Peptide Alcohols as Promoters of Nitrate and Ammonium Ion Uptake in Plants 1

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ABSTRACT

Several aryl-carbamoyl dipeptide alcohols increased the uptake of nitrate and ammonium ions into corn root segments by up to 50% and 90%, respectively. The most effective one was N-carbobenzoxy-L-prolyl-L-valinol. Foliage application of this compound to underfertilized corn plants caused an increase in the rate of plant growth in the greenhouse and provided modest (6-10%) corn yield increases in field tests in Delaware.

It is well established that crop yields can be increased by the use of fertilizer, especially nitrogen-containing fertilizers. Compounds which increase the transport of nitrate and ammonium ions into plant roots may increase the efficiency of fertilizer utilization by crops. Except for the reported ion-uptake enhancing activity (16, 17) of a cyclic peptide (HC-1 1 toxin) (3, 6, 10, 11, 14, 15), no other compounds exhibiting such type of activity have been reported.

This study reports on a series of synthetic dipeptide alcohols which increase the uptake of both nitrate and ammonium ion in vitro studies. The most active compound, (1) N-carbobenzoxy-L-prolyl-L-valinol produced modest increase in growth and yield when applied foliarly to nitrogen-deficient corn plants.

MATERIALS AND METHODS

Syntheses of Chemicals. The compounds reported here were synthesized by the reaction of the appropriate arylcarbamoyl proline-mixed carboxylic anhydride (1) with the appropriately substituted amino alcohol. Satisfactory C, H, and N analyses were obtained for all compounds. Properties are reported in Table 1.

Details are given for the synthesis of N-carbobenzoxy-L-prolyl-

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2 Abbreviations: HC-1, Helminthosporium carbonum race 1; THF, tetrahydrofuran; ADA, aryl-carbamoyl dipeptide alcohols.

O
\[\begin{align*}
\text{O} & \text{C} \quad \text{N} \\
\text{H} & \text{H} \\
\text{CH}_2 & \text{CH}_2 \text{OH} \\
\end{align*} \]

l-valinol (l) (9). A stirred solution of 162 g of N-carbobenzoxy-
l-proline and 65.8 g of N-methylmorpholine in 1 L of dry THF was cooled to -20°C under dry nitrogen and 88.8 g of isobutyl chloroformate was added, followed in 5 min with 67.1 g of L-valinol in THF. The mixture was stirred at 0°C for 30 min, solvent was evaporated under reduced pressure, and the residue was stirred with a 1:1 mixture of water and ethyl acetate. The ethyl acetate layer was separated, washed sequentially with 5% aqueous citric acid and 5% aqueous sodium bicarbonate and dried over MgSO4. The mixture was filtered, evaporated under reduced pressure, and the residue was crystallized from an ethyl acetate (1 L)-hexane (100 ml) mixture to give 187.5 (87%) of (1). A similar procedure was employed for the other compounds in Table 1. Compounds 13, 14, and 15 were prepared from the corresponding 4-substituted benzoxycarbonyl-l-proline derivatives (Tm oil, 138.5-140.5°C and 90-93.5°C, respectively) which were in turn prepared from the corresponding benzyl alcohols by the nitrophenyl carbonate procedure of Chamberlin (2).

In Vitro Ion Uptake into Root Sections. Corn (Pioneer Hybrid 3320), wheat (Arthur), sorghum (SC-599-6), barley (CM-721), rice (M101), and cotton (Tambot SP-37) seeds were surface sterilized with 1% NaOCl and placed in 20.3 x 30.5 cm x 5.1 cm Pyrex glass utility trays that were lined with four layers of bleached paper towels and irrigated with 150 ml of 0.2 mM CaCl2 solution. The trays were covered with plastic food wrap, perforated to permit air exchange, and placed in a dark growth room which was maintained at 30°C and 90% RH. About 48 h later, another 150 ml of 0.2 mM CaCl2 solution was added to each tray to maintain water saturation of the paper towels (12).

Two cm root segments were cut 0.5 to 2.5 cm from the tip of 3-d-old etiolated seedling roots. Batches of 20 (for corn and cotton), or 40 (for wheat, barley, sorghum, and rice) root sections were tied in cheesecloth bags, leaving a 20 to 30 cm length of string to facilitate movement between test solutions. Root tissue was washed at 30 ± 1°C by immersing the cheesecloth bags in a well-aerated 0.2 mM K2HPO4/KH2PO4 and CaCl2 solution (pH 6.0) for 4 h (12). During the last 30 min of this washing period, 1 mm potassium nitrate was introduced to induce nitrate reductase activity. For uniformity, 100 ml of washing solution was used for every bag of root segments (about 300 mg fresh tissue).

