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Comparison of Sorption and Diffusion by Pyridate and Its Polar Metabolite in Isolated Cuticular Wax of *Chenopodium album* and *Hordeum vulgare*

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Sorption and diffusion of the herbicide pyridate and its metabolite CL9673 were measured in reconstituted cuticular waxes isolated from *Chenopodium album* L. and *Hordeum vulgare* L. (cultivar Igri) leaves. The compounds have the same basic chemical structure, except that pyridate is characterized by a C_8-alkyl chain bound via a thioester to the ionizable hydroxyl group of CL9673. Sorption of the weak acid CL9673 from aqueous solutions into cuticular waxes was pH-dependent, and the apparent wax/water partition coefficients decreased with increasing pH. Wax/water partition coefficients of pyridate were not dependent on pH, and they were about 4 orders of magnitude higher as compared to the nondissociated species of CL9673. Diffusion coefficients measured in reconstituted cuticular wax for CL9673 fitted established models predicting diffusion coefficients in relation to molar volumes. However, this was not the case with pyridate, which was characterized by a self-accelerating effect leading to diffusion coefficients, which were up to 2 orders of magnitude higher than predicted from the molar volume. This is a remarkable result since pyridate represents a compound combining the properties of an active ingredient and of a plasticizer.

KEYWORDS: Active ingredient; cuticular permeability; cuticular wax; diffusion coefficient; foliar uptake; pyridate; partition coefficient

INTRODUCTION

The plant cuticle forms the interface between leaves and atmosphere. Diffusion of xenobiotic chemicals through cuticles is of major importance in the fields of agricultural and environmental sciences in relation to foliar uptake of plant protection agents (*1*) and foliar uptake of pollutants (*2*). Sorption and diffusion in cuticular wax represent the first steps of foliar uptake, and it is generally accepted that transport across the cuticular wax barrier is the rate-limiting step of uptake (*3*). Rates of foliar uptake can be determined by measuring the permeance P [m s^{-1}], which is a quantity composed of the diffusion coefficient D [m^2 s^{-1}] in the cuticular wax, the wax/water partition coefficient K_{ww}, and the thickness of the wax layer l [m]

$$P = \frac{DK_{ww}}{l} \quad (1)$$

Since P is a composite quantity, sorption and diffusion in cuticular wax should be considered separately when analyzing its dependence on the physicochemical properties of the respective xenobiotic chemicals of interest. Reconstituted cuticular waxes form a very useful tool for mechanistic studies and the establishment of quantitative structure−property relationships (*4*−*5*).

When plant protection agents are applied to leaf surfaces, rapid uptake into the leaf across the cuticle should occur to minimize loss to the environment. Accordingly, active ingredients are applied as formulations containing adjuvants (*6*) acting as accelerators of diffusion (*7*). This can significantly increase rates of cuticular permeability. Furthermore, the performance of an active ingredient can also be improved by chemical derivatization, provided that the biologically inactive derivative is subsequently metabolized to its active form again in the leaf, after foliar uptake. The herbicide pyridate and its metabolite CL9673 represent an example of this approach (*8*). The two compounds are characterized chemically by the same basic structure with the exception that pyridate contains a C_8-alkyl side chain bound via a thioester to the hydroxyl group of CL9673 (**Figure 1**). To act as an inhibitor of photosynthesis

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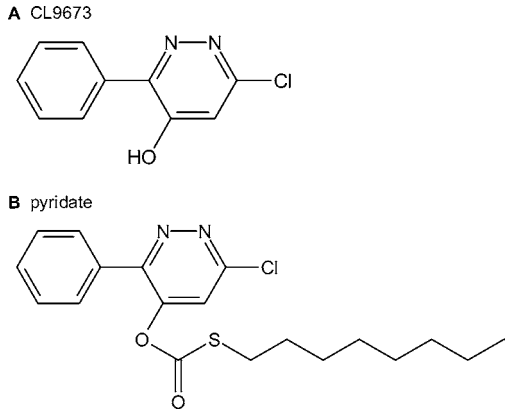

Figure 1. Chemical structures of CL9673 (**1A**) and pyridate (**1B**).

