Salicylic Acid Transport in *Ricinus communis* Involves a pH-Dependent Carrier System in Addition to Diffusion[1][OA]

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Despite its important functions in plant physiology and defense, the membrane transport mechanism of salicylic acid (SA) is poorly documented due to the general assumption that SA is taken up by plant cells via the ion trap mechanism. Using *Ricinus communis* seedlings and modeling tools (ACD LogD and Vega ZZ softwares), we show that phloem accumulation of SA and hydroxylated analogs is completely uncorrelated with the physicochemical parameters suitable for diffusion (number of hydrogen bond donors, polar surface area, and, especially, LogD values at apoplastic pHs and Δ LogD between apoplast and phloem sap pH values). These and other data (such as accumulation in phloem sap of the poorly permeant dissociated form of monohalogen derivatives from apoplast and inhibition of SA transport by the thiol reagent *p*-chloromercuribenzenesulfonic acid [pCMBS]) lead to the following conclusions. As in intestinal cells, SA transport in *Ricinus* involves a pH-dependent carrier system sensitive to pCMBS; this carrier can translocate monohalogen analogs in the anionic form; the efficiency of phloem transport of hydroxylated benzoic acid derivatives is tightly dependent on the position of the hydroxyl group on the aromatic ring (SA corresponds to the optimal position) but moderately affected by halogen addition in position 5, which is known to increase plant defense. Furthermore, combining time-course experiments and pCMBS used as a tool, we give information about the localization of the SA carrier. SA uptake by epidermal cells (i.e. the step preceding the symplastic transport to veins) insensitive to pCMBS occurs via the ion-trap mechanism, whereas apoplastic vein loading involves a carrier-mediated mechanism (which is targeted by pCMBS) in addition to diffusion.

Salicylic acid (SA), classified under the group of plant hormones, has been reported to induce heat production in inflorescences of thermogenic species, stimulate flowering and enhance flower longevity, inhibit ethylene biosynthesis, block the wound response, and reverse the effect of abscisic acid (Davies, 2004; Hayat et al., 2007). Moreover, considerable attention has been focused for about two decades on the role of SA in plant disease resistance, since the observation that acetylsalicylic acid (aspirin) induces resistance to viruses and is highly effective in activating pathogenesis-related genes (White, 1979). Further support for the role of SA in plant disease resistance comes from mutants and transgenic plants impaired in the accumulation of SA and thereby unable to develop efficient defense responses after infection (Gaffney et al., 1993; Ryals et al., 1996; Sticher et al., 1997) and measurements of endogenous SA levels in response to pathogen attacks (Malamy et al., 1990; Yalpani et al., 1991). Although free SA is not the primary signal that induces systemic acquired resistance (Rasmussen et al., 1991; Vernooij et al., 1994; Ryals et al., 1996; Maldonado et al., 2002; Park et al., 2007; Truman et al., 2007), it also moves from the infected leaf to the upper uninoculated leaves via the sieve tubes (Metraux et al., 1990; Yalpani et al., 1991; Shulaev et al., 1995; Molders et al., 1996). It may be supposed that, in mature infected tissues, SA transport to the sieve elements occurs via either the symplastic or the apoplastic pathway according to the species, as described in the past for assimilates (Bonnemain, 1975). In the case of apoplastic phloem loading, SA must cross the plasma membrane of the companion cell-sieve element complex. Similarly, exogenous SA and analogs applied to the leaf surface must cross the plasma membrane of a cell to reach the phloem sap. Because

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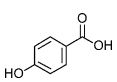
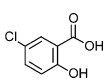
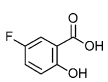
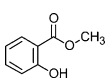
Table I. *Chemical structures and physicochemical descriptors (molecular mass, molecular volume, pKa, LogD, percentage of the protonated form in an octanol-water mixture, PSA, and HBD) of SA and other derivatives used in this work*

All parameters were computed using ACD LogD suite version 11.01 software except molecular volume and PSA, which were estimated using Vega ZZ version 2.3.1.

Feature	Salicylic Acid (SA)	Benzoic Acid (BA)	3-Hydroxybenzoic Acid (3-OHBA)	4-Hydroxybenzoic Acid (4-OHBA)	5-Chlorosalicylic Acid (5-ClSA)	5-Fluorosalicylic Acid (5-FSA)	Methyl Salicylate (MeSA)
Structural formula							
Molecular mass (D)	138.12	122.12	138.12	138.12	172.57	156.11	152.15
Molecular volume (Å^3)	115.9	108.6	116.1	115.7	130.0	121.0	132.6
pKa $_1$ (COOH)	3.01 ± 0.1	4.20 ± 0.1	4.08 ± 0.1	4.57 ± 0.1	2.64 ± 0.1	2.68 ± 0.1	–
LogD							
pH 4.6	−0.34 ± 0.36	1.02 ± 0.28	0.5 ± 0.30	1.01 ± 0.27	0.54 ± 0.39	0.045 ± 0.47	2.52 ± 0.24
pH 6.0	−1.06 ± 0.51	−0.23 ± 0.33	−0.78 ± 0.34	−0.16 ± 0.32	0.081 ± 0.62	−0.44 ± 0.69	2.52 ± 0.24
RCOOH O/W (%)							
pH 4.6	26.5	93.6	80.2	94.7	52.9	36.9	
pH 6.0	1.4	36.6	13.9	41.6	4.3	2.3	
PSA (Å^2)	61.1	42.0	67.0	67.0	61.0	61.1	44.6
HBD	2	1	2	2	2	2	1

SA is a weak acid, it is generally thought that SA phloem uptake occurs by diffusion of its lipophilic undissociated form, which dissociates into poorly permeating anions into the phloem symplast (Krasavina, 2007). According to Kleier's prediction model, the physical properties of this hormone, in terms of ionization constant in aqueous solution (pKa) values and octanol/water partitioning coefficient (log K_{ow}), are nearly ideal for phloem systemicity by way of the ion-trap mechanism (Yalpani et al., 1991).