Nitrate ion uptake was measured using radioactive chloride ion (35ClO3-) as a mimic for nitrate ion (5). 35ClO3- was prepared from 35Cl- by electrolysis as described previously (4). Uptake was measured by incubating 20 or 40 cheesecloth-bound washed root segments for 30 min in 100 ml of absorption solution. The absorption solution contained 0.5 mM potassium nitrate, 0.2 mM CaCl2, 5 mM Mes buffer, and 5 mg/l sodium molybdate. This solution was adjusted to pH 6 with potassium hydroxide, and labeled with 10,000 cpm/ml of carrier-free radioactive chloride ion. Absorption temperature was maintained at 30 ± 1°C in a
water bath, and moderate aeration was provided. All test compounds were added to the absorption solution. After the absorption period, the cheesecloth bag containing root segments was rinsed with distilled H$_2$O for 10 s and then immersed in 100 ml of ice-cold, aerated 0.2 mm CaCl$_2$ solution for 20 min to remove exchangeable components of adsorbed $^{36}$ClO$_3^-$ Root segments were then removed from the cheesecloth bags, blotted dry, placed in a preweighed scintillation vial, and weighed to determine fresh tissue weight. One ml of water and 10 ml of Scinti Verse I (Fisher Scientific Co.) scintillation fluid were added, and radioactivity was determined by scintillation counting. The rate of nitrate ($^{36}$ClO$_3^-$) uptake in $\mu$mol/g tissue fresh weight-h was calculated.

Nitrate ion uptake into corn roots was also determined by homogenizing root segments previously exposed to the nitrate absorption solution (without added $^{36}$ClO$_3^-$) with a precooled glass homogenizer in 5 ml of cold extraction medium. This medium contained 23 mm phosphate buffer (pH 8.8), 5 mm cysteine, 2.5 mm EDTA, and 0.1% Neutronex 600 (Onyx Chemical Co., Jersey City, NJ). The resulting homogenate was centrifuged at 30,000g for 15 min, and an aliquot of the resulting supernatant solution was reserved for nitrate ion determination with an Orion nitrate ion-specific electrode. The extraction procedure was substantially similar to that described for nitrate ion uptake, as above.

Nitrate reductase activity was determined by a modification of the procedure of Hageman and Hucklesby (8). A typical assay mixture contained 26 $\mu$mol K-phosphate (pH 7.5), 50 $\mu$mol potassium nitrate, 20 $\mu$mol nicotinamide adenine dinucleotide (reduced form) (NADH), 1.4 $\mu$mol EDTA, enzyme extract (tissue homogenate), and water to make a final volume of 2 ml. The reaction was terminated by adding 0.2 ml of 0.5 M zinec acetate plus 1.2 ml of phenazine methosulphate (46 mg/l). After 20 min at room temperature, extracts were centrifuged at 100g for 10 min. The resulting supernatant was used for determination of nitrate, as previously described (7).

**Greenhouse Tests.** Greenhouse tests were conducted by germinating corn seeds in a 25.4-cm pot containing a commercial potting soil mixture. After 2 weeks, seedlings were thinned to two plants in each pot. Distilled H$_2$O was applied to maintain moisture status and to leach carry-over nutrients from the potting soil mixtures. The remaining corn plants were randomized, divided into several groups, and sprayed (5 ml/plant) with test compounds dissolved in acetone, glycerol, water, and Tween 20 (459:20:520:1) mixture. Two hundred and fifty ml of a complete, nitrate ion-free, potassium ion-free, and phosphate ion-free Hoagland nutrient solution were added to each pot twice weekly. This nutrient solution was supplemented with distilled H$_2$O to maintain proper soil moisture. Growth and development of plants were observed in the greenhouse. Plants were allowed to grow to maturity and plant height and fresh weight were measured at the conclusion of each test.

**Field Tests.** Field tests were conducted in 1981 and 1982 in Delaware by seeding corn (Pioneer Hybrid 3535) at a rate sufficient to obtain an excess plant population. The resulting population was thinned after emergence, 14 d after planting, to a density of about 64,000 plants/ha, with plants about 20 cm apart in each row. The test population was subjected to nitrogen stress by applying about 67 kg nitrogen/ha (60 lb/acre) on the day before planting. Nitrogen stress symptoms were apparent on lower plant leaves 32 d after emergence. Test compounds were applied to plants 34 d after emergence (26 d before anthesis). Average canopy height at time of treatment was 85 cm.

