 7.10-7.55 (m, 4H, H₂ and C₆H₃); **NMR** ¹³C (CDCl₃) δ (ppm): 18.19 (CH₃), 52.92 (OCH₃), 57.61 (CH), 94.12 (C-CN), 115.52 (CN), 123.75 (C-C₆H₃), 121.45, 127.15, 127.51 and 129.51 (arom. CH); 131.18, 133.39 and 133.66 (arom. C), 170.14 (C=O); **IR**ν (cm⁻¹): 3150, 1410 (pyrrole), 3060, 1590, 795, 710 (C₆H₃ 1',2',3'-trisubstituted), 2220 (CN), 1750 (C=O), 1215, 1160 (COC); **MS** (EI), m/z: 322 (M⁺), 34%; 263, 100%; 228, 6%; 192, 11%; 138, 6%. 20

2.1.2 Synthesis of N-(1-carboxyethyl)-3-cyano-4-(2,3-dichlorophenyl)pyrrole (Fig 2, **2b**)^{7,8}

25 Compound **1b** (3.37 g, 10 mmol) and lithium iodide (9.3 g, 57 mmol) were refluxed in dimethylformamide (300 ml) for 18 h. After cooling to room temperature, the mixture was diluted with water (300 ml), acidified (pH ≈ 3) using hydrochloric acid (2 M), and then extracted with

dichloromethane (3 x 100 ml). The organic phase was washed with water (3 x 40 ml), dried over anhydrous magnesium sulfate, and the solvents were removed under reduced pressure to give **2b** as an oil which slowly crystallized. Purification was achieved by washing the product with dichloromethane (50 ml) and drying under vacuum. The purity of the compound was verified using HPLC (Discovery
5 C16 RP-amide column 250 x 4.6 mm [Supelco, Bellefonte, PA], eluting with acetonitrile + water containing 1 ml litre⁻¹ trifluoroacetic acid [1+1 by volume], flow 0.8 ml min⁻¹, detection wavelength 218 nm).

2b. Yield: 52 %; mp 181°C; Log Kow = 2.98 and pKa = 2.53 (calculated using ACD Log *D* v 8.0 software); **NMR** ¹H (d₆ DMSO) δ (ppm): 1.72 (d, 3H, CH₃, *J* 7.3Hz), 5.13 (q, 1H, CH, *J* 7.3Hz), 7.30-
10 8.30 (m, 6H, H₂, H₅, C₆H₃, OH); **NMR** ¹³C (d₆ acetone) δ (ppm): 17.78 (CH₃), 57.16 (CH), 92.16 (C-CN), 116.00 (CN), 122.47 (C-C₆H₃), 122.14, 128.05, 129.33, 129.63 and 130.14 (arom. CH), 130.35, 132.61 and 134.02 (arom. C), 171.70 (C=O); **IR**ν (cm⁻¹): 3300-2500 (OH), 3130, 1410 (pyrrole), 3060, 1587, 810, 785, 715 (C₆H₃ 1',2',3'-trisubstituted), 2220 (CN), 1740 (C=O); **MS** (EI), *m/z*: 236, 100 %; 201, 35%; 174, 39%; 166, 27%; 138, 18%; 100, 25%; 83, 31%.

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2.2 Tests of systemicity

2.2.1 Plant material

Castor bean seedlings (*Ricinus communis* L cv Sanguineus), obtained from Ball-Ducrettet (Margencel, France), were used 6 days after sowing as described previously.⁵

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2.2.2 Electrophysiological measurements

Transmembrane potential difference (PD) measurements of subepidermal cells in *Ricinus* hook were made according to the method previously described.^{5,9}

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2.2.3 Sap collection and analysis

Cotyledons were incubated in a buffered solution supplemented with the fenciclonil acidic derivative and the phloem sap was collected from the upper part of the *Ricinus* seedlings according to

the methods already described.^{5,10} Young *Ricinus* cotyledons isolated from endosperm are very sensitive to environmental parameters⁵: at pH 4.5, citrate cannot be used as a buffer due to its chelating effect toward Ca^{++} ; in absence of xenobiotics, a too acidic incubation medium (pH 4.0) inhibits phloem sap exudation whatever the buffer used. At pH 4.5, in presence of HEPES 20 mM used as a buffer, compound **2b** at 250 μM also altered phloem sap exudation. Consequently, the fenpiclonil derivative was used at 100 μM in these experiments. The phloem sap was analyzed by HPLC.