SA, under its pharmaceutical derivative (aspirin), is a popular drug. It was initially believed that monocarboxylic acid drugs were absorbed from the small intestine lumen by a passive diffusion mechanism depending on the degree of protonation of the carboxylate moiety and the lipid solubility of the unionized

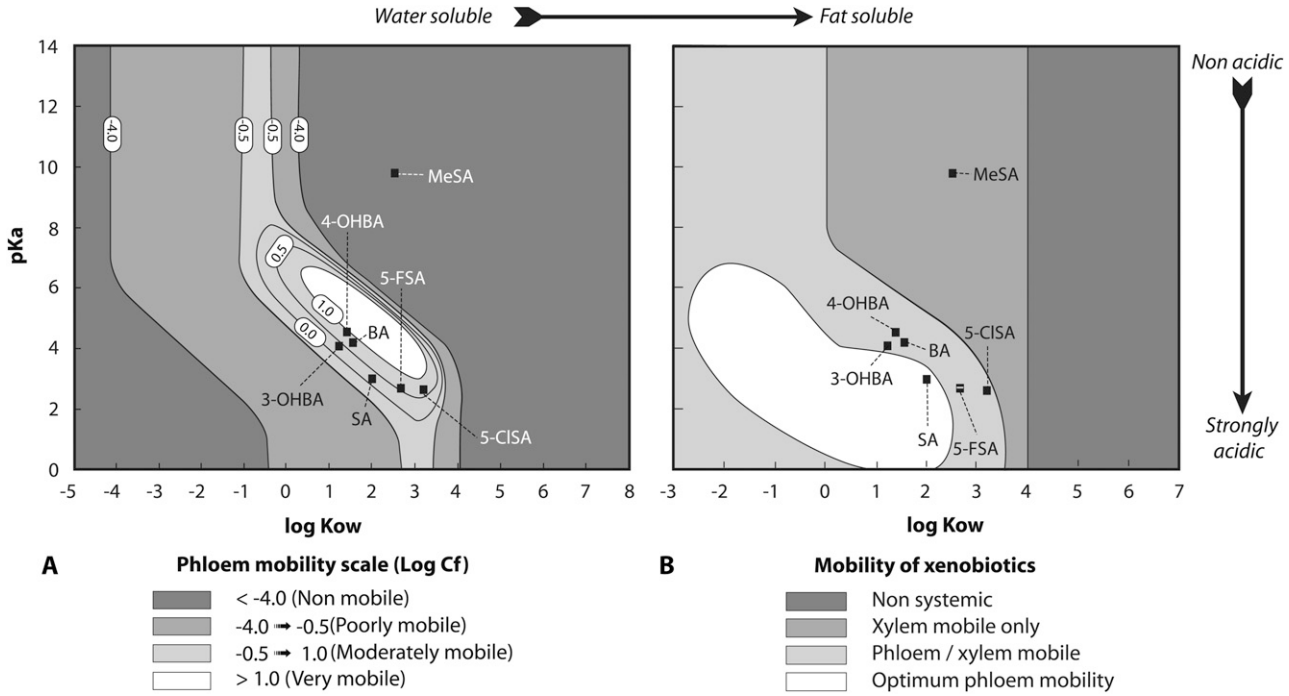


Figure 1. Prediction of phloem mobility of SA, 5-ClSA, 5-FSA, BA, 3-OHBA, 4-OHBA, and MeSA. A, Kleier map (log Cf as a function of log K_{ow} and pKa) according to Kleier and Hsu (1996). Plant parameters are for a short plant. B, Bromilow model (degrees of mobility as a function of log K_{ow} and pKa) according to Bromilow et al. (1991).

molecule (Brodie and Hogben, 1957) in the ''acid microclimate'' (pH 5.5–6.0) of the intestinal surface (Elbert et al., 1986). Then, experiments conducted with brush-border membrane vesicles from animal small intestine or the human colon adenocarcinoma cell line Caco-2, which exhibits several functional properties of small intestine, have demonstrated that transport of various monocarboxylic acids (acetic acid, nicotinic acid, benzoic acid [BA], and SA) occurs mainly via a pH-dependent and carrier-mediated transport mechanism (Simanjuntak et al., 1990; Takanaga et al., 1994; Tsuji et al., 1994). These works led to the characterization of the monocarboxylate transporter (MCT) family in animal cells (Garcia et al., 1994; Tamai et al., 1995; Enerson and Drewes, 2003).

In a recent paper (Rocher et al., 2006), we studied the ability of the phloem of *Ricinus communis* seedlings to load exogenous SA. It was shown that phloem loading of SA is dependent on the pH of the cotyledon-incubating medium and is highest at the most acidic values (pH 4.6 and 5.0). However, a residual phloem loading still occurs at pH values close to neutrality (i.e. when SA is only in its poorly permeant dissociated form). Furthermore, in contrast to the Kleier and Bromilow model predictions (Bromilow et al., 1990, 1991; Hsu and Kleier, 1996; Kleier and Hsu, 1996), dichlorinated analogs (with a log K_{ow} near 4) also moved in the phloem. These discrepancies may give rise to the hypothesis that SA and aromatic monocarboxylate analogs are taken up by a carrier system in addition to the ion-trap mechanism (Rocher et al., 2006). These data need further investigation. Particularly, log K_{ow} values refer to molecules in their neutral form that may not exist or may be present only in small part (as SA) at biological pH. In this regard, at this step of the study, important parameters controlling diffusion and the ion-trap mechanism (number of hydrogen bond donors, polar surface area, Δ LogD between apoplast and phloem sap pH values) were not taken into consideration, and there was a lack of direct physiological evidence that SA transport is carrier mediated. The purpose of this work is to test this hypothesis. Here, we demonstrate that phloem transport of SA and its halogen analogs is carrier mediated and put forward several functional properties of the carrier system, especially its substrate specificity and its sensitivity to the thiol reagent *p*-chloromercuribenzenesulfonic acid (pCMBS). We also give information about the tissue localization of this transporter and give details of the mechanisms of the two pathways involved in SA phloem loading.

