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Effect of introducing amino acids into phenazine-1-carboxylic acid on phloem mobility

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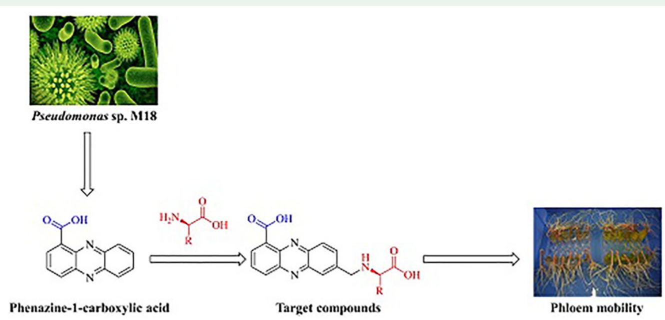
ABSTRACT
To develop new phenazine carboxylic acid derivatives with better phloem mobility, five novel 7-amino acid substituted phenazine-1-carboxylic acids were synthesised by introducing amino acids into PCA at the 7-position. The phloem mobility experiments in *Ricinus communis* seedlings showed that retaining the carboxyl group of PCA and conjugating amino acids to its phenazine ring can also endow PCA with phloem mobility. Comparing our previous research, we found the amino acids substituted at 7-position on phenazine ring of PCA could clearly enhance the phloem mobility of PCA than that of amino acids conjugated with carboxyl group. Especially, the phloem transport concentration of the compound 7-L-isoleucine substituted PCA (**7d**) was 21 times higher than PCA-L-isoleucine conjugate (**8d**). These data suggest that the introduction of amino acids at different structural sites on the phenazine ring could effectively enhance the phloem mobility of PCA and it is worth a further study.



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Phenazine-1-carboxylic acid; amino acid; synthesis; phloem mobility

1. Introduction

It is estimated that only a very small part of pesticides (less than 0.1%) actually reaches the sites of action, while the larger proportion is useless and causes serious environmental pollution (Wang and Liu 2007). As environmental and health issues become more serious, more attention is paid to reducing pesticide use and improving

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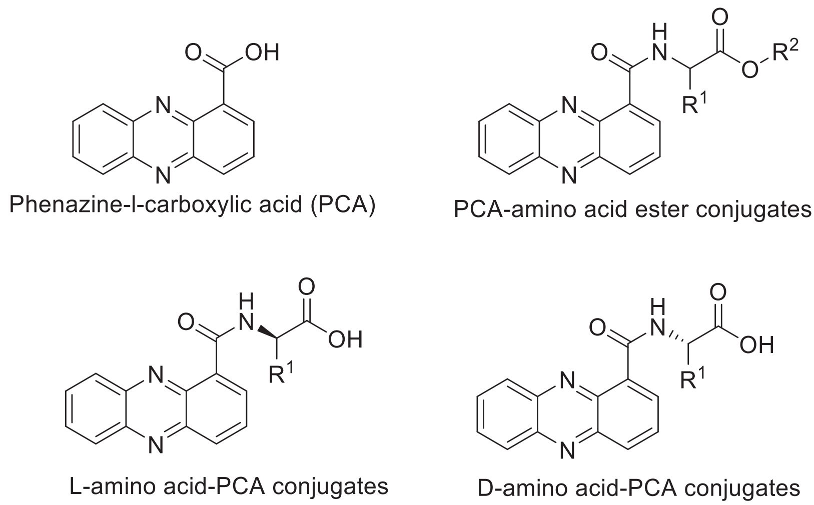


Figure 1. The structures of PCA and its derivatives.

the pesticide efficiency (Pimentel et al. 1993; Mehrazar et al. 2015). Fungicides with phloem mobility have obvious advantages in controlling root pathogens and vascular bundle pathogens, improving the effective utilisation rate of pesticides and changing the traditional application methods (Edgington 1981; Chollet et al. 2004). In order to obtain fungicides molecules with phloem mobility, an effective strategy is to change the physicochemical properties of the molecules (Chollet et al. 2005). Another strategy is to introduce endogenous small molecule such as a sugar or an amino acid that can be transported within plants into the non-phloem mobile molecules (Wu et al. 2012; Xie et al. 2016; Sheng et al. 2018).

Phenazine-l-carboxylic acid (PCA, Figure 1) is an antibiotic secreted by *Pseudomonas* sp. M18 (Ge et al. 2004; Shao et al. 2010), which is a broad-spectrum and highly effective fungicide that inhibits plant pathogenic fungi and promotes plant growth (Thomashow and Weller 1988; Lee et al. 2003). At present, PCA has registered a new microbial source fungicide on rice in China and has been widely promoted. However, PCA does not have phloem mobility (Niu et al. 2017; Yu et al. 2018).