2.3 *In vitro* fungicidal activity on *Eutypa lata*

2.3.1 *Fungal-growth conditions*

10 The effect of compound **2b** was studied on the strain (ELJFC 02) of *Eutypa lata* used previously.⁵ This pathogenic fungus, which destroys wood and phloem of vine stocks, was grown on a sterile culture medium solidified with agar (20 g litre⁻¹), containing malt extract (15 g litre⁻¹) and chloramphenicol (0.25 g litre⁻¹).

15 2.3.2 *Mycelium-growth assays*

The effects of compound **2b** on mycelial growth were measured on cultures made in 55 mm diameter wells.

3 RESULTS AND DISCUSSION

20 3.1 Predicting phloem mobility of compound **2b** and evaluation of its systemicity

Phloem mobility for compound **2b** ($\text{pK}_a = 2.53$, $\log K_{ow} = 2.98$) was predicted using the model of Bromilow¹¹ and the mathematical model of Kleier.³ In both models, the acidic fenpiclonil derivative ranged into the areas of moderately phloem mobile compounds (Fig 3). The predictions are almost the same as those of compound **2a** ($\text{pK}_a = 2.44$, $\log K_{ow} = 2.63$), the physicochemical properties of both acidic derivatives being nearly the same.

Before evaluating phloem systemicity of compound **2b** in the *Ricinus* system, its effect on the activity of the plasma membrane proton-pumping ATPase (PM H^+ -ATPase) was studied. This intrinsic

master enzyme generates an electrochemical proton gradient across this membrane¹² and is indirectly involved in maintaining acidic molecule influx through the lipidic bilayer.¹³ Values of transmembrane PD are a good indicator of the PM H⁺-ATPase activity. Compound **2b** at 250 μM did not depolarize the hypocotyl tissues. The transmembrane PD of subepidermal cells of the hook was indeed close to –
5 100 mV both in control (see Figure 6a of Ref. 5) and treated sets immediately after isolation of the upper part of the young plant (cotyledons and hook). However, hyperpolarization of transmembrane PD which usually takes place during ageing¹⁴ was weaker in the treated set than in the control (about –125 mV and –150 mV after 6 h of incubation respectively).

When cotyledons were incubated in acidic solution (pH 5.0) in presence of compound **2b** at
10 100 μM, this derivative was found in the phloem sap (Fig 4) and no fenpiclonil could be detected. This indicates that, just as compound **2a**⁵, compound **2b** is not degraded to the parent compound in the sieve-tubes. Its concentration in the phloem sap increased for 150 min before reaching a plateau (Fig 5).

Although compounds **2a** and **2b** exhibit almost similar physicochemical properties (Fig. 3), the
15 percentage of their respective undissociated forms may differ markedly at apoplastic pH values (according to ACD logD v 8.0 software predictions). The range of these values is estimated to be between 5.0 and 6.0 in the leaf apoplast. For instance, leaf tissues of *Vicia faba*, an apoplastic loader, acidify the incubation medium at pH 5.4 before ageing.¹⁵ However, expression of the PM H⁺-ATPase is higher in the phloem than in the mesophyll.^{16,17,18} This suggests that the micro-environment of the
20 plasma membrane must be more acidic in the former tissue than in the latter in spite of the passive influx of protons driving the phloem loading processes. During ageing, a phase characterized by an increasing activity of the PM H⁺-ATPase within the mesophyll, broad bean leaf tissues acidify the incubation medium at pH 5.0 or even at more acidic values.^{19,20} All these data suggest that the pH values of the phloem apoplast must be close to 5.0, at least in apoplastic loaders. The concentration
25 factor of compound **2b** (ie the concentration in the phloem sap related to that of the incubation medium), similar to that of compound **2a** at pH 4.6 (0.4), became higher at pH 5.0 (0.33 and 0.17 respectively) in accordance with the predictions of ACD Log D v 8.0 software (compare Fig. 6 of this

paper and Fig. 9 in Ref. 5). However the concentration factor of compound **2b** (0.09) in the phloem sap did not differ significantly from that of compound **2a** (0.07) at pH 5.5. Similarly to compound **2a**, compound **2b** uptake was marginal at pH 6.0 and could not be detected at neutral and alkaline pH values. These data indicate that compound **2b** phloem uptake occurs by diffusion of its lipophilic undissociated form which dissociates into poorly permeant anions into the phloem symplast, ie a mechanism elegantly demonstrated for herbicides in the eighties.^{21,22}