The treatments were applied from an acetone/water (1:3) solution with 0.2% added nonionic surfactant, Tween 20, using a hand-held nitrogen-pressurized sprayer at a 5 ml/plant application rate. One day after treatment, an application of about 34 kg/ha (30 lb/acre) ammonium nitrate was broadcast on the soil surface and immediately leached into the root zone by irrigation.

**RESULTS AND DISCUSSION**

Evaluation of N-carbobenzyox-y-L-prolyl-L-valinol and N-carbobenzyox-y-L-prolyl-L-leucinol at varying test concentrations indicated that a concentration of about 50 ppm was optimal for stimulation of nitrate ion uptake by immersed corn root sections (Fig. 1). In addition, nitrate ion uptake stimulation by the test compounds was more pronounced when nitrate ion concentration was low (Fig. 2). This observation suggested that these ADA would provide increased yields in nitrate-deficient soils.

Experimental results obtained at a 50 ppm concentration of

![FIG. 1. Effect of N-carbobenzyox-y-L-prolyl-L-valinol (compound 1) on the NO$_3^-$ uptake into corn root segments. Control value was 0.65 $\mu$mol NO$_3^-$/g fresh weight-h. NO$_3^-$ concentration in the uptake solution was 1 mm.]
NITRATE AND AMMONIUM UPTAKE PROMOTERS

Table I. Ion Uptake by Corn Root Sections Treated with Test Compounds

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Tm°</th>
<th>Nd 25b</th>
<th>Substituents</th>
<th>NO3⁻ Reductase (Increase over Control)</th>
<th>Ion Uptake (Increase over Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>°C</td>
<td></td>
<td>X Y Z R</td>
<td>%</td>
<td>NH₄⁺</td>
</tr>
<tr>
<td>1 Z-l-prolyl-L-valinol</td>
<td>112.5-114</td>
<td>-67.5</td>
<td>CH₃ H H i-Pr</td>
<td>7</td>
<td>100</td>
</tr>
<tr>
<td>2 Z-l-prolyl-L-leucinol</td>
<td>95-95.5</td>
<td>-72*</td>
<td>CH₃ H H i-Bu</td>
<td>-1</td>
<td>80</td>
</tr>
<tr>
<td>3 Z-l-(β-hydroxyethyl)amide</td>
<td>99.4-99.9</td>
<td>-45.4</td>
<td>CH₃ H H H</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>4 Z-l-prolyl-L-alaninol</td>
<td>126.5-127.6</td>
<td>-48.9</td>
<td>CH₃ H H CH₃</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5 Z-l-prolyl-L-butyrylmin</td>
<td>105.4-106.3</td>
<td>-69.7</td>
<td>CH₃ H H C₂H₅</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6 Z-l-prolyl-L-butyrylmin</td>
<td>99.1-105.1</td>
<td>-28.5</td>
<td>CH₃ H H C₂H₅</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7 Z-l-prolyl-D-valinold</td>
<td>118.0-118.2</td>
<td>-27.6</td>
<td>CH₃ H H i-Pr</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8 Z-d-prolyl-L-valinol</td>
<td>117.5-118.6</td>
<td>+26.7</td>
<td>CH₃ H H i-Pr</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9 Z-d-prolyl-D-valinold</td>
<td>113-114</td>
<td>+67.6</td>
<td>CH₃ H H i-Pr</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0 Z-l-γ-thiapropyl-L-valinol</td>
<td>92-94</td>
<td>-123.7</td>
<td>S H H i-Pr</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1 Z-l-γ-oxypropyl-L-valinol</td>
<td>118-119.5</td>
<td>-82.6</td>
<td>O H H i-Pr</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2 Z-5R-methyloxazolidine-4S-carbonyl-L-valinol</td>
<td>98-101</td>
<td>-75.3</td>
<td>O H CH₃ i-Pr</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3 p-Fluorobenzyloxy carbonyl-L-prolyl-L-valinol</td>
<td>123-124</td>
<td>-60.2</td>
<td>O p-F H i-Pr</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4 p-Chlorobenzyloxy carbonyl-L-prolyl-L-valinol</td>
<td>143-144.5</td>
<td>-54.2</td>
<td>CH₂ p-Cl H i-Pr</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5 p-Methylbenzyloxy carbonyl-L-prolyl-L-valinol</td>
<td>122-123.5</td>
<td>-61.9</td>
<td>CH₂ p-CH₃ H i-Pr</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Melting points in °C after two recrystallizations.
  † Specific rotation in acetone; concentration, 1.0 g/100 ml.
  ‡ A dash indicates not determined.
  § Purified by HPLC from Z-l-prolyl-DL-valinol.
  ¶ Purified by repeated fractional crystallization of Z-d-prolyl-DL-valinol.