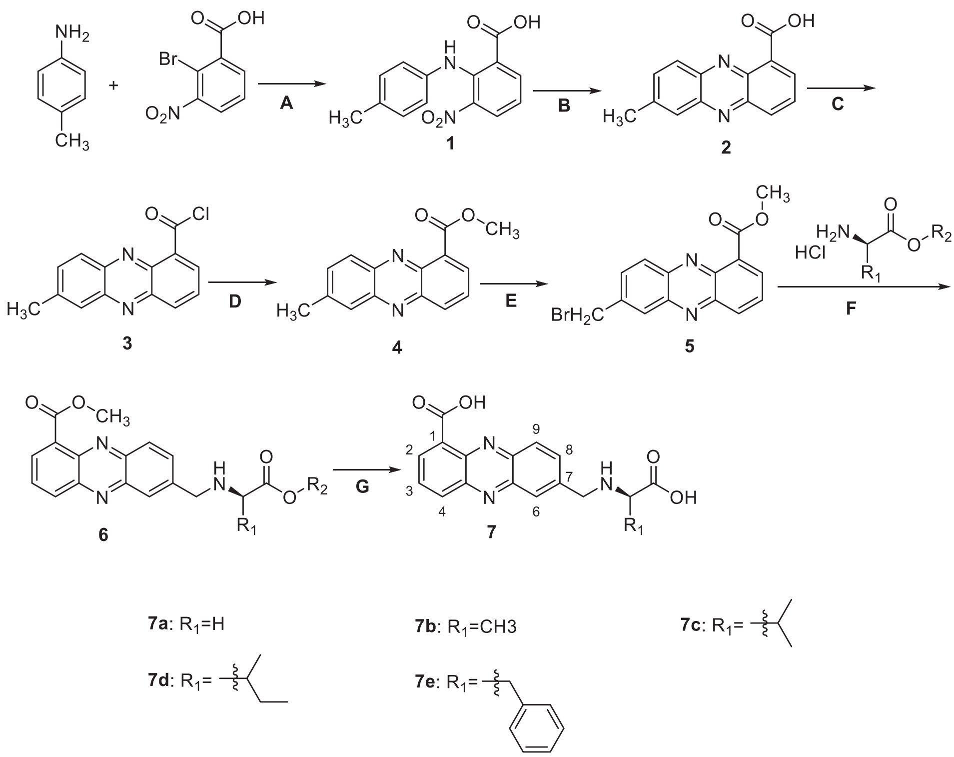
In our previous work, in order to improve the phloem mobility of PCA, a series of PCA-amino acid ester conjugates were designed and synthesised. Unfortunately, all conjugates did not have phloem mobility (Niu et al. 2016). Soon after, our research group synthesised a series of PCA-amino acid conjugates (Yu et al. 2018). Among them, most conjugates exhibited phloem mobility. In addition, we found that different steric conformational PCA-amino acid conjugates differ in phloem mobility (Zhu et al. 2019). Furthermore, our work on the structural modification of PCA is based on the carboxyl group of PCA (Xiong et al. 2017; Lu et al. 2018; Han et al. 2019; Qin et al. 2019; Zhu et al. 2019).

Based on our previous research work, we suspect that the introduction of amino acids from the phenazine ring of PCA can also improve the phloem mobility of PCA. Five novel 7-amino acid substituted phenazine-1-carboxylic acids were designed and synthesised. In addition, the experiments in *Ricinus communis* seedlings were performed to verify whether the compounds had phloem mobility after the amino acids were conjugated to the phenazine ring.

2. Results and discussion

2.1. Chemical synthesis

The synthetic route of the intermediate and 7-amino acid substituted phenazine-1-carboxylic acids **7a–7e** is shown in Scheme 1. Intermediate **1** was obtained by an Ullmann reaction in yield of 75%, followed by reduction with sodium borohydride to give the intermediate **2** in yield of 81%. The pure compounds obtained in the above two steps can be obtained by a simple post-treatment. Intermediate **2** was subjected to acid chloride reaction to obtain intermediate **3** in yield of 98%, which was then esterified with methanol to obtain intermediate **4** in yield of 92%. Intermediate **5** was obtained by bromination of intermediate **4** in yield of 73%. Then, a nucleophilic reaction with the hydrochloride of the amino acid ester got the intermediate **6** in yields ranging from 76% to 88%. Finally, the target compounds **7a–7e** was obtained by hydrolysis of the corresponding intermediate **6** in good yields ranging from 74% to 82%. The structures of the target compounds **7a–7e** were confirmed by ^{1}H, ^{13}C nuclear magnetic resonance (NMR) spectra and high-resolution mass spectra (HRMS). (Analysis data of all the synthesised compounds are available in supplementary information).



