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Inhibition of Ethylene Production in Sunflower Cell Suspensions by a Novel Oxime Ether Derivative

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Abstract. During the incubation of undifferentiated cell suspensions of sunflower (*Helianthus annuus* L. cv. Spanners Allzweck) ethylene production was effectively inhibited by the novel oxime ether derivative LAB 181 508, [[(Isopropyliden)-amino]-oxy]-acetic acid-2-(methoxy)-2-oxoethylester (PACME). The compound was most active during the first 6 days of incubation exhibiting a value of 50% inhibition at 9.5×10^{-6} mol $\times$ L^{-1}. The pattern of changes in the internal 1-aminocyclopropanecarboxylic acid (ACC) and N-malonyl-ACC (MACC) levels paralleled the influence on ethylene formation. While the addition of ACC fully restored ethylene production, applied S-adenosyl-L-methionine (SAM) was not effective. Experiments with [^{14}C]indole-3-acetic acid (IAA) revealed that LAB 181 508 did not affect auxin uptake into suspension cells of sunflower. The results suggest that LAB 181 508 reduces ethylene formation by inhibiting the conversion of SAM to ACC in the biosynthetic pathway. In comparison to the structurally related inhibitor of ACC synthase, aminoethoxyvinylglycine (AVG), LAB 181 508 reduced growth and viability of the suspension cells only slightly. Low phytotoxicity of LAB 181 508 combined with a less complicated chemical synthesis might offer interesting aspects for physiological research and horticultural and agricultural practice.

Ethylene influences many processes in the development and yield formation of plants (e.g., fruit set and ripening, senescence, and stress reactions) (Abeles 1973). Control of its synthesis can therefore be of economic importance in agriculture and horticulture. As a target for regulation, the pyridoxalphosphate-linked 1-aminocyclopropanecarboxylic acid (ACC) synthase (Boller et al. 1979) functions as a key enzyme in the biosynthetic pathway of ethylene (Yang and Hoffman 1984). Two types of pyridoxal enzyme inhibitors are used to block ACC synthase (Yang and Hoffman 1984). The first are represented by vinylglycine analogs with rhizobitoxine (Owens et al. 1971) and aminoethoxyvinylglycine (AVG; Amrhein and Wenker 1979) being the most active compounds. The second group includes hydroxylamine derivatives, such as L-canaline (Murr and Yang 1975) and aminooxyacetic acid (AOA; Amrhein and Wenker 1979). However, the known inhibitors are of limited usefulness for physiological research, as well as for application, because of toxic side effects and chemically complicated synthesis resulting in a high price of the compounds. Recently, derivatives of substituted oxime ethers were found to inhibit effectively the ethylene production in leaf discs and intact plants of various species (Kirchner 1991, J. Kirchner et al. in preparation, Schulz et al. 1987). The readily obtainable synthetic compounds also retarded the senescence of cut carnation flowers. Since these oxime ethers are structurally related to the vinylglycine- and hydroxylamine-type inhibitors of ACC synthase, a similar mode of action might be expected.

As previously shown, undifferentiated, auxin-dependent cell suspension cultures of sunflower are suitable to analyze the effects of compounds on cellular ethylene biosynthesis (Sauerbrey et al. 1987). Data suggested that at least the final steps in the ethylene synthesis of the cells are identical to those of intact plants (Sauerbrey et al. 1988).

In the present report we have characterized the influence of the oxime ether LAB 181 508 (Fig. 1)

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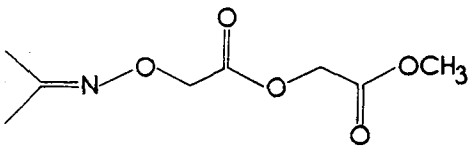

LAB 181508

Fig. 1. Structural formula of LAB 181 508.

on ethylene production, content of ACC and N-malonyl-ACC (MACC), and growth of sunflower suspension cells. The results were compared with the effects of AVG and AOA.

Materials and Methods

Chemicals

The following compounds were used: [[(Isopropyliden)-amino]oxy]-acetic acid-2-(methoxy)-2-oxoethylester (LAB 181 508, PACME, Fig. 1, Schulz et al. 1987) from BASF Aktiengesellschaft, Ludwigshafen, FRG. 1-Aminocyclopropanecarboxylic acid (ACC) was obtained from Calbiochem (La Jolla, CA, USA), aminoethoxyvinylglycine (AVG) from Fluka AG (Buchs, Switzerland), and S-adenosyl-L-methionine (SAM, p-toluenesulfonate salt) from Sigma.