(*9*−*10*), pyridate must be converted to CL9673 by hydrolysis of the ester side chain.

Most studies on derivatives of herbicides focused on the enzymatic metabolization and the physiological action in the leaf after foliar uptake (*11*−*13*). Normally, less information is available regarding the initial steps of cuticular uptake. Thus, it was the intention of this study to analyze in which way sorption (K_{ww}) and mobility (D) are affected by derivatization of CL9673 leading to pyridate. *Chenopodium album* was selected as a potential target species of the active ingredients in the field. *Hordeum vulgare* was chosen as a reference, to be able to compare the data obtained with CL9673 and pyridate with sorption and diffusion of a series of other active ingredients in barley wax (*14*).

MATERIALS AND METHODS

Plant Material. Cuticular wax was isolated from the leaves of *C. album* L. and *H. vulgare* L. (cultivar Igri) plants grown in the greenhouse. Leaves were harvested from plants at the three-leaf growth stage, and cuticular wax was extracted by dipping the leaves in chloroform (purity 99%, Riedel de Haën, Seelze, Germany) for 5 s. The final concentration of the wax extract was adjusted to about 50 mg mL^{-1} by evaporation of the solvent. For the experiments, wax layers were prepared as described by Schreiber and Schönherr (*4*). Aluminum disks (radius 0.4 mm) were immersed in the wax extracts, and the wax layer was made homogeneous by heating the disks to 100 °C for 5 min. The wax amount covering both sides of the disk was determined by weighing the disk before and after wax crystallization (microbalance S3D, Satorius, Göttingen, Germany). Wax amounts per disk were in the range of 150−250 μg, which corresponded to a thickness of the wax layer in the range of 1.7−2.8 μm assuming a wax density of 0.9 g cm^{-3} (*15*).

Chemicals. The active ingredients pyridate (CL11344, 6-chloro-3-phenylpyridazin-4-yl-*S*-octyl thiocarbonate, CAS RN 55512-33-9, specific activity 297 GBq mol^{-1}, radiochemical purity 97%) and CL9673 (6-chloro-3-phenylpyridazin-4-ol, CAS RN 40020-01-7, specific activity 576 GBq mol^{-1}, radiochemical purity 98.5%) were used as ^{14}C-ring-labeled compounds (Syngenta Crop Protection, Basel, Switzerland) (**Figure 1** and **Table 1**). Radiochemical and chemical purities of the compounds were checked by radio-thin-layer chromatography (Berthold, Wildbad, Germany) and gas chromatography (HP 5890 II, Hewlett-Packard). These methods were also used to ensure that the compounds were stable under the selected experimental conditions. Active ingredients were dissolved in deionized water buffered with a citric buffer (10 mmol kg^{-1}; Sigma, Deisenhofen, Germany; pH 2−6), hepes buffer (10 mmol kg^{-1}; Sigma; pH 7), and borate buffer (10 mmol kg^{-1}; Sigma; pH 8). pH values were adjusted with HCl and KOH. Sodium azide (1 mmol kg^{-1}; Merck-Schuchardt, Hohenbrunn, Germany) was added to prevent the growth of microorganisms.

Table 1. Molar Weights (MW), Molar Volumes (MV), Water Solubilities (WS), and Melting Points (MP) of Compounds CL9673 and Pyridate[a]

Compound	MW [g mol^{-1}]	MV [cm^3 mol^{-1}]	WS [g kg^{-1}]	MP [°C]
CL9673	206.6	142.3	0.06	225
Pyridate	378.9	287.0	0.0015	27.0

[a] MV was calculated according to Abraham and McGowan (*32*). WS and MP were taken from the data sheet obtained from the manufacturer (Syngenta Crop Protection, Basel, Switzerland) for CL9673 and from Budavari et al. (*33*) for pyridate.