Scheme 1. Synthetic route of target compounds **7a–7e**. Reagents and conditions: (**A**) cuprous chloride, copper powder, N-ethylmorpholine, 2,3-butanediol, 70 °C, 15 h; (**B**) Sodium borohydride, sodium ethoxide, ethanol, reflux; (**C**) oxalyl chloride, CH_2Cl_2, reflux, 8 h; (**D**) methanol, 0 °C, 6 h; (**E**) N-bromosuccinimide, dibenzoyl peroxide, CCl_4, reflux, 4 h; (**F**) N,N-diisopropylethylamine, 60 °C, 4 h; (**G**) lithium hydroxide, 1,4-dioxane/H_2O (v/v = 1:1), room temperature, 5 h.

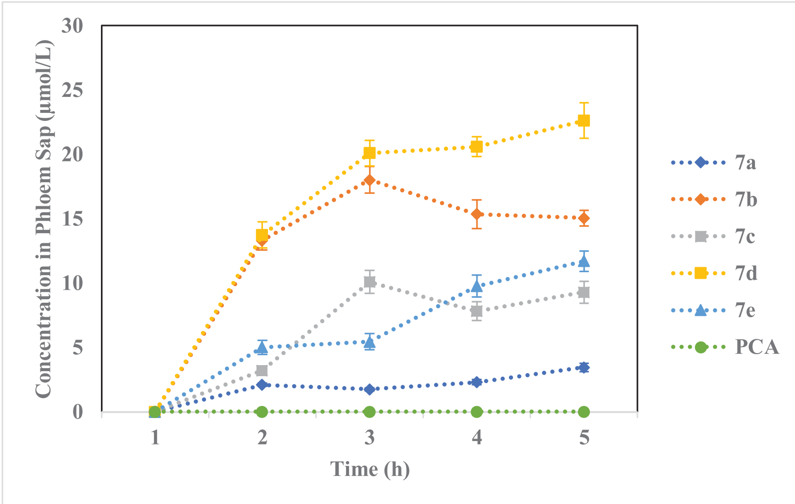


Figure 2. Phloem mobility of **7a–7e** and PCA. Phloem sap was collected at a 1 h interval for 5 h. Each datapoint is the mean of 12 seedlings ± SE ($n = 3$).

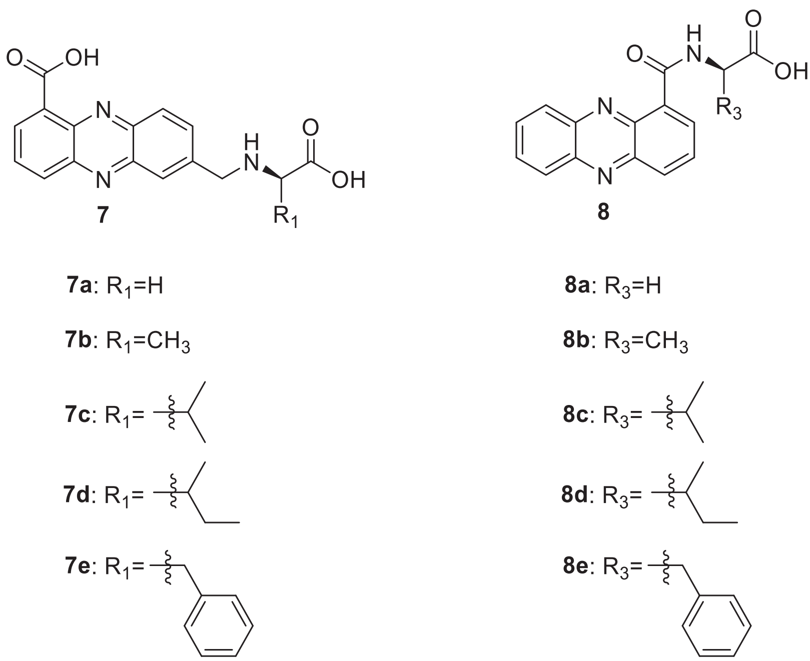


Figure 3. Compounds **7a–7e** are different amino acids conjugated to the 7-position of the phenazine ring of PCA. Compounds **8a–8e** are different amino acids conjugated to the carboxyl group of PCA.