3.2 Biological activity of compound **2b**

The fungicidal activities of fenpiclonil and compound **2b** on mycelial growth of *Eutypa lata* were compared using a large range of concentrations. In the presence of fenpiclonil at 1 mM, the mycelium grew vertically from the inoculum after a latency period of a few days and then covered the culture medium slowly and compactly (Fig 7A). By contrast, the pathogenic fungus growth was completely inhibited by compound **2b** at the same concentration (Fig 7F). In the presence of fenpiclonil at 50 μ M, the response of the mycelium was practically the same as that described at 1 mM (Fig 7B). *Eutypa lata* development was no longer totally inhibited by compound **2b** when used at 50 μ M, but a large inner part of the colony became necrosed around the inoculum disk after a three-week culture (Fig 7G) while the outer part was reduced to a thin halo. The necrosis of the older part of the mycelial colony was still observed after a three-week culture both in the presence of fenpiclonil or compound **2b** at 10 μ M (Fig 7C and H). However, the growth of the mycelial halo was less inhibited by contact with **2b**. Finally, at 1 μ M, fenpiclonil and the acidic derivative reduced the thickness of *Eutypa lata* mycelium (Fig. 7D and I) to a similar degree compared to the control (Fig. 7E and J).

Compound **2b** exhibits a fungicidal effect greater than that of the recently described **2a**.⁵ The latter acidic derivative is not active at 10 μ M, whereas compound **2b** still exhibits some fungistatic effect at 1 μ M. Taking into account the pH values of the culture medium of *E. lata* (from 5.0 to 5.5)⁵ and the calculated percentage of the undissociated form of the two acidic derivatives under these conditions (Fig 6 of this paper and Fig 9 in Ref. 5), uptake of compound **2b** may be in theory more efficient than that of compound **2a**. However, this point, supported only in part in the *Ricinus* system

(Fig 6), cannot alone explain the difference in biological activity observed (compare Figure 7 of this paper with Figure 3 of Ref. 5). The fungicidal activity of the *N*-substituted derivatives may also depend on their ability to release the parent compound in CH₃CN/H₂O (3/7) as suggested by Nyfeler and Ackermann.²³ Compound **2b** (as compound **2a**) is not degraded to fenpiclonil in a detectable amount in the phloem sap. A detailed analysis of what happens to it in plant tissues and fungus is now needed to establish whether this acidic derivative is a phloem mobile pro-fungicide or a new phloem mobile fungicide.

4 CONCLUSION

Unsuccessful attempts made in the seventies to synthesize a phloem mobile fungicide from a non-mobile or apoplastic fungicide have discouraged further works in this research area. In the last two decades, except the work on cymoxanil acidic derivatives,⁴ no data were published in this field to our knowledge, the main challenge being to maintain the fungicidal activity of the parent compound. Fenpiclonil, an analogue of a metabolite produced by several *Pseudomoniae*, was selected as a model molecule as a first approach. It has proved possible to synthesize many derivatives from the parent compound and, in this way, to manipulate the physicochemical properties of the phenylpyrrole molecules. In this paper, we show that addition of a methyl group at the alpha-carbon adjacent to the carboxyl function of *N*-carboxymethyl-3-cyano-4-(2,3-dichlorophenyl)pyrrole, recently described, improves the phloem systemicity at pH 5.0, ie a pH value close to that of the phloem apoplast in apoplastic loaders. Furthermore, the new *N*-(1-carboxyethyl)-3-cyano-4-(2,3-dichlorophenyl)pyrrole exhibited an apparent fungicidal activity on *Eutypa lata* better than that of fenpiclonil at high concentration (1 mM) and a biological effect almost similar at the lower concentrations (10 and 1 μM) tested. In this case, the problem due to the conflicting requirements for activity and phloem mobility was solved. Because of lack of information concerning the selectivity of the biological effect of phenylpyrroles, this paper must be considered only as a fundamental work on phloem systemicity of fungicides.