The monodisperse alcohol ethoxylate tetraethylene glycol monooctyl ether (C_8E_4, Fluka, Neu-Ulm, Germany) was chosen as a powerful accelerator of diffusion in cuticular wax (*14*). C_8E_4 was dissolved in deionized water, and a concentration 10-fold above the critical micelle concentration (cmc = 2.0 g kg^{-1}) was used to achieve maximum effects (*16*).

Determination of K_{ww}. Disks covered with the wax samples were immersed in aqueous solutions of the active ingredients in 20 mL glass vials. Equilibrium was achieved within 24 h by rotating the vials for 24 h at 25 °C in the dark. After equilibration, wax samples were washed in deionized water for 5 s to remove donor solution adhering to the wax surface and carefully blotted on filter paper. Subsequently, wax samples were put in scintillation vials and dissolved in chloroform, and 5 mL of scintillation cocktail (Ultima Gold XR, Packard, Meriden, CT) was added. Radioactivity was determined by liquid scintillation counting (Counter 1409, Wallac, Turku, Finland). A suitable aliquot (100 μL) of the external donor solution was also sampled and counted.

In the case of CL9673, the apparent wax/water partition coefficients (K_{ww}^{pH}) at the different pH values were calculated from its concentration in the wax (c_{wax}) divided by the concentration in the external donor phase (c_{water})

$$K_{ww}^{pH} = \frac{c_{wax}}{c_{water}} \quad (2)$$

Apparent partition coefficients were corrected for the degree of ionization of CL9673 to obtain the partition coefficient for the nonionized species of the compound (K_{ww})

$$K_{ww} = K_{ww}^{pH}(1 + 10^{pH-pKa}) \quad (3)$$

The pK_a value and corrected K_{ww} for the nonionized species of CL9673 were determined by regression analysis after the transformation of eq 3 in its linear form (for details of the mathematical procedure, see ref *17*). Care was taken that the correction for the apparent partition coefficient was not affected by radiochemical impurities (*2*). In the basic range (pH > 8), the degree of ionization of CL9673 exceeded 99%. Since the dissociated species exhibited only a weak sorption (**Figure 2**), the detection of radioactivity in the wax samples at basic pH values most probably must have been be due to the sorption of lipophilic contaminants.

Determination of D in Cuticular Wax. For diffusion experiments, wax samples were again equilibrated with the radiolabeled active ingredients dissolved in their aqueous donor solutions. After equilibration, the wax samples were carefully washed in water for 5 s to remove small drops of donor solution adhering to the surface and blotted on dry filter paper. Radiolabeled compounds were desorbed again from the wax samples in 5 mL glass vials rotated at 25 °C in the dark on a rolling bench either using a phospholipid suspension (PLS, 10 g kg^{-1}, soybean lecithin; Roth, Karlsruhe, Germany) as the control (*18*) or C_8E_4 as an active desorption medium. PLS has been shown to be an inert desorption medium not altering transport properties of isolated cuticles (*18*), whereas alcohol ethoxylates such as C_8E_4 have been shown to significantly decrease cuticular barrier properties (*1*). The desorption medium was replaced at regular intervals. Plotting relative amounts desorbed (M_t/M_0) versus the square root of time t [s], the desorption kinetics can be linearized, and D [m^2 s^{-1}] can be calculated from the slope of regression lines fitted to the linearized parts of the desorption