2.2. Phloem mobility in Ricinus communis seedlings

Phloem mobility of **7a–7e** and PCA were measured by the castor bean system which is widely employed to study the phloem mobility of xenobiotics (Rocher et al. 2009; Sheng et al. 2018). The cotyledons of *Ricinus communis* were incubated with each compound of 0.2 mmol/L for 2 h. Then, phloem sap was collected and analysed using Ultra High Performance Liquid Chromatography-Mass Spectrometer (UHPLC-MS).

As shown in Figure 2, the cotyledons incubated with PCA, the fungicide was not detected in phloem sap even after 5 h. In contrast, when the cotyledons were incubated with compounds **7a–7e**, these compounds were clearly detected in phloem sap. This confirmed our hypothesis that retaining the carboxyl group of PCA to conjugate an amino acid to its phenazine ring can also endue PCA with phloem mobility.

Time-course experiments were performed within 5 h to evaluate the phloem mobility of compounds **7a–7e** over time. Figure 2 shows that compounds **7a–7e** were not detected at 1 h. The concentrations of compounds **7a**, **7d** and **7e** in the phloem sap increased with test time. Among them, compound **7d** was found to have the highest concentration in phloem sap of 22.63 ± 1.39 μmol/L at 5 h, indicating that it had the best phloem mobility. The phloem sap concentration of compound **7c** was 10.09 ± 0.15 μmol/L at 3 h, approximately three times higher than that at 2 h.

In our previous work, our group conjugated 12 amino acids to the carboxyl group of PCA (Yu et al. 2018), five of which are identical to the amino acids conjugated to the phenazine ring in this study (Figure 3). Therefore, we put the two results together for comparison. As shown in supplementary information Figure S1, all PCA-amino acid conjugates have phloem mobility. In addition, it can be clearly seen in supplementary information Figure S1 that there is a large difference in the phloem mobility of amino acid conjugated at different locations of PCA. Among them, the concentration in the phloem sap of the compound 7-L-isoleucine substituted PCA (**7d**) was 21 times higher than PCA-L-isoleucine conjugate (**8d**). For most compounds, the phloem mobility of the same amino acid conjugated to the 7-position of the phenazine ring of PCA is significantly better than that of the amino acid conjugated to the carboxyl group of PCA.

2.3. Prediction of phloem mobility using the Kleier model

The Kleier model was widely used to predict whether have the phloem mobility of xenobiotics based on the physicochemical properties (log K_{ow} and pKa) (Hsu et al. 1995; Hsu and Kleier 1996; Kleier and Hsu 1996). The experimental data results of many xenobiotics were consistent with the theoretical predictions of the Kleier model. Thus, the physicochemical properties of the compounds **7a–7e** and PCA were listed in supplementary information Table S1. Based on their physicochemical properties, we marked the compounds on the predicted phloem mobility may in supplementary information Figure S2.

As shown in Figure S2, all compounds **7a–7e** and PCA were predicted to have possibly mobile. Consistent with the results of the castor bean system, all compounds have phloem mobility in addition to PCA. In addition, Xu et al. found that the mobility of glycine-fipronil in castor seedlings is mediated by the amino acid carrier system (Xie et al. 2016). While compounds **7a–7e** are amino acid-PCA conjugates similar to glycinergic-fipronil conjugate, which may also involve the participation of amino acid carriers. The vector-mediated mechanism of these amino acid-PCA conjugates requires further investigation.

3. Conclusions

Five novel 7-amino acid substituted phenazine-1-carboxylic acids **7a–7e** were designed and synthesised by amino acid substitution in C-7 position of phenazine ring. The phloem mobility test results showed that all of the synthesised target compounds **7a–7e** have phloem mobility. In addition, we compared the PCA-amino acid conjugates of the previously reported amino acids conjugated to the carboxyl group of PCA and found that most amino acid is conjugated to the phenazine ring for better phloem mobility. Furthermore, the results of the phloem sap experimental data are consistent with the theoretical predictions of the Kleier model. Overall, this study provides an example of the conjugating of amino acids to different locations of existing fungicide molecules, which can also endue it with phloem mobility. In order to obtain compounds with better phloem mobility, it is the focus of our future research on the effects of the same amino acid conjugated to different positions of PCA.

Disclosure statement

No potential conflict of interest was reported by the authors.

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