Novel inhibitors of ethylene production in higher plants

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Of a number of O-substituted hydroxylamine derivatives, N-benzyloxy carbonyl-1-α-aminooxy-propionic acid and α-aminooxyacetic acid inhibited ethylene production by etiolated mung bean hypocotyls by 50% at 3 and 6 μM concentrations, respectively. Their potency is thus similar to that of aminoethoxyvinylglycine (50% inhibition at 2 μM), the most potent inhibitor of ethylene production hitherto known. Methionine partially alleviated inhibition of ethylene production by α-aminooxy-acetic acid. The results are in agreement with the postulated involvement of pyridoxal phosphate in ethylene biosynthesis.

Key words: Ethylene — Mung beans — Aminooxy compounds — Pyridoxal phosphate.

Involvement of pyridoxal phosphate in the formation of ethylene from methionine in higher plant tissues was postulated on the basis of inhibition experiments with the phytotoxin rhizobitoxine (α-aminooxy-2′-aminooxy-3′-hydroxypropoxy)-trans-β-butenoic acid) (15) and the L-ornithine analogue L-canaline (α-aminooxy-γ-aminooxybutyric acid) (14). Recently, the sequence methionine → S-adenosylmethionine → 1-aminooxy-cyclopropane-1-carboxylic acid → ethylene has been established as the pathway of ethylene biosynthesis in apple tissue (1), and the rhizobitoxine analogue L-α-aminooxy-γ-(2′aminooxy-ethoxy)-trans-β-butenoic acid (α-aminooxyvinylglycine, AVG), as well as L-canaline, were shown to inhibit the activity of the enzyme forming 1-aminooxy-cyclopropane-1-carboxylic acid from tomato fruits (8). L-Canaline inhibits various enzymes which require pyridoxal phosphate as a cofactor (16), and formation of an oxime-type compound between canaline and pyridoxal phosphate was suggested as the mechanism of L-canaline-mediated inhibition of ornithine transamination (12, 17).

We recently became interested in aminooxy compounds as inhibitors of phenylpropanoid metabolism in higher plants (2–7, 10). α-Aminooxy-acetic acid (AOA) (5) and L-α-aminooxy-β-phenylpropionic acid (AOPP) (3, 7) block phenylpropanoid synthesis in buckwheat and other tissues, and AOPP is an extremely potent competitive inhibitor of phenylalanine ammonia-lyase (PAL) both in vitro (3) and in vivo (10). PAL does not require pyridoxal phosphate as a coenzyme, but carries an essential carbonyl-like dehydro-alanine residue at its active site (9) and is thus subject to inhibition by carbonyl reagents. We have assessed the effect of a large

Abbreviations: AOA, α-aminooxy-acetic acid; AOPP, L-α-aminooxy-β-phenylpropionic acid; AVG, amino-ethoxy-vinylglycine (=L-α-aminooxy-2′-aminooxyethoxy)-trans-β-butenoic acid.)
number of aminooxy compounds on phenylpropanoid synthesis in vivo and the deamination and transamination of phenylalanine in vitro (Amrhein et al., in preparation). Among these compounds, L-canaline showed relatively poor inhibitory activity in our test systems. As it had been reported that low levels of L-canaline inhibited the auxin-induced ethylene production by mung bean hypocotyls (14), we decided to investigate the effect of the compounds, which were at our disposal, on ethylene production in this system and compare their action with those of known inhibitors of ethylene formation.

Materials and methods

Plant materials

Mung bean (Phaseolus aureus Roxb.) seeds were obtained from Conimex, Baarn, The Netherlands, and buckwheat (Fagopyrum esculentum Moench) seeds from Landwirtschaftliche Bezugs- und Absatzgenossenschaft, Bochum. Golden Delicious apples of South African origin were obtained locally. Mung bean seedlings were grown and prepared for the experiments essentially as described by Murr and Yang (14), except that hypocotyl sections were cut in dim daylight. Buckwheat seedlings were grown for 6 days in the dark as described in ref. 3.