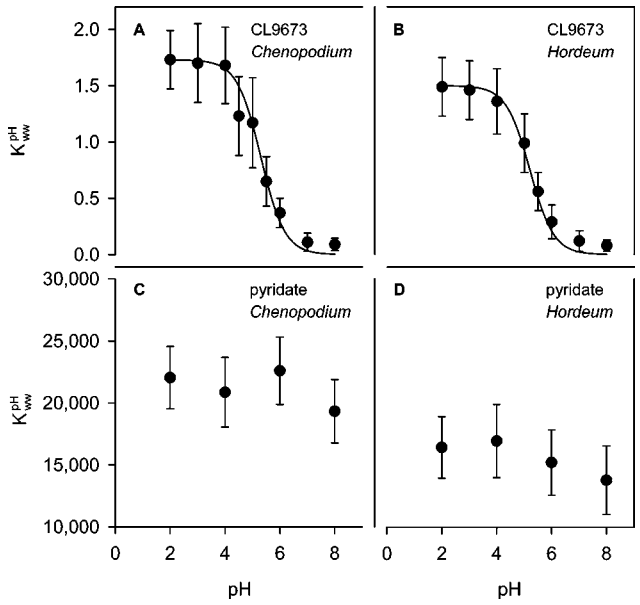

Figure 2. pH dependence of the apparent wax/water partition coefficient (K_{ww}^{pH}) for CL9673 (**2A** and **2B**) and pyridate (**2C** and **2D**) in the cuticular waxes of *C. album* and *H. vulgare*. Results are means ($n = 10$) with 95% confidence intervals.

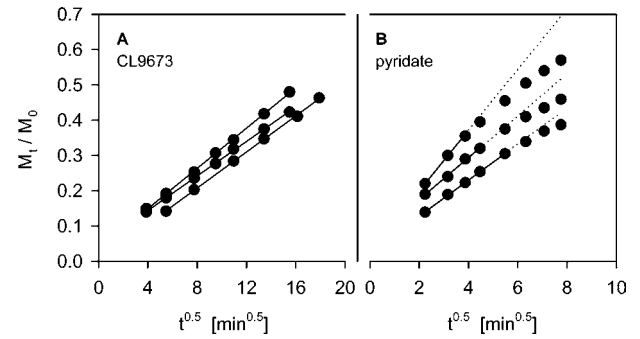

Figure 3. Three representative examples of desorption kinetics for CL9673 (**3A**) and pyridate (**3B**) in reconstituted cuticular waxes of *H. vulgare* using phospholipid suspension as desorption medium. Relative amounts desorbed (M_t/M_0) is plotted vs the square root of time (t).

kinetics (*4*) by eq 4 with l [m] representing the thickness of the wax layer

$$\frac{M_t}{M_0} = \frac{4}{l}\sqrt{\frac{Dt}{\pi}} \quad (4)$$

Desorption kinetics of CL9673 could be linearized for 50% of the relative amounts desorbed, which is characteristic of concentration-independent diffusion (*19*). Desorption kinetics of the pyridate deviated from linearity well before 50% of the relative amount desorbed (**Figure 3**). This indicated that D for pyridate was dependent on the concentration of pyridate in the wax. Therefore, D of pyridate had to be calculated from the initial linear parts of the desorption kinetics regression. Different pyridate concentrations in the wax were investigated.

Statistics. K_{ww} and D are given as means of 10 replications with 95% confidence intervals. Statistically significant differences were calculated on the basis of the Student's t-test with a significance level of 5%.

RESULTS

Values of K_{ww}^{pH} for CL9673 were strongly affected by the pH (**Figure 2**). They ranged from 1.7 at pH 2 to 0.09 at pH 8 in *C. album* wax (**Figure 2A**) and from 1.5 at pH 2 to 0.08 at pH 8 in *H. vulgare* wax (**Figure 2B**). Since **Figure 2A**,**B** shows titration curves of CL9673, the inflection points of the curves represent the pK_a value of CL9673, and the partition coefficient of the nondissociated species can be obtained from the left plateau of the curve at the low pH values (**Table 2**). Sorption of pyridate into cuticular waxes of both species was not affected by pH (**Figure 2**). Values of K_{ww} for pyridate were on average 4 orders of magnitude higher as compared to the nondissociated species of CL9673 (**Table 2**).