RESULTS AND DISCUSSION

Predicting Phloem Mobility of SA Analogs Using the Kleier and Bromilow Models

The phloem mobility of three types of SA analogs was studied (Table I): monohalogenated derivatives

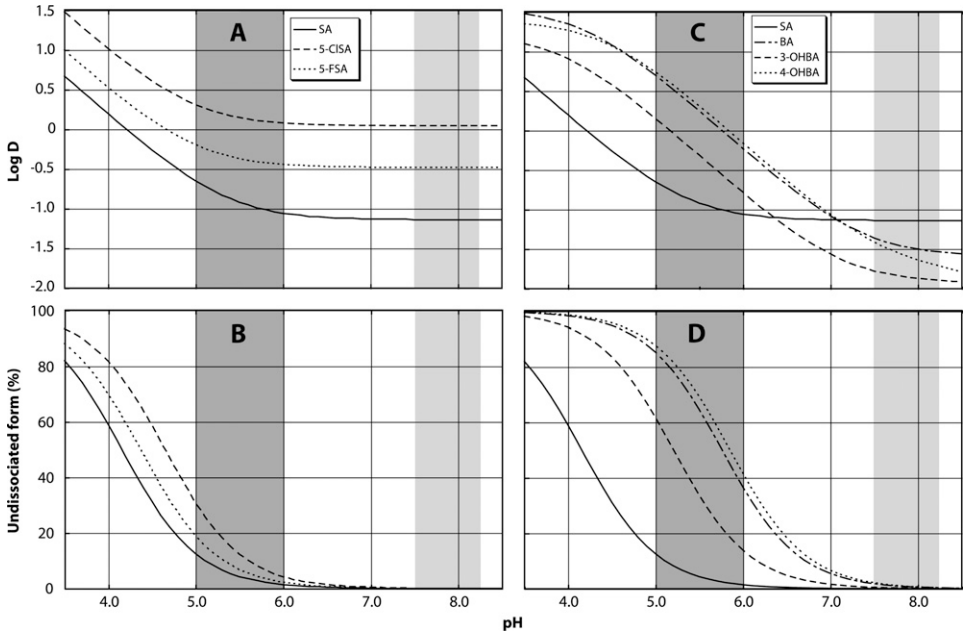


Figure 2. Physicochemical properties of SA and SA derivatives. LogD of SA, 5-ClSA, and 5-FSA (A) and SA, BA, 3-OHBA, and 4-OHBA (C) and percentage of undissociated forms of SA, 5-ClSA, and 5-FSA (B) and SA, BA, 3-OHBA, and 4-OHBA (D) in an octanol-water mixture as a function of pH. The apoplast and phloem sap pH values are represented by gray and light gray areas, respectively. Note that Δ LogD variations between pH 5.0 and 8.0 are small for SA and halogen derivatives (about 0.5 and 0.25 unit, respectively) and high for BA, 3-OHBA, and 4-OHBA (about 2.5 units). The apoplastic pH values are from Giaquinta (1977), but more acidic values are possible in phloem apoplast, taking into consideration the high expression of the plasma membrane H^+-ATPase in phloem cells, especially in companion cells (Bouche-Pillon et al., 1994). SA LogD values and percentage of undissociated forms calculated by ACD LogD version 11.01 are similar to those calculated by version 9.0 (Rocher et al., 2006).

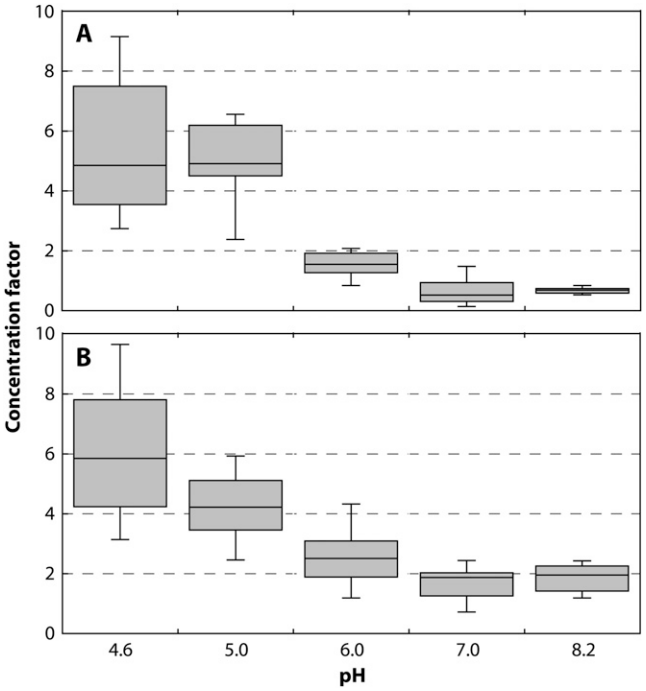


Figure 3. Phloem mobility of SA halogen derivatives. Concentration factors of 5-ClSA (A) and 5-FSA (B) added to the incubation solution at 10 μM final concentration in phloem sap of *Ricinus* as a function of the pH of the incubation medium. The sap was collected during the third and fourth hours of incubation. The concentration factor was the ratio [5-ClSA or 5-FSA]sap/[5-ClSA or 5-FSA]medium. For box plots, $10 \leq n \leq 17$.

with a halogen addition in position 5; BA and BA derivatives differing from SA by the position of the hydroxyl group; and an endogenous compound with the carboxylic function masked by a methyl group (i.e. methyl salicylate [MeSA]).

As previously (Rocher et al., 2006), phloem mobility was predicted using the models of Kleier (Kleier, 1988; Hsu and Kleier, 1996) and Bromilow (Bromilow et al., 1991). These models (Fig. 1) indicate two physicochemical properties (pKa and log K_{ow} values) of xenobiotics and natural compounds required for phloem accumulation (Hsu and Kleier, 1996) or various types of systemic behavior (Bromilow et al., 1991). Because several parameters, such as penetration across the leaf cuticle, metabolism, and cell compartmentation, are not taken into consideration, the aim of these models is to give useful information of the systemic ability of compounds rather than accurate predictions of mobility (Bromilow and Chamberlain, 1995; Hsu and Kleier, 1996). In this regard, *Ricinus* seedlings, with their thin and highly permeable cuticle, which facilitates molecule diffusion to and therefore within the cotyledon apoplast, is a suitable plant material to test the information from the predicting models. These models have also been used to design phloem-mobile propesticides (Kleier and Hsu, 1996; Kleier, 2001; Chollet et al., 2005). Furthermore, strong discrepancies between predictions and experimental data may indicate the involvement of a carrier system instead of diffusion through the plasma membrane (Oparka, 1991).

According to the calculations of pKa and log K_{ow} made by ACD LogD Sol Suite version 11.01 software, all of the SA analogs exhibited a phloem mobility near that of SA in both models (Fig. 1), except MeSA, which was predicted to be nonmobile (Fig. 1A) or mobile only in the xylem (Fig. 1B).