Incubation of plant material

Hypocotyl segments (mung beans: 20; buckwheat: 40; 1.5 cm long, cut 1 cm below the hook) were incubated for 17 hr at 25°C in the dark with shaking in 5 ml of 5 mM potassium phosphate buffer, pH 6.1, containing 30 μM IAA, 100 μM kinetin, 2% sucrose and inhibitor at the indicated concentrations in 30-ml Erlenmeyer flasks sealed with rubber serum caps. Apple plugs (8 mm in diameter and 10 mm long) were cut with a cork borer and razor blade and were incubated in 1-g equivalents for 1 hr under aeration in 4 ml 2% KCl containing the various inhibitors at the appropriate concentrations. They were then transferred to 30-ml Erlenmeyer flasks and incubated in the sealed flasks for a further 3 hr, during which ethylene production was monitored every 30 min.

Gas analysis

Samples of the gas phase in the flasks were removed with a hypodermic syringe and analyzed for their ethylene content in a Varian 1400 gas chromatograph equipped with a Poropak R 80/100 column and a flame ionization detector.

Chemicals

The following compounds were obtained commercially: α-aminooxy-acetic acid semihydrochloride, L-canaline and O-benzylhydroxyamine hydrochloride from Sigma, St. Louis, Mo.; the hydrochlorides of hydroxylamine, O-methylhydroxyamine and N-methylhydroxyamine, as well as pyridine-4-carboxylic acid hydrazide from Merck, Darmstadt; O-ethylhydroxyamine hydrochloride from Eastman-Kodak, Rochester, N.Y.; phenylethylhydrazine from K & K Rare and Fine Chemicals, Plainview, N.Y. The following compounds were obtained as gifts: β-aminooxy-propionic acid hydrochloride (Dr. P. W. Connell, Upjohn Company, Kalamazoo, Michigan); L-α-amino-γ-alkoxy-butenoic acids and the corresponding saturated
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Results

For each of the putative inhibitors a dose-response curve was recorded in the mung bean hypocotyl system and for some inhibitors also in the apple system. Representative dose-response curves for some inhibitors of ethylene production in mung beans are given in Fig. 1. \( I_{50} \) values, defined as those inhibitor concentrations which cause a 50% inhibition of ethylene production, are given in Table 1 for various hydroxylamine derivatives. The parent compound, hydroxylamine, is a poor inhibitor, and \( N \)-methylation decreases, while \( O \)-methylation increases its inhibitory activity. A comparison of \( O \)-methyl- and \( O \)-ethyl-hydroxylamine with \( O \)-carboxymethyl-hydroxylamine (\( \alpha \)-aminoxyacetic acid) shows that introduction of the carboxyl group increases the inhibitory activity of the compound approximately 30-fold (Fig. 1 and Table 1).

![Dose-response curves for selected inhibitors of ethylene production in mung bean hypocotyl sections.](image-url)
Table 1 *Inhibition of ethylene production by hydroxylamine derivatives*<sup>a</sup>