Table 2. Wax/Water Partition Coefficients (K_{ww}) of CL9673 and Pyridate in Reconstituted Cuticular Wax of *C. album* and *H. vulgare* and Dissociation Constant (pK_a) of CL9673 Obtained from Inflection Points of Figure 2A,B[a]

Compound	cuticular wax	K_{ww}	pK_a
CL9673	*C. album*	1.73 ± 0.02	5.26 ± 0.25
Pyridate	*C. album*	21620 ± 1260	
CL9673	*H. vulgare*	1.49 ± 0.03	5.17 ± 0.32
Pyridate	*H. vulgare*	15580 ± 1120	

[a] K_{ww} of CL9673 was corrected for fraction of ionized CL9673 according to eq 3. Results are given as means ($n = 10$) with 95% confidence intervals.

Table 3. Diffusion Coefficients (D) of CL9673 in Reconstituted Cuticular Wax of *C. album* and *H. vulgare* Using Either a Phospholipid Suspension (PLS) or a Micellar Solution of the Alcohol Ethoxylate C_8E_4 as Desorption Medium[a]

Species	desorption medium	CL9673D × 10^{-16} [m^2 s^{-1}]
C. album	PLS	0.124 ± 0.018
C. album	C_8E_4	1.63 ± 0.45
H. vulgare	PLS	0.106 ± 0.034
H vulgare	C_8E_4	1.78 ± 0.53

[a] Results are given as means ($n = 10$) with 95% confidence intervals.

In reconstituted wax of *C. album*, the D value for CL9673 was 1.24 × 10^{-17} m^2 s^{-1} (**Table 3**) and increased by a factor of 13 when CL9673 was desorbed into the medium with C_8E_4 instead of PLS (**Table 3**). In the wax of *H. vulgare*, the D value was 1.1 × 10^{-17} m^2 s^{-1} and using desorption media with C_8E_4 caused a 17-fold increase of D (**Table 3**). Diffusion of pyridate was dependent on the concentration of pyridate in the wax, and it increased D in *C. album* wax by a factor of 5.0 from 3.2 × 10^{-17} to 1.6 × 10^{-16} m^2 s^{-1}, when the pyridate concentration in *C. album* wax was increased from 0.18 to 4.9 g kg^{-1} (**Figure 4**). In *H. vulgare* cuticular wax, D increased by a factor of 6.7 from 2.7 × 10^{-17} to 1.8 × 10^{-16} m^2 s^{-1}, when the concentration was increased from 0.37 to 5.4 g kg^{-1} (**Figure 4**). When pyridate was desorbed into the C_8E_4 solution instead of PLS, no concentration dependence of diffusion was observed (**Figure 4**), and the use of C_8E_4 did not increase pyridate diffusion even at the highest pyridate concentration.

DISCUSSION

The pH dependence of the apparent wax/water partition coefficients for CL9673 resulted in a titration-like curve where the point of inflection gave a pK_a value of 5.26 (**Figure 2** and **Table 2**). At pH values below the pK_a, the majority of the molecules are nonionized, and the partition coefficient reached a maximum at pH 2. At high pH values, the majority of the molecules are ionized, and the lowest K_{ww}^{pH} was observed at pH 8 (**Figure 2**). This behavior proves that only the nondissociated species of CL9673 is lipophilic enough to be soluble in the cuticular waxes, whereas the ionized form is not soluble in wax. This observation corresponds with results comparing