Cell Culture

Suspension cultures of sunflower (*Helianthus annuus* L. cv. Spanners Allzweck) derived from shoots were cultivated in a Murashige-Skoog medium (1962) modified according to Seitz and Richter (1970) with amino acids (including 0.05 mg $\times$ L^{-1} DL-methionine), vitamins, 2 mg $\times$ L^{-1} indole-3-acetic acid, 0.2 mg $\times$ L^{-1} kinetin, and 0.1 mg $\times$ L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). The cells, grown in culture for several years, were subcultured at intervals of 7 days in the exponential growth phase under the following conditions: 250-ml Erlenmeyer flasks containing 80 ml of cell suspension were shaken at 110 rpm in the dark at 25°C. Solutions of chemical compounds were prepared in acetone and were added to the flasks shortly before inoculation. After acetone had volatilized, cell suspensions diluted with fresh medium were poured in the flasks. At various times after treatment, fresh weight, viability of the cells (Grossmann et al. 1983), ethylene production, and the contents of ACC and MACC were determined in three parallel flasks. Mean values $\pm$ SE are given. All experiments were repeated with reproducible trends, and results of a representative experiment are shown.

Determination of Ethylene

Suspended cells (2 ml) were incubated for 6 h in sterile plastic tubes (three replicate tubes per flask) capped with a rubber septum and shaken at 400 rpm and 25°C in the dark. A 1-ml gas sample of head space was withdrawn with a syringe, and ethylene was quantified by gas chromatography (alumina column, flame ionization detector, see Sauerbrey et al. 1987). Ethylene production was linear during the 6-h incubation period. The cell material per tube was harvested by filtration and was weighed.

Determination of ACC and MACC

ACC and MACC were determined in 2–3 g of washed and filtered cells which were homogenized with mortar and pestle in 10 ml 70% ethanol. After centrifugation, a 5-ml aliquot of the supernatant was concentrated in vacuo to dryness, then brought to a volume of 5.5 ml with 4 ml bidistilled water and 1.5 ml 2 N KOH. ACC content was assayed by converting it to ethylene according to Lizada and Yang (1979). MACC was hydrolyzed to ACC in 1 ml of the supernatant under addition of 5 ml 2 N HCl at 110°C for 4 h. The concentration of ACC was similarly assayed (Lizada and Yang 1979). Ethylene was determined by gas chromatography as described above.

Uptake of [1-^{14}C]Indole-3-acetic acid (IAA)

Cell suspensions of sunflower were subcultured and treated with 10^{-4} mol $\times$ L^{-1} LAB 181 508 in three parallel Erlenmeyer flasks as described above. At various times after treatment, 2-ml aliquots (four replicates) of each flask were transferred into sterile plastic tubes. The cells were collected by centrifugation, washed three times with 4 ml of medium (without IAA and 2,4-D), and resuspended in 2 ml medium supplemented with 3.7 KBq [1-^{14}C]IAA (2183 MBq/mmol, Amersham Buchler, Braunschweig, FRG) and 10^{-4} mol $\times$ L^{-1} LAB 181 508. In controls the compound was omitted. The tubes were kept on a rotary shaker at 400 rpm for 1 h at 25°C in the dark (Grossmann 1988). Then the cells were washed four times with 4 ml of complete medium and recovered by centrifugation (4°C). The cells were lysed with 0.2 ml of 1% sodium dodecylsulfate overnight and radioactivity was counted after addition of 7 ml Minisolve scintillation fluid (Zinsser, Frankfurt, FRG).