<table>
<thead>
<tr>
<th>Compound</th>
<th>( I_{50} ) (m)</th>
<th>Mung bean</th>
<th>Apple</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxylamine</td>
<td>( 9 \times 10^{-4} )</td>
<td>2 \times 10^{-3}</td>
<td></td>
</tr>
<tr>
<td>N-Methyl-hydroxylamine</td>
<td>( 7 \times 10^{-3} )</td>
<td>6 \times 10^{-3}</td>
<td></td>
</tr>
<tr>
<td>O-Methyl-hydroxylamine</td>
<td>( 2 \times 10^{-4} )</td>
<td>5 \times 10^{-3}</td>
<td></td>
</tr>
<tr>
<td>O-Ethyl-hydroxylamine</td>
<td>( 3 \times 10^{-4} )</td>
<td>2 \times 10^{-3}</td>
<td></td>
</tr>
<tr>
<td>O-Benzyl-hydroxylamine</td>
<td>( 1 \times 10^{-4} )</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>( \alpha )-Aminooxy-acetic acid</td>
<td>( 6 \times 10^{-6} )</td>
<td>9 \times 10^{-5}</td>
<td></td>
</tr>
<tr>
<td>L-( \alpha )-Aminooxy-propionic acid</td>
<td>( 2 \times 10^{-5} )</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>( \beta )-Aminooxy-propionic acid</td>
<td>( 2 \times 10^{-5} )</td>
<td>2 \times 10^{-5}</td>
<td></td>
</tr>
<tr>
<td>( d,l,\alpha )-Aminooxy-pentanoic acid</td>
<td>( 9 \times 10^{-6} )</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>( d,l,\alpha )-Aminooxy-isopentanoic acid</td>
<td>( 2 \times 10^{-5} )</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>( d,l,\alpha )-Aminooxy-hexanoic acid</td>
<td>( 2 \times 10^{-5} )</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>( d,l,\alpha )-Aminooxy-octanoic acid</td>
<td>( 2 \times 10^{-5} )</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>( d,l,\alpha )-Aminooxy-dodecanoic acid</td>
<td>( 2 \times 10^{-5} )</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>L-( \alpha )-Aminooxy-( \beta )-phenylpropionic acid</td>
<td>( 4 \times 10^{-5} )</td>
<td>4 \times 10^{-5}</td>
<td></td>
</tr>
<tr>
<td>( d,\alpha )-Aminooxy-( \beta )-phenylpropionic acid</td>
<td>( 3 \times 10^{-5} )</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>( N )-Benzyloxycarbonyl-hydroxylamine</td>
<td>( 2 \times 10^{-4} )</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>( N )-Benzyloxycarbonyl-L-( \alpha )-aminooxy-propionic acid</td>
<td>( 3 \times 10^{-6} )</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>( N )-Benzyloxycarbonyl-L-( \alpha )-aminooxy-( \beta )-phenylpropionic acid</td>
<td>( 9 \times 10^{-6} )</td>
<td>3 \times 10^{-4}</td>
<td></td>
</tr>
<tr>
<td>( N )-Benzyloxycarbonyl-d-( \alpha )-aminooxy-( \beta )-phenylpropionic acid</td>
<td>( 1 \times 10^{-5} )</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>( N )-Benzyloxycarbonyl-L-phenylalanine</td>
<td>( 5 \times 10^{-4} )</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>( d )-Cycloserine</td>
<td>( 1 \times 10^{-3} )</td>
<td>7 \times 10^{-3}</td>
<td></td>
</tr>
<tr>
<td>L-Canaline</td>
<td>( 7 \times 10^{-5} )</td>
<td>3 \times 10^{-4}</td>
<td></td>
</tr>
<tr>
<td>( d,\alpha )-Aminooxy-( \beta )-aminooxy-propionic acid</td>
<td>( 8 \times 10^{-5} )</td>
<td>n.d.</td>
<td></td>
</tr>
</tbody>
</table>

n.d. = not determined

* The mung bean hypocotyl segments produced approximately 120 nl ethylene in the controls, while the apple plugs produced ethylene at a rate of 45 to 90 nl/g fresh weight/hr. A control was run for each individual inhibitor. Standard deviations of \( I_{50} \) values in triplicate experiments were in the range of 16 ± 10%.

Compounds having the general structure \( H_2N-OCHR-CO_2H \) (\( R \)=aliphatic side chain) exhibit rather similar potencies, and the length of the side chain is apparently not critical. The position of the aminooxy group in the \( \alpha \)- or \( \beta \)-position is also not critical, as a comparison of the aminooxy-propionic acids shows (Table 1). Compounds in which the aminooxy group is protected by the \( N \)-benzyloxycarbonyl residue generally are more inhibitory than unsubstituted compounds. \( N \)-Benzyloxycarbonyl-L-aminooxy-propionic acid is, in fact, nearly as inhibitory as the very potent AVG (Fig. 1, Tables 1 and 4). Plant tissues probably are able to release the free aminooxy compound from the substituted compounds (7). It might be worthwhile to test aminooxy compounds with substituents other than the \( N \)-benzyloxycarbonyl group for their inhibitory activity. Free \( N \)-benzyloxycarbonyl-hydroxylamine and \( N \)-benzyloxycarbonyl-L-phenylalanine were inhibitory only at much higher concen-
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| Table 2  Effect of L-methionine on inhibition by α-amino-oxy-acetic acid of ethylene production by mung bean hypocotyls |
|---------------------------------|----------------------------------|----------------------------------|
| α-Amino-oxy-acetic acid (m) | L-Methionine (m) | Ethylene production (in % of control) |
| 0 | 0 | 100 |
| 1×10^{-5} | 0 | 20±8a |
| 5×10^{-5} | 0 | 4 |
| 1×10^{-5} | 10^{-3} | 39±7a |
| 1×10^{-5} | 10^{-2} | 61±12a |
| 5×10^{-5} | 10^{-3} | 3 |
| 5×10^{-5} | 10^{-2} | 4 |
| 0 | 10^{-3} | 105b |
| 0 | 10^{-2} | 112b |

a Standard deviations calculated from 6 to 8 separate experiments.
b Values not significantly different from control at 95% confidence level.