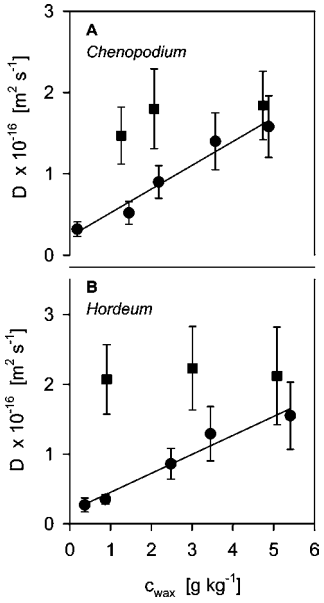

Figure 4. Correlation between diffusion coefficients (*D*) for pyridate in cuticular waxes of *C. album* (**4A**) and *H. vulgare* (**4B**) and the concentration of pyridate in the wax (c_{wax}). *D* was calculated from desorption kinetics using phospholipid suspension as desorption medium (circles) or an aqueous solution of the alcohol ethoxylate C_8E_4 (squares). Results are given as means ($n = 10$) with 95% confidence intervals.

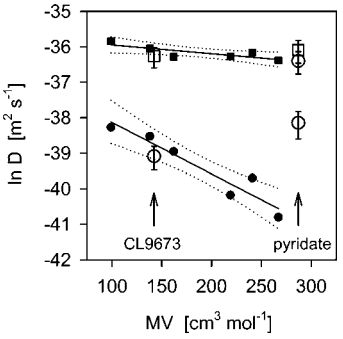

Figure 5. Correlation between the logarithms of the diffusion coefficients (ln *D*) for different compounds in the cuticular wax of *H. vulgare* with molar volumes (MV) of these compounds (data were taken from ref *14*). The lower curve (small black circles) represents desorption with phospholipid suspension (PLS), whereas the upper curve (small black squares) represents desorption with the alcohol ethoxylate tetraethylene glycol monooctyl ether (C_8E_4). Large white circles give ln *D* measured for CL9673 and pyridate (both symbols correspond to lowest and highest internal wax concentrations used in the desorption experiments) when PLS was used as a desorption medium. Large white squares give ln *D* for CL9673 and pyridate when C_8E_4 was used as desorption medium. Results are means ($n = 10$) with 95% confidence intervals.

cuticular permeability of nonionized and ionized species of the same molecule (*20*−*23*). It was shown that permeability across isolated cuticular membranes (*20*−*21*) and uptake into leaves (*22*−*23*) were only measurable for the nondissociated species of ionizable compounds. Pyridate is the C_8-alkyl ester of CL9673, which increases lipophilicity and removes the pH dependent sorption trait. The K_{ww} for pyridate is more than 4 orders of magnitude higher than the K_{ww} for CL9673 (**Figure 2** and **Table 2**). It was shown previously that the logarithms of partition coefficients in the cutin polymer (*16*, *24*) and in reconstituted cuticular wax (*14*) increased by a value of 0.5 with each additional methylene group added to the molecule.

First indications that diffusion of pyridate depended on pyridate concentration in wax were evident from the shape of the desorption kinetic lines since they deviated significantly from linearity before 50% of the relative amount had been desorbed (**Figure 3B**), which was not the case with CL9673 (**Figure 3A**). This conclusion was further confirmed by the observation that *D* of pyridate increased with increasing pyridate concentrations in the wax (**Figure 4**). Thus, pyridate must be considered as a compound with a pronounced self-accelerating property, which means that pyridate itself has a plasticizing effect on the wax phase in which it is diffusing, thus increasing *D*. Such a self-accelerating effect of a diffusing molecule was first described by Baur et al. (*25*) for chlorofenvinphos diffusing across isolated cuticular membranes. An important prerequisite for such a self-acceleration of diffusion is a sufficiently high concentration in wax, which is in fact the case for pyridate characterized by a K_{ww} between 15 000 and 20 000 (**Table 2**). Because of the high K_{ww} value, the pyridate concentration in the wax was 5 g kg^{-1}, which is exactly in the range of concentrations where accelerating effects of alcohol ethoxylate adjuvants on diffusion in wax have been described (*14*).