Predicting Transport of SA and SA Analogs Using Diffusion Predictors

Two parameters are now intensively used for the prediction of the diffusion of small molecules (less than 500 D) through human membranes: the polar surface area (PSA) and the number of hydrogen bond donors (HBD; Winiwarter et al., 2003; Ertl, 2008). HBD has been also assayed in algae (Raevsky and Schaper, 1998). PSA is defined as the sum of surfaces of polar atoms in a molecule, usually oxygen and nitrogen and the hydrogens bonded to these atoms (Ertl et al., 2000). This descriptor has been found to correlate well with passive drug permeability through the plasma membrane and, therefore, allows reliable predictions of transport properties of drug candidates in clinical development (Palm et al., 1998; Winiwarter et al., 1998), except for actively transported molecules (Palm et al., 1997; Winiwarter et al., 1998). To diffuse easily through the intestinal barrier, molecules must exhibit a PSA of less than 60 Å^2.

The second descriptor for the prediction of absorption of drugs is the hydrogen-bonding capacity and

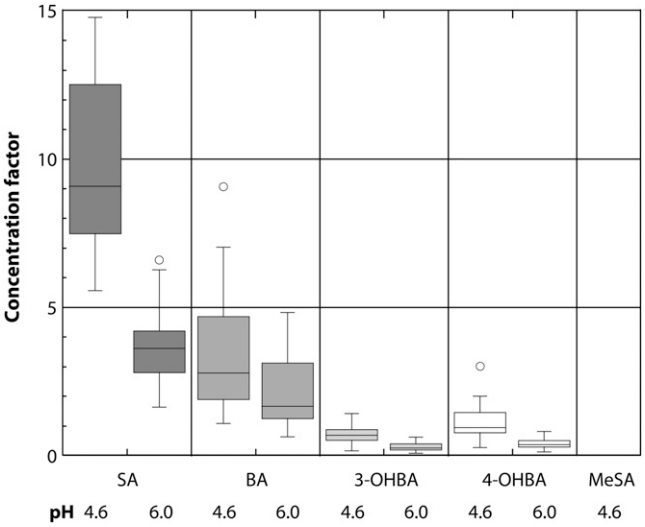


Figure 4. Phloem mobility of SA and SA analogs. Concentration factors of SA, BA, 3-OHBA, 4-OHBA, and MeSA added to the incubation medium at 10 μM final concentration in phloem sap of *Ricinus*. Cotyledons were incubated in a buffered solution at pH 4.6 or 6.0. The sap was collected during the third and fourth hours of incubation. The concentration factor was the ratio [studied product]sap/[studied product]incubation medium. MeSA could not be detected. For box plots, $16 < n < 29$.

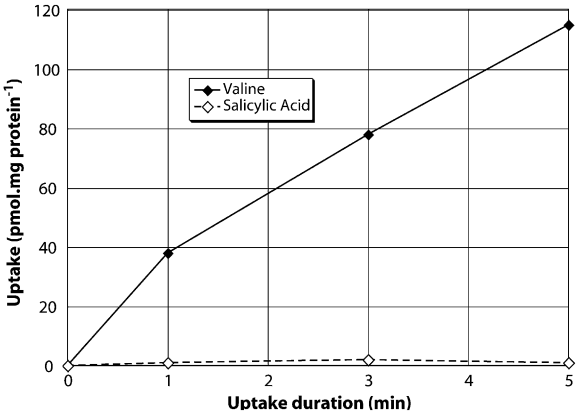


Figure 5. Time-dependent uptake of 100 μM [^{3}H]Val and 50 μM [^{14}C]SA by yeast (*Saccharomyces cerevisiae*) stain JT-16 expressing the amino acid transporter ANT1 from Arabidopsis (Chen et al., 2001). Data are means of four replicates and are expressed as the difference between uptake in JT-16/ANT1 and JT-16/pYES2 (empty plasmid) to measure transport due to ANT1.

particularly the number of HBD (i.e. a hydrogen atom attached to a relatively electronegative atom). HBD was successfully used for the correlation with permeability and absorption data for numerous chemicals and drugs in human cells (Raevsky and Schaper, 1998; Winiwarter et al., 1998, 2003) and algae (Raevsky and Schaper, 1998). Passive absorption is more likely when there are less than five (Lipinski et al., 1997) or 10 (Palm et al., 1997) HBD per molecule.

SA and SA analogs have low PSA and HBD values, consistent with efficient diffusion (Table I). Moreover, these values are identical (HBD) or very close (PSA) for SA, 3-hydroxybenzoic acid (3-OHBA), and 4-hydroxybenzoic acid (4-OHBA). If the passive transport was the only mechanism involved in *Ricinus* phloem uptake, the concentration factor should be in the same range for all three positional isomers.

PSA and HBD are considered more convenient diffusion predictors than LogD from intestinal lumen (Winiwarter et al., 2003). There is an interesting analogy about the pH gradient values between the surface of intestinal cells (pH 5.5–6.0) and the blood (pH 7.35–7.45) and the pH gradients between the phloem apoplast and the phloem sap. In absence of buffers, the tissues of the *Ricinus* cotyledons stabilize the incubation medium at pH 5.2 (our measurements), while the pH values of the *Ricinus* phloem sap vary from 7.5 to 8.2 (Hall and Baker, 1972; Vreugdenhil and Koot-Gronsveld, 1989). Taking into account these latter high pH gradients, LogD of ionizable compounds and Δ LogD variations from apoplast to phloem sap pH values (Δ $LogD_{A\text{-}P}$) need special attention.

Structure-Phloem Mobility Relationships

Halogen addition in position 5 on the aromatic ring, which enhances elicitor response against pathogens (Kauss et al., 1993) slightly (5-chlorosalicylic acid [5-ClSA]) or marginally (5-fluorosalicylic acid [5-FSA]), increases molecular mass and the molar volume. These molecules are less hydrophilic and less dissociated than SA at acidic pH values, but LogD variations from apoplast to phloem sap pH values are very small (Δ $LogD_{A\text{-}P} \approx 0.25$; Table I; Fig. 2, A and B). They exhibited good phloem mobility, although less than that of SA (concentration factor near 5 at pH 4.6 and 5.0 instead of 9 for SA; Fig. 3). Phloem mobility of both compounds was pH dependent (i.e. influenced by pH of the solution; Fig. 3) but not correlated to the percentage of the permeant undissociated form at the octanol-water interface (Fig. 2B), as calculated with ACD LogD software. A residual phloem transport (5-ClSA) or a clear phloem accumulation (5-FSA; concentration factor = 2) was noted at external pH 7.0 and 8.2 when these molecules were fully dissociated and at their minimum permeability for partitioning into the bilayer. This accumulation of 5-FSA in its anionic form in the phloem sap is completely inconsistent with diffusion. A residual uptake of BA and SA at pH values close to neutrality was also noted in intestinal cells (Takanaga et al., 1994; Tsuji et al., 1994). By contrast, phloem uptake of acid derivatives of fenpiclonil in *Ricinus* occurred only in their permeant undissociated form (Chollet et al., 2004, 2005).