Results

Cell suspensions of sunflower produce peak levels of ethylene in the middle of the exponential growth phase (Fig. 2). The oxime ether LAB 181 508 inhibited this ethylene production most effectively during the first days of incubation. At 7 days after treatment a clear rise in ethylene formation followed especially at 10^{-6} mol $\times$ L^{-1} LAB 181 508 possibly indicating an inactivation of the compound. The rate of ethylene production under the influence of LAB 181 508 was paralleled by the pattern of changes in the internal ACC and MACC levels. The reason might be an inhibition of a step prior to the conversion of ACC to ethylene in the biosynthetic pathway. This assumption could be confirmed by a simultaneous treatment of sunflower cell suspensions with 3×10^{-5} mol $\times$ L^{-1} LAB 181 508 and increasing concentrations of ACC (Table 1). Here, the effect of LAB 181 508 on ethylene formation

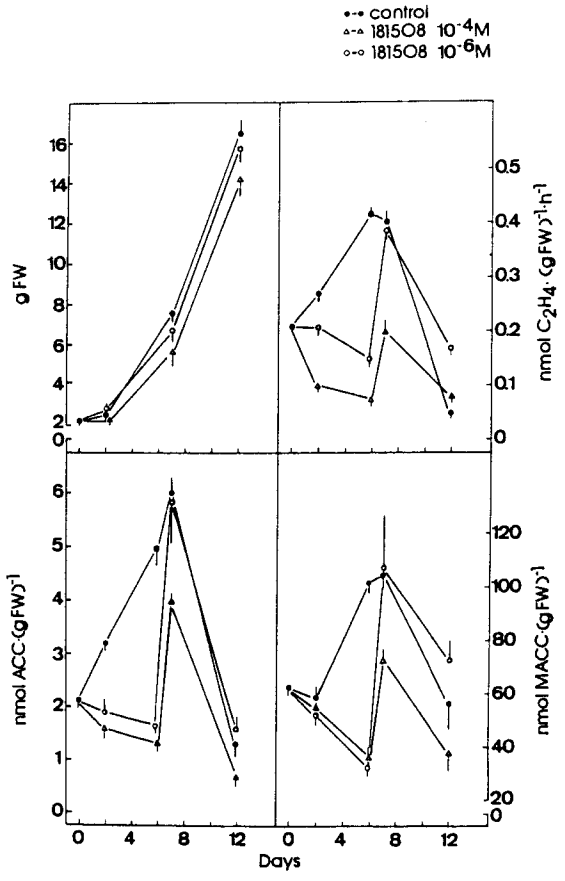

Fig. 2. Effect of LAB 181 508 on growth (measured by fresh weight) and production of ethylene and content of ACC and MACC in sunflower cell suspensions. ●, control; ○, 10^{-6} mol × L^{-1}; △, 10^{-4} mol × L^{-1} LAB 181 508. Vertical bars represent SE of the mean.

Table 1. Effects of ACC and SAM on the inhibition of ethylene production in sunflower cell suspensions caused by LAB 181 508.

Compound, concentration (mol × L^{-1})	Ethylene production (nmol × g fresh wt^{-1} × h^{-1})
ACC	
Control	0.435 ± 0.004 (100)
LAB 181 508, 3×10^{-5}	0.116 ± 0.004 (27)
LAB 181 508, 3×10^{-5} + ACC, 10^{-6}	0.105 ± 0.004 (24)
LAB 181 508, 3×10^{-5} + ACC, 10^{-5}	0.135 ± 0.008 (31)
LAB 181 508, 3×10^{-5} + ACC, 10^{-4}	0.570 ± 0.015 (131)
SAM	
Control	0.754 ± 0.015 (100)
LAB 181 508, 3×10^{-5}	0.038 ± 0.004 (5)
LAB 181 508, 3×10^{-5} + SAM, 10^{-6}	0.053 ± 0.004 (7)
LAB 181 508, 3×10^{-5} + SAM, 10^{-4}	0.041 ± 0.004 (5)
LAB 181 508, 3×10^{-5} + SAM, 10^{-3}	0.075 ± 0.011 (10)
LAB 181 508, 10^{-5}	0.439 ± 0.011 (58)
LAB 181 508, 10^{-5} + SAM, 10^{-6}	0.521 ± 0.008 (69)
LAB 181 508, 10^{-5} + SAM, 10^{-4}	0.439 ± 0.011 (58)
LAB 181 508, 10^{-5} + SAM, 10^{-3}	0.446 ± 0.015 (59)

Simultaneously with the application of LAB 181 508, ACC or SAM was added. Formation of ethylene was determined after 5 days (ACC treatment) or 6 days (SAM treatment) of incubation. Data are means of four replicates ± SE. The values in parentheses represent the percentage of ethylene production to control.