The alcohol ethoxylate C_8E_4, which has been described as a very efficient plasticizer increasing diffusion of active ingredients in wax (*14*), did not have any effect on the diffusion of pyridate in wax at highest pyridate concentrations (**Figure 4**). This is good indication that both types of molecules must have a similar mode of action. To explain the effect of an accelerator on the structure of cuticular wax, a comparison with the action of plasticizers in synthetic polymers is helpful (*26*). According to this comparison, it must be postulated that accelerators have a plasticizing effect by weakening the van der Waals forces between the wax molecules, and thus, the wax becomes more fluid (*27*). This, in turn, results in an increase of the free volume available for diffusion (*7*, *14*) and a decrease of the activation energy needed for diffusion (*28*).

It is suggested that the melting point (MP) of a compound is a useful parameter for estimating the potential of a compound having a plasticizing effect. A compound could induce plasticization of cuticular waxes if the MP of the compound is lower than the MP of the wax. Aliphatic cuticular waxes melt in the range of 60−90 °C (*29*), and the MP of pyridate (27 °C) is significantly lower than that of CL9673 (225 °C; **Table 1**). This behavior follows the general observation that esterification of organic compounds causes a significantly lower MP temperature (*30*). These principles are confirmed by other studies that have shown self-accelerating properties for chlorofenvinphos (*25*) and permethrin (*7*), compounds also with low MPs in the same range as that of pyridate. Thus, the prerequisites for self-accelerating effects are high internal concentrations in the cuticular wax and a low MP. These prerequisites also apply to classical accelerators such as alcohol ethoxylates (*7*, *14*) and tributyl phosphate (*28*) leading to an efficient plasticization of the cuticular wax barrier.

The *D* value of active ingredients in the cuticular wax of *H. vulgare* can be modeled and predicted according to the free volume theory (*14*). It predicts that *D* decreases exponentially with increasing molar volumes (MV) of diffusing compounds. However, *D* of pyridate was higher than *D* of CL9673, although the molar volume (MV) of pyridate was significantly larger, which conflicts with the established model. Using the model, a *D* value of 1.4×10^{-17} m^2 s^{-1} was predicted for CL9673, which is in good agreement with the measured value (**Table 3** and **Figure 5**). However, for pyridate, a *D* value of 1.6×10^{-18} m^2 s^{-1} was predicted from the model, which is 2 orders of magnitude lower than experimentally determined values (**Figure 4**). Even an extrapolated *D* of pyridate at a theoretical pyridate

concentration in wax of zero (see **Figure 4**) was 1.3×10^{-17} m^2 s^{-1}, which is still 10 times higher than the predicted value (**Figure 5**). This significant deviation of the measured *D* of pyridate from the predicted *D* (**Figure 5**) is further good evidence for the self-accelerating effect of pyridate. *D* of active ingredients in cuticular wax of *H. vulgare* can also be predicted when plasticized by C_8E_4 (**Figure 5**). In this situation, there is only a weak dependence of *D* on the molar volume (*14*). Predicted *D* values of CL9673 (2.2×10^{-16} m^2 s^{-1}) and pyridate (1.5×10^{-16} m^2 s^{-1}) in the presence of C_8E_4 both are in good agreement with measured *D* (**Figure 5**).

In conclusion, CL9673 was characterized by low sorption and diffusion in cuticular wax, whereas pyridate showed a high K_{ww} and *D* value. Thus, the introduction of an alkyl substituent by esterification of the hydroxyl group provides an efficient strategy enhancing transport in the cuticular wax barrier since the pH-dependence disappears and lipophilicity of the compound is considerably increased. In addition, pyridate exhibits a self-accelerating effect on its diffusion, which further enhances diffusion in wax. However, due to the high K_{ww} of pyridate, it could accumulate in the cuticle (*31*) because its solubility in the aqueous cell wall would be low. Thus, to guarantee its activity in a plant, a high rate of metabolism is required converting pyridate to CL9673 (*11*).

ABBREVIATIONS USED

D, diffusion coefficient; K_{ww}, wax/water partition coefficient; MP, melting point.

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