BA, 3-OHBA, and 4-OHBA exhibited a much better physicochemical profile than SA for diffusion through a phospholipidic layer in response to a transmembrane pH gradient in terms of LogD at acidic pH values, LogD variations from apoplast to phloem sap pH values (Δ $LogD_{A\text{-}P} \approx 2.5$ instead of 0.5 for SA), and percentage of the permeant undissociated form at acidic pH values (Table I; Fig. 2, C and D). It may be added that in a pure water medium, the percentage of

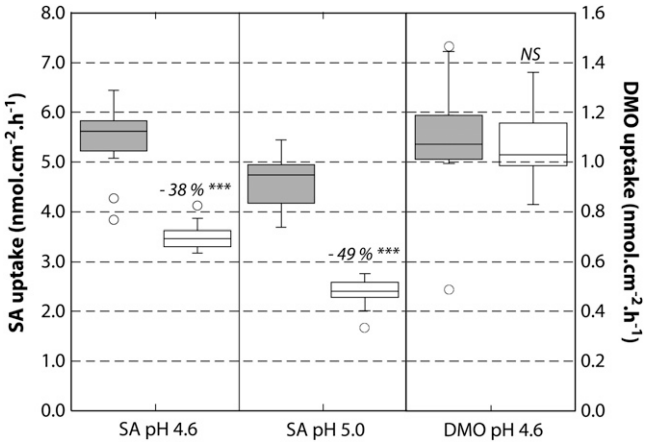


Figure 6. Effect of 1 mM pCMBS on 10 μM SA or 10 μM DMO uptake by discs from *Ricinus* cotyledons. Discs were preincubated in a buffered solution (pH 4.6 or 5.0) for 30 min and then transferred to the incubation medium for 1 h (the same solution containing [^{14}C]SA or [^{14}C]DMO without [gray boxes] or with [white boxes] pCMBS). The Mann-Whitney *U* test was used to assess statistically significant differences (*** $P < 0.001$; NS, not significant). For box plots, $10 < n < 16$.

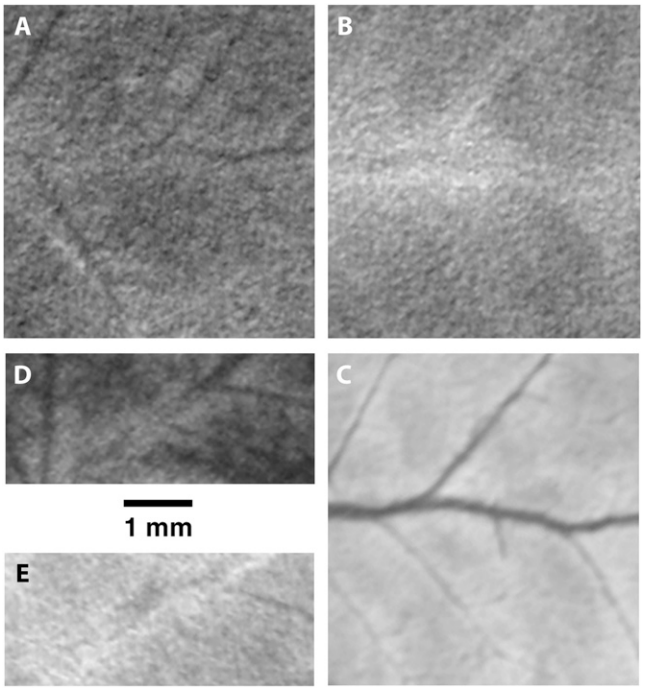


Figure 7. Autoradiographic images of the lower side of *Ricinus* cotyledons. Discs were incubated for 1 h in a buffered solution (pH 5.0) containing either 10 μM [^{14}C]SA (A and B; exposure time, 15 h) or 100 μM [^{14}C]Suc (D and E; exposure time, 51 h) without (A and D) or with (B and E) 1 mM pCMBS. C, Photograph of the major veins of the cotyledon area autoradiographed in B. Radioactivity appears in black.

the permeant undissociated form was 29%, 19%, and 41% at pH 4.6 for BA, 3-OHBA, and 4-OHBA, respectively, while SA monohalogenated analogs were only in their dissociated form according to the calculations made with ACD LogD Sol Suite software. However, the phloem loading of these compounds, especially that of 3-OHBA (concentration factor = 0.70 at pH 4.6) and 4-OHBA (concentration factor = 0.95 at pH 4.6), was dramatically reduced in comparison with that of SA, 5-ClSA, and 5-FSA (Figs. 3 and 4), and this cannot be explained by PSA and HBD values (Table I), which are the same for the three molecules as mentioned above. These strong discrepancies, and those mentioned above, between predictions of diffusion through the plasma membrane (Table I; Figs. 1 and 2) and the experimental data (Figs. 3 and 4) strongly indicate that SA phloem loading involves a pH-dependent carrier system in addition to the ion-trap mechanism.

MeSA can diffuse through the membrane, taking into account its PSA and HBD values (Table I), but similarly in both directions because the molecule is not ionizable at biological pH values (Δ $LogD_{A\text{-}P}$ = 0). In agreement with the predicting models of phloem mobility (Fig. 1), MeSA concentration in the phloem sap was so low that it could not be detected by HPLC in the *Ricinus* phloem sap (under our experimental conditions, the detection limit for MeSA was 0.25 μM). This is consistent with recent measurements of MeSA levels by gas chromatography-mass spectrometry in tobacco (*Nicotiana tabacum*) leaves. MeSA concentrations vary from about 3 to 10 nM in the phloem sap according to the plant material and treatments and are about 100-fold lower than those noted in the leaf tissue (Park et al., 2007). By contrast, after leaf inoculation with the *Tobacco necrosis virus*, SA concentrations vary from about 0.5 to 7 μM in the mesophyll and from 2 to 9 μM in the phloem sap (Malamy et al., 1990; Metraux et al., 1990), despite SA metabolism and SA 2-*O*-β-D-glucoside sequestration in the vacuoles. SA levels can even reach 200 to 500 μM in the sap after *Pseudomonas* infection of cucumber (*Cucumis sativus*) leaves (Rasmussen et al., 1991).