Test compound are summarized in Table I. Test compounds are identified in Table I by the identity of substituents X, Y, Z, and R in the following formula:

![Chemical structure](image)

Table I shows that certain structural features of ADA were significant: an aryl carbamoyl group (e.g., benzoyloxy carbonyl) was necessary at the amino terminus, and a free primary alcohol residue was necessary at the carbinol terminus. Activity was observed with proline (X = CH₃) as well as with hetero-analogs of proline (X = O, S) when a small branched aliphatic residue (R = isopropyl or isobutyl) was adjacent to the carbinol terminus.

Table II. Nitrate Ion Uptake by Plant Root Sections Treated with 50 ppm N-Carbobenzoxy-L-prolyl-L-valinol

<table>
<thead>
<tr>
<th>Crop</th>
<th>Ion Uptake (Increase over Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
</tr>
<tr>
<td>Corn</td>
<td>65</td>
</tr>
<tr>
<td>Barley</td>
<td>75</td>
</tr>
<tr>
<td>Wheat</td>
<td>55</td>
</tr>
<tr>
<td>Rice</td>
<td>10</td>
</tr>
<tr>
<td>Sorghum</td>
<td>-5 (decrease)</td>
</tr>
<tr>
<td>Cotton</td>
<td>-10 (decrease)</td>
</tr>
</tbody>
</table>

A spray containing 50 ppm N-carbobenzoxy-L-prolyl-L-leucinol (2) stimulated height (15-25% over the control) of 2-week-old corn plants deficient in nitrate ion. A similar stimulating effect was observed with N-carbobenzoxy-L-prolyl-L-valinol (1) (data not shown).

Enhancement of nitrate ion uptake by N-carbobenzoxy-L-prolyl-L-valinol (1) was evaluated with other selected crops, and results are summarized in Table II. Uptake was significantly stimulated in corn, barley, and wheat, whereas rice, sorghum, and cotton root tissues were insensitive.

The specificity of the effect on nitrate and ammonium ion uptake is shown for two of the better ADA in Table III. These...
ADA show no effect on Cl−, K+, and Pi; they produced a slight inhibition of urea uptake into corn root segments and an increase in NO3− and NH4+ uptake by 40 to 60% and 80 to 100%, respectively. Table III also shows that the plasmalemma ATPase and glutamate synthase activities were stimulated by 30 to 60% with no effect on nitrate reductase activity. We do not know the effect of ADA on the total nitrogen metabolism in the tissue.

Field tests conducted in Delaware in 1981 showed a 6.3% (statistically significant [P = 0.05]) increase in grain yield of field-grown corn plants (248.4 bushels/ha versus 232.1 bushels/ha for the control) when test plants were sprayed with 500 ppm (= 1/8 lb/acre) N-carbobenzoxy-l-prolyl-l-valinol. A 10% yield increase was observed in 1982 when test plants were sprayed with 125 ppm (= 1/32 lb/acre) of (1) (Fig. 3). Figure 3 also shows that the compound (1) is more effective at lower concentrations which is in agreement with in vitro uptake tests (Fig. 1).

The mechanism of the activity has not been determined. The observation that foliar application of these compounds causes an increased flux of ions in the root suggests that these compounds are translocated from the site of application, leaves and stems, to the site of action, roots.

The synthesized aryl-carbamoyl dipeptide alcohols (ADA) selectively enhance uptake of nitrate and ammonium ions by plant roots and enhance rate of plant growth. This effect would be useful in reducing the fertilizer requirements of certain plant varieties, especially those which require large quantities of nitrogen or where enhanced rates of nitrate run-off occur as a result of no-tillage farming techniques (13). The enhanced nutrient ion uptake has been demonstrated in laboratory tests and confirmed in selected greenhouse and field tests.

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LITERATURE CITED