...trations. Both preparations of L-canaline which we tested, i.e., the commercial product and the sample obtained from Dr. Rosenthal, were less inhibitory than described previously in the mung bean hypocotyl system (14). All amino-oxy compounds were generally more efficient inhibitors of ethylene production in mung bean hypocotyls than in apple tissue (Table 1).

Reversibility of AOA-mediated inhibition of ethylene production was tested by incubating mung bean hypocotyl sections in the presence and absence of 10^{-5} M AOA for 5 hr. Inhibition of ethylene production was 75%. The sections were then thoroughly rinsed in incubation medium without AOA, and the incubation was continued for 12 hr. Ethylene production was still inhibited 74%. Therefore, the inhibitor can not be washed out. Exogenous L-methionine, added simultaneously with 10^{-5} M AOA, partially alleviated the inhibition, but the effect of 5×10^{-5} M AOA could not be overcome by methionine (Table 2). Murr and Yang (14) state that methionine did not reverse the inhibition by L-canaline, but did not give the concentrations of the two compounds in this particular experiment.

Of the hydrazine derivatives, only D-α-hydrazino-β-phenylpropionic acid produced relatively effective inhibition (Table 3), but the limited availability of such compounds did not allow a close comparison with the amino-oxy counterparts. The

| Table 3  Inhibition of ethylene production by hydrazine derivatives |
|---------------------------------|---------------------------------|---------------------------------|
| Compound | Is0 (m) | Is0 (m) |
| | Mung bean | Apple |
| Hydrazine | >5×10^{-3} | >5×10^{-3} |
| Phenylethylhydrazine | 5×10^{-3} | n.d. |
| L-α-Hydrazino-β-phenylpropionic acid | 1×10^{-4} | 1×10^{-4} |
| D-α-Hydrazino-β-phenylpropionic acid | 4×10^{-5} | 6×10^{-6} |
| L-α-Hydrazino-β-(3,4-dihydroxyphenyl)-propionic acid | 4×10^{-3} | n.d. |
| Pyridine-4-carboxylic acid hydrazide | 3×10^{-3} | n.d. |

n.d. = not determined
Table 4  Inhibition of ethylene production by mung bean hypocotyls by unsaturated amino acids and their saturated analogues

<table>
<thead>
<tr>
<th>Compound</th>
<th>$I_{50} \text{ (m)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>d,L-$\alpha$-Amino-$\beta$-butenoic acid (Vinylglycine)</td>
<td>$1 \times 10^{-2}$a</td>
</tr>
<tr>
<td>d,L-$\alpha$-Amino-$\gamma$-pentynoic acid (Propargylglycine)</td>
<td>$4 \times 10^{-3}$a</td>
</tr>
<tr>
<td>L-$\alpha$-Amino-$\gamma$-(2'-aminoethoxy)-trans-$\beta$-butenoic acid (AVG)</td>
<td>$2 \times 10^{-6}$</td>
</tr>
<tr>
<td>L-$\alpha$-Amino-$\gamma$-(2'-aminoethoxy)-butanoic acid</td>
<td>$6 \times 10^{-6}$a</td>
</tr>
<tr>
<td>L-$\alpha$-Amino-$\gamma$-methoxy-trans-$\beta$-butenoic acid</td>
<td>$2 \times 10^{-5}$</td>
</tr>
<tr>
<td>L-$\alpha$-Amino-$\gamma$-methoxy-butanoic acid</td>
<td>$2 \times 10^{-8}$a</td>
</tr>
</tbody>
</table>

a extrapolated

relative inhibitory activities of the two enoether amino acids and their saturated analogues (Table 4) agreed well with those previously reported for apple tissue slices (13), and the parent compound vinylglycine as well as propargylglycine inhibited ethylene production only at very high concentrations (Table 4). We recently found that the two enoether amino acids are very potent competitive inhibitors of transaminase activity in mung bean acetone powder extracts, and a report on this finding will be published elsewhere (N. Amrhein and O. Akinpelu, submitted for publication).