Our data show that efficient phloem transport of BA derivatives requires a free carboxyl group on the aromatic ring and a hydroxyl group in position 2. It has recently been demonstrated that some Suc carriers (AtSUC9 and LjSUT4) transport several phenyl glucosides, including salicin [2-(hydroxymethyl)phenyl-β-D-glucopyranoside; Sivitz et al., 2007; Reinders et al., 2008], which is metabolized to SA in the human body, but not SA (Sivitz et al., 2007). Furthermore, complementary experiments indicated that SA was not translocated by ANT1, an aromatic and neutral amino acid carrier also transporting nonhydroxylated monocarboxylic acids such as auxin and 2,4-dichlorophenoxyacetic acid (Chen et al., 2001; Fig. 5). This is in agreement with the conclusion that specific structural requirements must be met for efficient transport of aromatic monocarboxylic acids (Fig. 4). By contrast, the animal MCT1, which translocates SA, is also implicated in intestinal absorption of several endogenous and exogenous compounds differing in size and structure, from unbranched aliphatic monocarboxylates such as

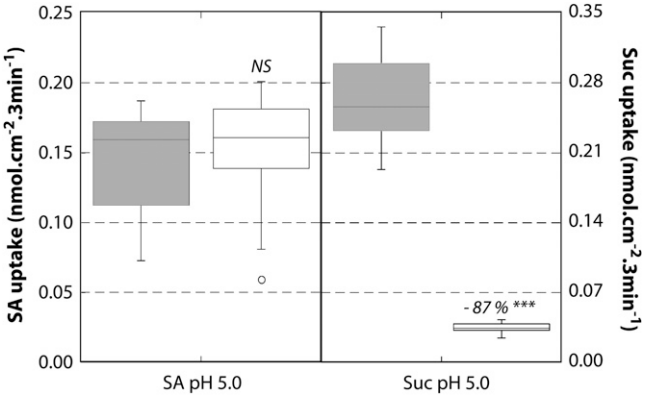


Figure 8. Effect of 1 mM pCMBS on 10 μM SA or 100 μM Suc uptake by discs from *Ricinus* cotyledons for 3 min. Discs were floated on a buffered standard solution (pH 5.0) for 30 min and then on the same medium without or with 1 mM pCMBS for 15 min. After the pretreatment, tissues were transferred to the incubation medium (the same solution containing either 10 μM [^{14}C]SA or 100 μM [^{14}C]Suc without [gray boxes] or with [white boxes] pCMBS) for 3 min. The Mann-Whitney *U* test was used to assess statistically significant differences (*** $P < 0.001$; NS, not significant). For box plots, $n = 10$.

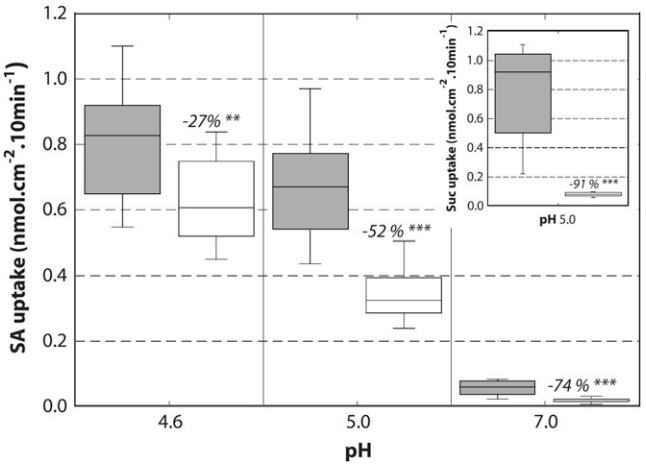


Figure 9. Effect of 1 mM pCMBS on 10 μM SA uptake or 100 μM Suc uptake (inset) by discs from *Ricinus* cotyledons for 10 min. Discs were preincubated in a buffered standard solution for 30 min and then on the same medium without or with 1 mM pCMBS for 15 min. After the pretreatment, tissues were transferred to the incubation medium (the same solution containing 10 μM [^{14}C]SA or 100 μM [^{14}C]Suc without [gray boxes] or with [white boxes] pCMBS) for 10 min. The Mann-Whitney *U* test was used to assess statistically significant differences (*** $P < 0.001$, ** $P < 0.01$). For box plots, $12 < n < 16$.

acetate and propionate to much larger molecules such as statins (Enerson and Drewes, 2003; Halestrap and Meredith, 2004).

Effect of pCMBS on SA Uptake by *Ricinus* Cotyledons

pCMBS has been widely used to study nutrient uptake for a long time (Giaquinta, 1977). Due to its physicochemical properties, it is considered a poorly permeant or nonpermeant tool, and this is consistent with experimental data indicating that pCMBS is membrane impermeable (Bourquin et al., 1990; Bush, 1993). For instance, infiltration of broad bean (*Vicia faba*) leaf apoplast by 2 mM pCMBS completely blocks phloem loading of assimilates without significantly affecting CO_2 assimilation or the transmembrane potential difference generated by the plasma membrane H^+-ATPase (Bourquin et al., 1990). pCMBS is known as a potent inhibitor of Suc and oligopeptide carriers by reacting with Cys residues in the external part of these transporters (Delrot et al., 1980; Bush, 1993; Jamai et al., 1994; Orlich et al., 1998; Lemoine, 2000; Knop et al., 2004). This inhibiting effect cannot be generalized to other nutrient carriers, such as monosaccharide transporters (Noiraud et al., 2001), and depends on the location of Cys residues in the extracellular domains (Ramsperger-Gleixner et al., 2004).