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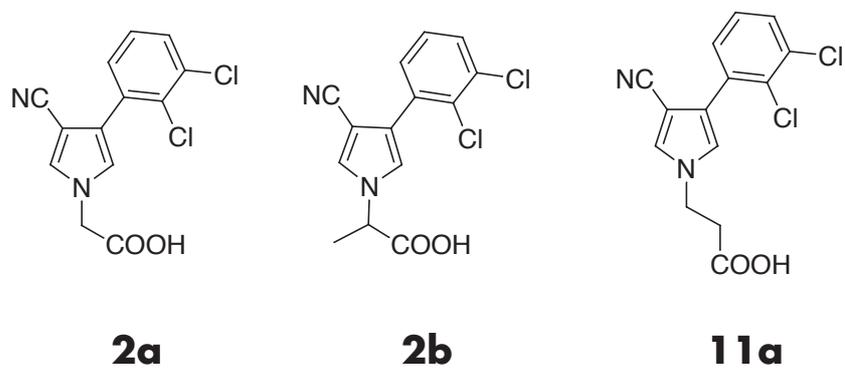


Figure 1. Acidic derivatives of fenpiclonil exhibiting a good profile for phloem systemicity.

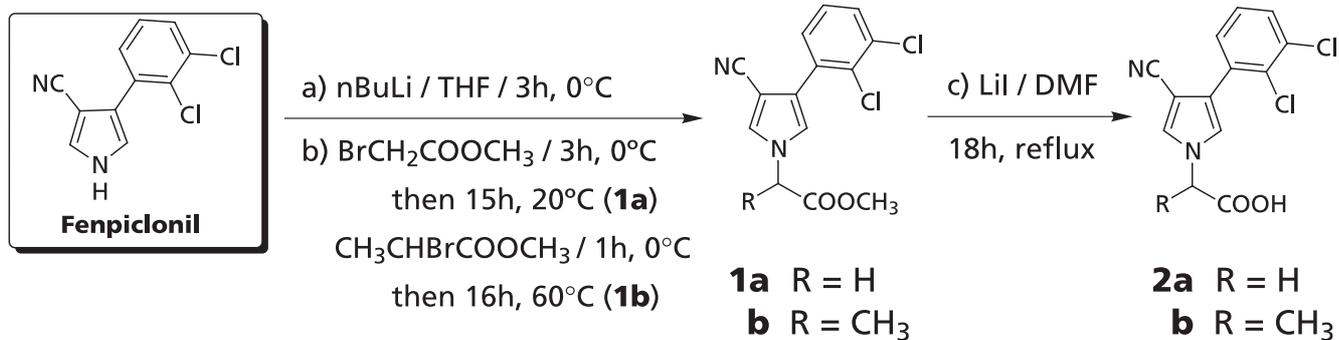
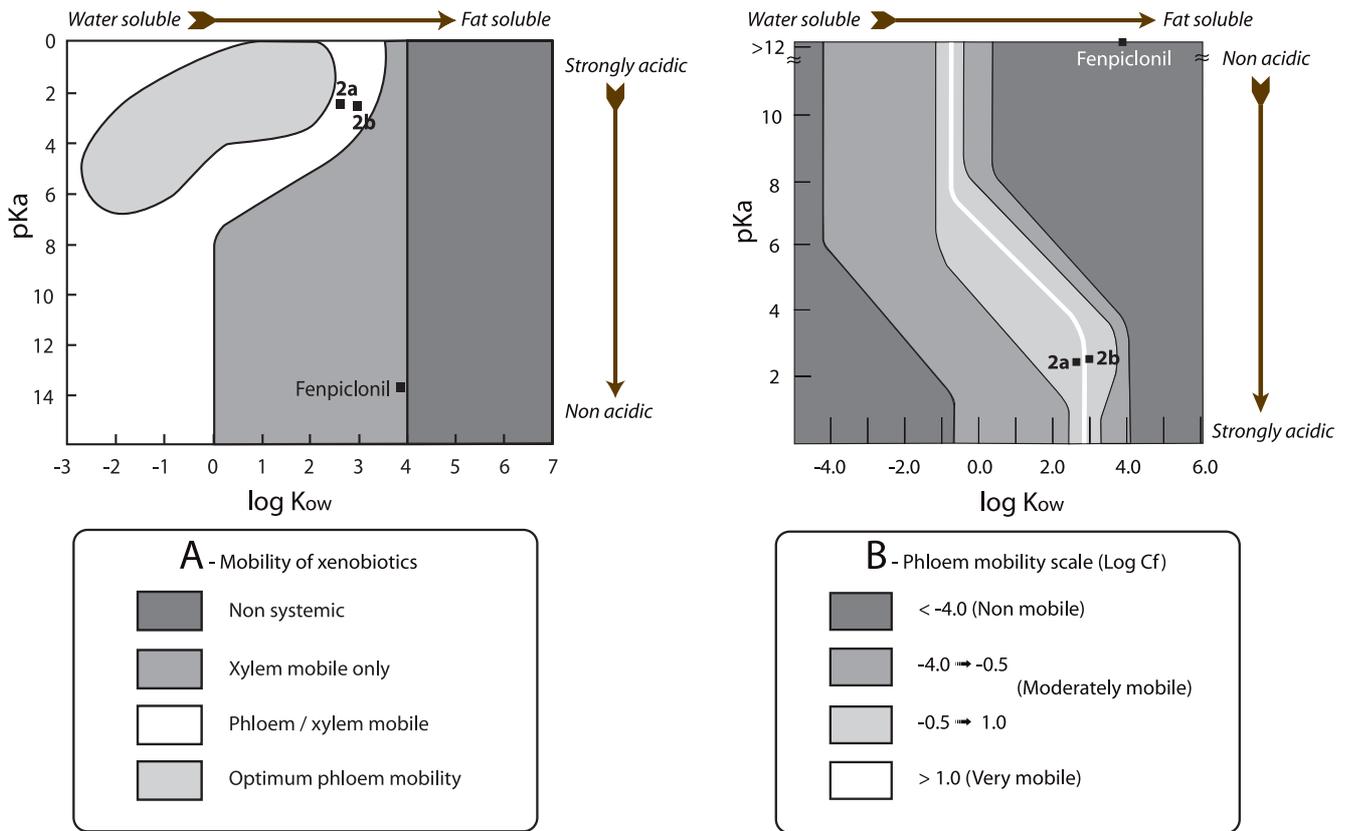