was completely reversed by the biosynthetic precursor. In contrast, application of SAM, the substrate of ACC synthase (Yang and Hoffman 1984), was not effective (Table 1). Hence, LAB 181 508 presumably inhibited the conversion of SAM to ACC mediated by ACC synthase. However, the reduction of ethylene levels caused by LAB 181 508 might also be explained by an influence on auxin transport into the cells. As previously shown, the supply of auxins is essential for the growth and ethylene formation of the sunflower cells under investigation (Sauerbrey et al. 1987). Thus, pulse experiments on the uptake behavior of [^{14}C]IAA into sunflower cells were performed (Fig. 3). During the first 6 days of incubation with increased ethylene production, 10^{-4} mol × L^{-1} LAB 181 508 did not change the rate of IAA uptake which transiently increased with a maximum at day 1 (Fig. 3). This indicates that LAB 181 508 does not inhibit ethylene production via depriving cellular auxin supply.

Concomitant with the strong reduction in ethylene formation, the increase in fresh weight of sunflower cell suspensions was only slightly influenced by LAB 181 508 (Fig. 2). At 6 days after treatment with 10^{-4} mol × L^{-1}, cell culture growth was reduced by approximately 20% and the number of viable cells by only 10% compared to control (data not shown). The concentration of LAB 181 508 necessary for a 50% inhibition of ethylene formation was 9.5×10^{-6} mol × L^{-1} (Table 2). Thus, LAB 181 508 was more active than AOA, but less active than AVG, which decreased cellular ethylene formation more effectively throughout the incubation period (Table 2, Fig. 4). However, growth reduction was approximately 60% accompanied by the same decrease in the amount of viable cells measured after 6 days of treatment (data not shown). It is concluded that AVG as a purported inhibitor of ACC synthase (Yang and Hoffman 1984) additionally causes certain cell-damaging effects.

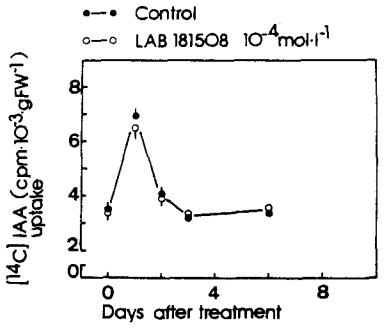

Fig. 3. Uptake rate of [1-^{14}C]IAA into suspension cells of sunflower in dependence of treatment with LAB 181 508. ●, control; ○, 10^{-4} mol × L^{-1} LAB 181 508. Vertical bars represent SE of the mean.

Table 2. Concentrations of different compounds required for a 50% inhibition ($[I]_{0.5}$) of ethylene production in sunflower cell suspensions determined after 6 days of treatment.

Compound	$[I]_{0.5}$ (M)
LAB 181 508	9.5×10^{-6}
AOA	3.0×10^{-5}
AVG	1.7×10^{-6}

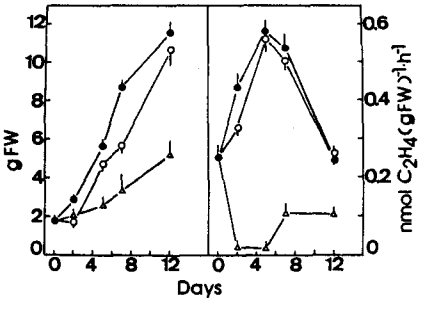

Fig. 4. Effect of AVG on growth (measured by fresh weight) and production of ethylene in sunflower cell suspensions. ●, control; ○, 10^{-6} mol × L^{-1}, △, 10^{-4} mol × L^{-1} AVG. Vertical bars represent SE of the mean.

Conclusion

The substituted oxime ether derivative LAB 181 508, a new type of ethylene inhibitor, has been shown to be a potent inhibitor of ethylene synthesis in sunflower cell suspensions. The compound did not exhibit excessive phytotoxic side effects. Low phytotoxicity combined with a less complicated chemical synthesis may offer interesting aspects for the use of LAB 181 508 both in physiological research and agricultural practice. The results presented here and the structural relationships to the vinylglycine- and hydroxylamine-type inhibitors of ACC synthase suggest a similar mode of action. It will be the aim of further detailed studies to verify this enzymatic target.

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