At acidic pH values, SA uptake by *Ricinus* cotyledons was significantly inhibited by 1 mM pCMBS (Fig. 6). By contrast, diffusion of the weak acid 5,5′-dimethyloxazolidine-2-^{14}C,4-dione ([^{14}C]DMO), used as an internal pH probe, through the plasma membrane in response to the transmembrane pH gradient was exactly the same in control and treated sets (Fig. 6). This indicates that the ion-trap mechanism of weak acids is not affected in the presence of pCMBS and that a protein-mediated translocation pathway for SA is targeted by the thiol reagent. The localization of [^{14}C]SA (and labeled metabolites) in cotyledon tissues was very similar to that of [^{14}C]Suc. It has been previously shown that [^{14}C]Suc was taken up not only by the phloem but also by the epidermal cells of *Ricinus* seedlings (Martin and Komor, 1980; Weig and Komor, 1996). Consequently, the labeling of cotyledon epidermis and mesophyll masked the minor veins on autoradiographic images (Martin and Komor, 1980). The distribution of [^{14}C]SA exhibited a similar pattern (Fig. 7, compare A and D), and the minor vein network was hardly perceptible. pCMBS affected the general labeling of the tissues, but particularly the vein network. Most of the major veins were no more perceptible (Fig. 7, B and C). Suc phloem loading was also inhibited by pCMBS, as was Suc uptake by external tissues (Fig. 7, D and E). These data indicate that SA transport in *Ricinus* tissues involves a carrier system sensitive to pCMBS. Within the animal MCT family, some carriers such as MCT1 are sensitive to pCMBS while others are not (Halestrap and Meredith, 2004).

Localization of the SA Carrier Targeted by pCMBS: Mechanisms of the Two Routes of SA Phloem Loading

Ricinus is a symplastic-apoplastic loader (Orlich and Komor, 1992). This means that endogenous molecules from seedling endosperm (or exogenous compounds from an incubation solution) found in the phloem sap may come, via the symplastic pathway, from the transfer cells of the lower epidermis or may be taken up directly from the phloem apoplast. This is the case with Suc, which is translocated by the Suc transporters RcSCR1 and RcSUT1, the latter being located in both the lower epidermis and the phloem (Weig and Komor, 1996; Bick et al., 1998). In this regard, the time course of

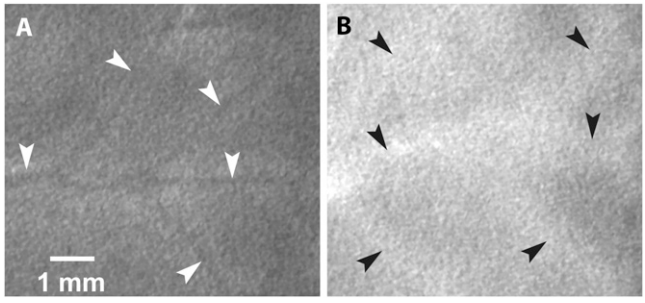


Figure 10. Autoradiographic images of the lower side of *Ricinus* cotyledons. Discs were floated for 30 min on a standard preincubation medium and then on the same medium for an additional 15 min without (A) or with (B) 1 mM pCMBS. After the pretreatment, tissues were incubated for 10 min in the same buffered solution (pH 5.0) containing 10 μM [^{14}C]SA without (A) or with (B) 1 mM pCMBS. Exposure time was 100 h. Radioactivity appears in black. Major veins are indicated by arrowheads.

[^{14}C]Suc uptake into *Ricinus* cotyledons, as measured by autoradiography, suggests that the epidermis primarily takes up Suc. The labeling of the major veins could be detected only after a 5-min incubation period and became obvious after 10 min (Martin and Komor, 1980). On this basis, the effect of pCMBS on [^{14}C]SA uptake by cotyledons tissues was studied after a 3-min incubation period (i.e. when the labeled molecules diffused mainly within the apoplast of the external cell layers) and after a 10-min incubation period.

Under the shortest experimental conditions, [^{14}C]SA uptake was similar in the control and in the presence of pCMBS, while [^{14}C]Suc uptake was dramatically inhibited by the thiol reagent (Fig. 8). This means that the SA carrier targeted by pCMBS is not located in the outer cells of *Ricinus* cotyledons (and especially in the lower epidermis transfer cells), contrary to the Suc carrier system. By contrast, after a 10-min incubation, both SA and Suc uptake were inhibited (about 50% and 90%, respectively, at pH 5.0) by the thiol reagent (Fig. 9). Moreover, autoradiographs showed that the major vein labeling was practically abolished in the treated set (Fig. 10). These data indicate that the SA carrier system targeted by pCMBS is mainly located in the vein area. A complementary experiment was conducted at pH 7.0 to examine the pH dependence of the inhibiting effect on SA uptake of pCMBS. The apparent inhibition varied from 27% (pH 4.6) to 74% (pH 7.0; Fig. 10). This suggests that the true inhibition of the SA carrier system by the thiol reagent at acidic pH values, especially at pH 4.6, is masked by the contribution of the ion-trap mechanism to the overall uptake. At pH 5.0 (i.e. a pH value close to that of cotyledon epidermis apoplast), the contribution of the ion-trap mechanism to the overall uptake seems not very different from that of the carrier-mediated mechanism (Fig. 9). The strong inhibition of SA uptake at pH 7.0 also supports that the carrier can manipulate SA in its anionic form.

Finally, our data indicate that SA molecules moving from epidermal transfer cells to the phloem via the symplastic route are taken up by a mechanism insensitive to pCMBS (Fig. 8), logically the ion-trap mechanism. The molecules that are not metabolized and sequestered in the vacuole during their transport from cell to cell can reach the phloem. SA molecules that are directly taken up from the apoplast in the vein areas are translocated by a carrier system sensitive to pCMBS (Fig. 9) and accumulate in the phloem. The ion-trap mechanism contribution to direct phloem loading of the conducting cells from the phloem apoplast appears to be a minor component of the overall phloem loading, taking into account the dramatic inhibition of conducting tissue labeling noted in the presence of pCMBS (Fig. 10).

CONCLUSION

Combining (1) the predictions of phloem mobility of SA and SA analogs from the Kleier and Bromilow models; (2) software calculations of physicochemical parameters of these molecules, such as PSA, HBD, and especially LogD, as well as percentage of the undissociated form and their variations according to the pH values of the apoplastic compartment and phloem sap; (3) analyses of phloem sap from a simple plant model without a cuticle barrier, which gives access to phloem plasma membrane properties; and (4) the use of a thiol reagent, it has been possible to demonstrate that SA transport involves a pH-dependent carrier system that is sensitive to pCMBS. This is the first time, to our knowledge, that the occurrence of a carrier system in plant tissues has been predicted by analysis of the discrepancies between the predictions of computational models and the actual results of the experiments. Furthermore, the rather poor phloem mobility of 3-OHBA and 4-OHBA indicates that this carrier system exhibits high substrate specificity with regard to the relative positions of the carboxyl and hydroxyl groups.