Figure 2. Reaction scheme for the synthesis of *N*-(1-carboxyethyl)-3-cyano-4-(2,3-dichlorophenyl)pyrrole (compound **2b**).



5 **Figure 3.** Prediction of phloem mobility of fenpiclonil and its two acidic derivatives using the model of Bromilow (A: mobility as a function of log K_{ow} and pKa) and the Kleier map (B: log Cf as a function of log K_{ow} and pKa; plant parameters are for a short plant⁶).

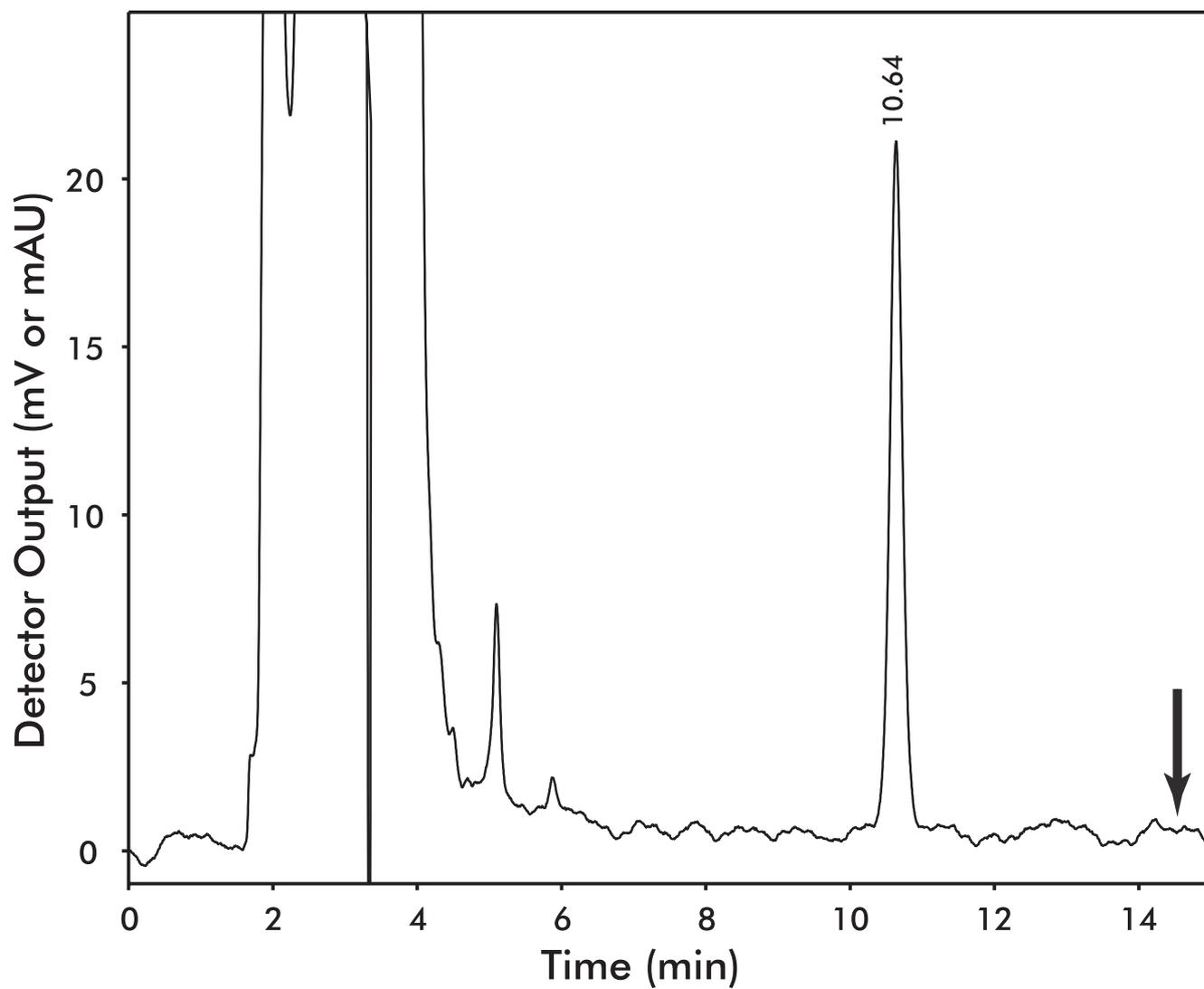


Figure 4. *Ricinus* sap analysis by HPLC. *Ricinus* cotyledons were incubated in the standard medium with compound **2b** (RT: 10.64 min) at 100 μ M concentration. The arrow indicates the retention time of fenpiclonil.