Discussion

The present investigation showed that compounds carrying aminoxy groups effectively inhibit ethylene production. Some of the compounds have a potency very close to that of the $\alpha$-amino-$\gamma$-alkoxy-butenoic acids. AOA is relatively toxic to plants and is patented as an agent for controlling plant growth (19), and the concentrations required to block ethylene production are quite low (Fig. 1). Other aminoxy compounds, possibly with a suitable protective group for the aminoxy group, might be even more active, as the example of L-$\alpha$-aminoxy-propionic acid and its $N$-benzyloxy-carbonyl-derivative shows (Table 1). Testing the aminoxy analogue of L-methionine as an inhibitor of ethylene production should be very interesting, but to our knowledge this compound has not been synthesized.

The general reactivity of AOA towards pyridoxal phosphate-dependent enzymes is well documented (11), and inhibition of ethylene production by this and other aminoxy compounds support the proposal that pyridoxal phosphate (or, more generally, a factor carrying a carbonyl group) is involved in the formation of ethylene in higher plant tissues. We have recently found that ethylene production of mung bean hypocotyls and tomato leaves in response to exogenous L-amino-cyclopropane-1-carboxylic acid is not subject to inhibition by AOA (N. Amrhein and D. Schneebeck, unpublished). AOA, therefore, appears to interfere with the conversion of methionine into the cyclic intermediate, presumably with the step from $S$-adenosylmethionine to L-amino-cyclopropane-1-carboxylic acid (8).

The results of this study are of significance for our experiments, in which we block phenylpropanoid synthesis with AOPP (4, 6, 7). Clearly, at the concentrations used (0.1–1 mM), ethylene production by the plant materials will be severely
Inhibitors of ethylene production

Table 5  Inhibition of ethylene production in buckwheat hypocotyl segments

<table>
<thead>
<tr>
<th>Compound</th>
<th>$I_{50}$ (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-a-Amino-γ-(2'-aminoethoxy)-trans-β-butenolic acid (AVG)</td>
<td>$1 \times 10^{-6}$ (2 x $10^{-6}$)</td>
</tr>
<tr>
<td>a-Aminoxy-acetic acid</td>
<td>$4 \times 10^{-6}$ (6 x $10^{-6}$)</td>
</tr>
<tr>
<td>L-a-Aminoxy-β-phenylpropionic acid</td>
<td>$4 \times 10^{-5}$ (4 x $10^{-5}$)</td>
</tr>
<tr>
<td>D-a-Aminoxy-β-phenylpropionic acid</td>
<td>$2 \times 10^{-5}$ (3 x $10^{-5}$)</td>
</tr>
</tbody>
</table>

* For comparison, values for mung bean hypocotyls are given in parentheses.

affected, and this might give rise to secondary effects not related to the inhibition of phenylpropanoid synthesis. Our standard in the phenylpropanoid studies is the buckwheat hypocotyl, and Table 5 shows that the ethylene production of buckwheat hypocotyl sections is affected by some selected inhibitors with the same sensitivity as that of mung bean hypocotyl sections. However, AVG does not interfere with phenylpropanoid synthesis in buckwheat (N. Amrhein, unpublished) or gherkin (6) seedlings, while it does inhibit ethylene synthesis. Therefore, inhibition of phenylpropanoid synthesis by AOPP is not secondary to the inhibition of ethylene synthesis. In studies on the effects of the aminooxy compounds on growth (7) one should, however, take inhibition of ethylene synthesis into account.

Addendum

After submission of the manuscript an article by Y.-B. Yu, D. O. Adams and S. F. Yang appeared in Arch. Biochem. Biophys. 198: 280–286 (1979), in which it was shown that 1-aminocyclopropanecarboxylate synthase from tomatoes is activated by pyridoxal phosphate and competitively inhibited by a-aminoxy-acetic acid. This finding agrees with our proposal.

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References