Then, combining time-course experiments, pCMBS used as a tool, and autoradiographic inhibition studies, it has been possible to pinpoint the tissue localization of the SA carrier in *Ricinus* cotyledons. Contrary to the Suc carrier system, which is expressed in both the epidermis and the veins (Bick et al., 1998), the SA carrier targeted by pCMBS is located only in the inner tissues, especially in the veins. As *Ricinus* is an apoplastic-symplastic loader (Orlich and Komor, 1992), SA phloem loading involves three components: (1)

Table II. *Chromatographic data for tested products*
Delivery was at 0.8 mL min^{-1}.

Product	Mobile Phase		Column		Detection UV	Retention Time
	Water + 0.1% Trifluoroacetic Acid	CH_3CN	Supelco Discovery	Merck Chromolith		
					nm	*min*
SA	50	50	+		210	8.31
BA	50	50	+		229	6.6
3-OHBA	70	30	+		212	8.1
4-OHBA	70	30	+		255	7.8
5-ClSA	65	35		+	212	3.5
5-FSA	60	40		+	233	4.3
MeSA	50	50	+		210	10.6

symplastic transport from the outer cells, especially the epidermis transfer cells; (2) uptake from the phloem apoplast via the ion-trap mechanism; and (3) uptake from apoplast mediated by a carrier system that is targeted by pCMBS.

MATERIALS AND METHODS

Plant Material

Ricinus communis 'Sanguineus' seeds, obtained from Ball-Ducrettet, were placed in wet cotton wool for 24 h at 27°C ± 1°C prior to sowing in vermiculite watered with tap water. Seedlings were grown in a humid atmosphere (80% ± 5%) at 27°C ± 1°C.

Phloem Sap Collection and Analysis

The sap collection method was similar to that recently described (Rocher et al., 2006). The phloem sap was analyzed by HPLC after dilution with ultra-high-quality water (18.2 MΩ cm^{-1}; 1:9, v/v). We employed reverse-phase chromatography using a Discovery RP-amide C16 column (length, 250 mm; i.d., 4.6 mm; Supelco) or a Chromolith performance RP 18e column (length, 100 mm; i.d., 4.6 mm; Merck) in accordance with the procedure set out in Table II. Results were processed with PC 1000 software version 3.5 from Thermo Fisher Scientific.

Uptake in Cotyledon Discs

Discs (1.13 cm^2 surface) were obtained with a 12-mm-diameter cork borer from *Ricinus* cotyledons. Then, they were floated on a preincubation medium containing 20 mM HEPES (pH 4.6, 5.0, and 7.0) as buffer, 0.5 mM $CaCl_2$, and 0.25 mM $MgCl_2$. After a 30-min preincubation period, the discs were incubated in the same buffered solution containing 10 μM [^{14}C]SA, 10 μM [2-^{14}C]DMO, or 100 μM [^{14}C]Suc with or without pCMBS at 1 mM concentration. Incubation was run under mild agitation on a reciprocal shaker at room temperature. After 3, 10, or 60 min of incubation, the disc apoplast was rinsed (3 × 2 min) in a solution similar to the preincubation medium. Then, each disc was digested overnight at 55°C in a mixture of perchloric acid (65%; 25 μL), hydrogen peroxide (33%; 50 μL), and Triton X-100 (1 g L^{-1}; 50 μL). After adding 4 mL of scintillation liquid (Ecolite+; MP Biomedicals), the radioactivity was counted by liquid scintillation spectrometry (Packard Tricarb 1900TR). In other sets, discs were incubated with 10 μM [^{14}C]SA or 100 μM [^{14}C]Suc with or without pCMBS. After 10 or 60 min of incubation, the discs were rinsed, dry-ice frozen, lyophilized, and autoradiographed (Kodak Biomax MR film).

Uptake in *Saccharomyces* Expressing ANT1

SA transport by an aromatic and neutral amino acid transporter from Arabidopsis (*Arabidopsis thaliana*) was tested. For this purpose, the ANT1 transporter expressed in yeast (*Saccharomyces cerevisiae*) strain JT16 was used (Chen et al., 2001). Experiments were conducted as described by Noiraud et al. (2001) with [^{14}C]SA at an external concentration of 50 μM. [^{3}H]Val at a 100 μM concentration was used as a control. Results were the difference between the uptake of yeast expressing ANT1 and yeast transformed with the empty plasmid. Each experimental point was repeated four times.

Chemicals

The compounds to be added to incubation solutions were from Acros Organics (SA, BA, 5-ClSA, 5-FSA, MES, and HEPES), from Alfa-Aesar (3-OHBA and 4-OHBA), from Sigma-Aldrich Chimie (SA-carboxy-^{14}C), from American Radiolabeled Chemicals (DMO), and from Toronto Research Chemicals (pCMBS).

Physicochemical Properties

Physicochemical properties and descriptors of SA and other ionizable molecules were predicted using ACD LogD Sol Suite version 11.01 software from Advanced Chemistry Development. This package of programs calculates log K_{ow} (the pH-independent octanol-water partition coefficient), pKa (the ionization constant in aqueous solution), solubility and dissociation in water at any pH, LogD (the pH-dependent log K_{ow}), and the number of HBD. To calculate LogD (the partition coefficient for almost any drawn organic compound at any pH), the software uses both pKa and log K_{ow} information, as already mentioned (Rocher et al., 2006). The algorithms for the predictions are based on contributions of separate atoms, structural fragments, and intramolecular interactions between different fragments. LogD is a very important parameter for bioavailability and absorption studies of drugs (van de Waterbeemd et al., 2003) and agrochemicals (Chollet et al., 2004, 2005). The calculation of confidence limits for LogD (δ LogD) is not implemented into ACD software but can be evaluated for a monoacid with the following formula:

$$\delta \log D = \delta \log Pion \cdot \left| \frac{10^{\log Pion}}{10^{\log Pion} + 10^{(pKa - pH + \log P)}} \right| + \delta pKa. \left| \frac{10^{(pKa - pH + \log P)}}{10^{\log Pion} + 10^{(pKa - pH + \log P)}} - \frac{10^{(pKa - pH)}}{1 + 10^{(pKa - pH)}} \right| + \delta \log P \cdot \left| \frac{10^{(pKa - pH + \log P)}}{10^{\log Pion} + 10^{(pKa - pH + \log P)}} \right|$$

where logP is the octanol-water partition coefficient for uncharged species and logPion is the octanol-water partition coefficient for negatively charged species.

Molecular volume and PSA were computed after running Mopac semi-empirical calculations with PM3 parameters using Vega *ZZ* version 2.3.1 software from Drug Design Laboratory.

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