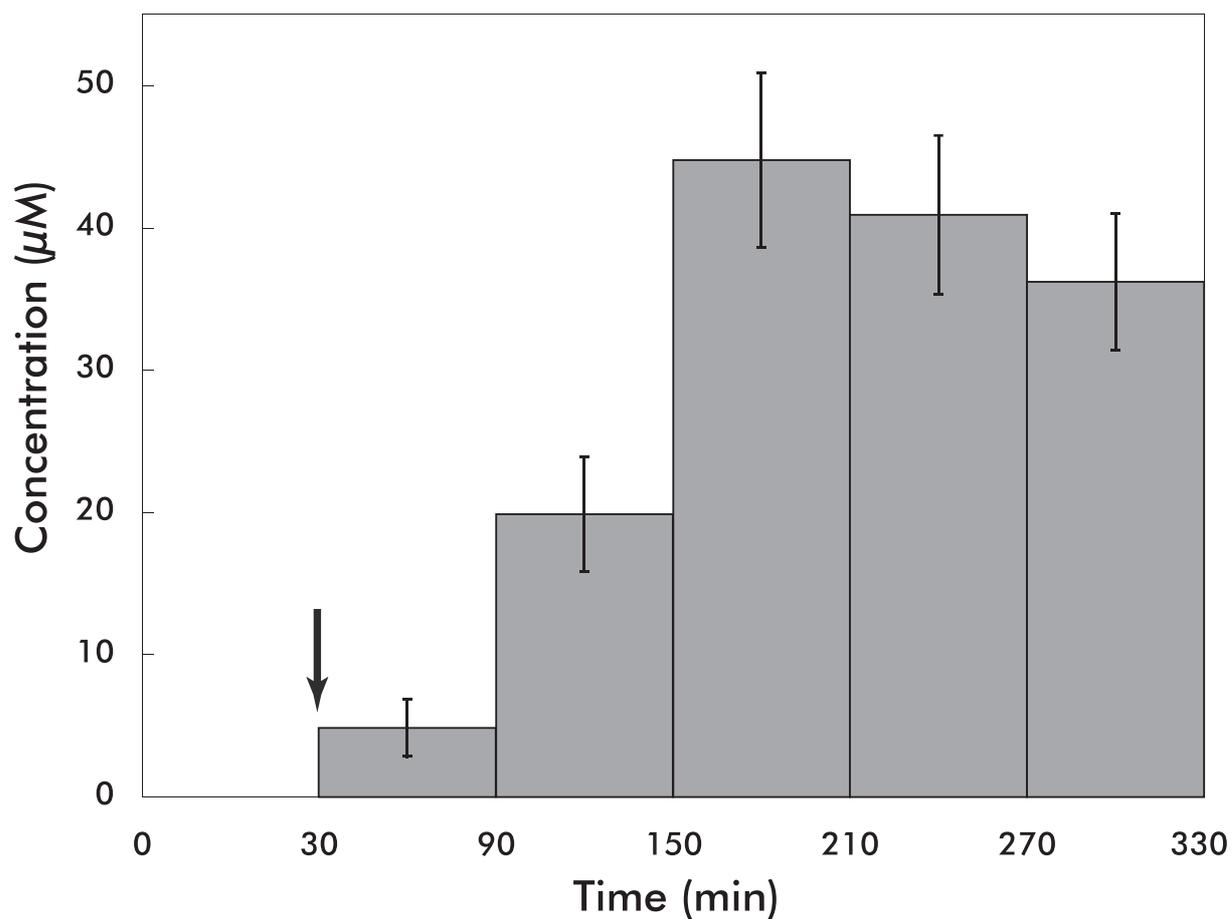


Figure 5. Time course of compound **2b** concentration in phloem sap of *Ricinus*. At time 0, compound **2b** (100 µM, final concentration) was added to the standard medium buffered at pH 5.0. The hook was severed at time 30 min (arrow). Mean of twelve plants \pm SE.

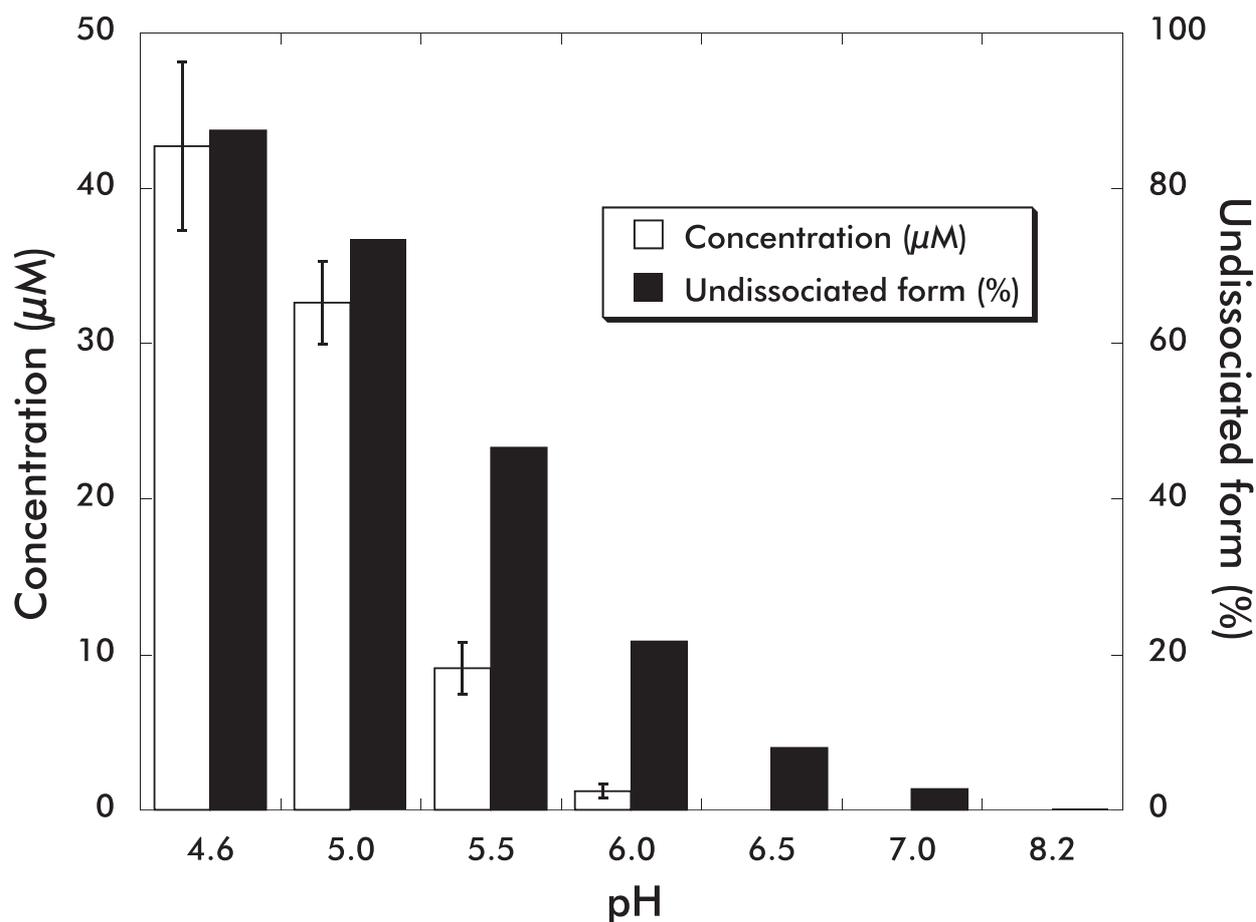
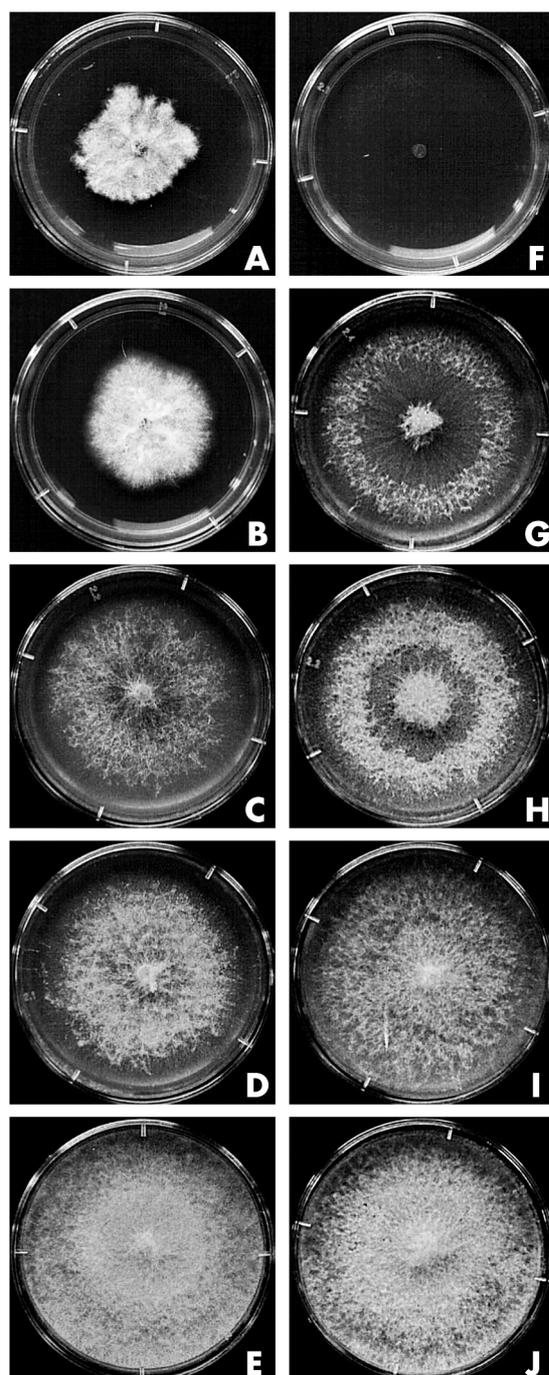


Figure 6. Concentration of compound **2b** (100 μM concentration in the external solution) in phloem sap of *Ricinus* as a function of the pH of the incubation medium and the percentage of its undissociated form at the apoplast/plasma membrane interface according to ACD log D v 8.0 software predictions. The sap was collected during the third and fourth hours of incubation. Mean of 52 plants (pH 4.6), 58 plants (pH 5.0), 88 plants (pH 5.5), 39 plants (pH 6.0), 22 plants (pH 6.5), 12 plants (pH 7.0) and 11 plants (pH 8.2) \pm SE.



5 **Figure 7.** Long-term effect (21 days of culture) of fenpiclonil (A-E) and compound **2b** (F-J) on *Eutypa lata* mycelial growth. The products were used at 1 mM (A, F), 50 μ M (B, G), 10 μ M (C, H), 1 μ M (D, I) and 0 mM (E, J) concentrations. The experiment was repeated six times with similar results.