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SHORT COMMUNICATION

EVIDENCE FOR D-CONFIGURATION OF *N*-MALONYLMETHIONINE, THE METABOLITE OF D-METHIONINE IN *NICOTIANA RUSTICA**

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Abstract—Evidence for the D-configuration of *N*-malonylmethionine-methyl-^{14}C isolated from tobacco plant fed with D-methionine-methyl-^{14}C was obtained by acid hydrolysis of the conjugate and subsequent deamination of methionine with D-amino acid oxidase. Parallel experiments with synthetically prepared *N*-malonyl-D-methionine were carried out.

OUR STUDIES on the metabolism of optical isomers of methionine in tobacco plant have shown[1] that, under identical experimental conditions, the D-isomer is metabolized into the corresponding *N*-malonyl conjugate while this pathway is lacking with its enantiomorph. In the present communication, evidence for the D-configuration of *N*-malonylmethionine formed in tobacco plant is presented.

A large amount of D-methionine-methyl-^{14}C was applied to five intact *Nicotiana rustica* plants and *N*-malonylmethionine-methyl-^{14}C was isolated from the root extracts by the procedure described previously.[1] Chromatographically and radiochemically pure metabolite was subjected to acid hydrolysis and aliquots were tested as substrates for L- and D-amino acid oxidase. For comparison, *N*-malonyl-D-methionine was prepared and all assays on the metabolite were carried out in parallel with the synthetically obtained conjugate. Since, α-keto-γ-methylthiobutyric acid, the deamination product of methionine, is an unstable compound especially susceptible to oxidation to the sulphoxide stage[1-3] the amount of methionine in the incubation mixture before and after the enzymatic reaction was quantitatively determined.

The results of the experiments clearly show that methionine originating from the *N*-malonyl conjugate formed in the plant is not a substrate for L-amino acid oxidase while with D-amino acid oxidase it undergoes a complete deamination (Table 1). The resulting keto acid was identified as the 2,4-dinitrophenylhydrazone derivative. However, during the isolation procedure, some decomposition of the acid took place and a great part of the hydrazone,

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when chromatographed on paper, underwent oxidation to the corresponding sulphoxide; the radioactivity of both the hydrazones accounted for 61–67% of the initial substrate activity.

The present finding is an additional support for the presumption[1,3,4–6] that *N*-malonylation of all D-amino acids in plants proceeds under retention of configuration.

TABLE 1. OXIDATION OF METHIONINE BY THE ACTION OF D-AMINO ACID OXIDASE

Sample originating from	Amount tested I (μg)	Amount tested II (counts/min)	Amount recovered I (μg)	Amount recovered II (counts/min)	Total deamination calculated I (%)	Total deamination calculated II (%)
N-Malonylmethionine-methyl-^{14}C,	232	14,100	8	630	96·6	96·2
from plant	224	13,620	10	650	95·5	95·2
N-Malonyl-D-methionine,	270	—	10	—	96·2	—
synthesized	270	—	13	—	95·2	—
D-Methionine	150	—	4	—	97·3	—

EXPERIMENTAL

Materials and Methods

D-Methionine-methyl-^{14}C (90 mg, spec. act. 56·14 μc/m-mole, $[\alpha]_D$ +9·9°, in water) was administered in one dose to five hydrocultured intact *Nicotiana rustica* plants which were then allowed to metabolize for 6 days. *N*-Malonylmethionine-methyl-^{14}C was isolated from the MeOH root extracts as already described,[1] the final purification of the metabolite being performed[1] by fractionation on a Sephadex G-25 column. D-Amino acid oxidase (from hog kidney, Fluka, pract. and Koch-Light 16 units/mg) and L-amino acid oxidase (from *Crotalus adamanteus*, Koch-Light) were used.

Paper chromatography, electrophoresis and radioactive counting procedures were performed as already described.[1] Methionine was determined by the ninhydrin method following essentially the standard procedure;[7] extinction was measured on a Beckman DU spectrophotometer at 510 mμ.

Configuration of C-2 in N-Malonylmethionine

Samples of the conjugate were hydrolysed in 2 N HCl at 100° for 2 hr. Hydrolysates were evaporated to dryness *in vacuo*, the residues were dried over NaOH and dissolved in water to a known volume. Portions of each sample were applied to Whatman paper, previously washed with *n*-BuOH–acetic acid–H_2O (60:15:25), chromatographed in the same solvent and scanned for radioactivity; the methionine peak was either eluted with 50% EtOH and checked for radioactivity, or developed with ninhydrin (0·5% in acetone–0·05 M phosphate buffer pH 7·0, 9·5:0·5, v/v), the spot eluted with EtOH–H_2O (3·5:1, v/v plus 5 mg $CuSO_4 \times 5H_2O$) and the extinction of the solution was measured.

Incubations were performed with 1–2 μmole of methionine, 0·05 M pyrophosphate buffer, pH 8·2, and 0·7–1·0 mg of D-amino acid oxidase in a total volume of 2·0 ml at 37° for 10–16 hr. Reaction mixtures were deproteinized by addition of 10 ml EtOH, followed by centrifugation. Aliquots of the supernatant were evaporated to dryness *in vacuo*, the residue was dissolved in about 1 ml of water and passed through a column of Dowex 50X-8 H^+ followed by water. Elution was carried out with N NaOH, the amino acid fraction was neutralized with 0·1 N acetic acid and concentrated *in vacuo* to known volume: aliquots were then taken for spectrophotometric and radioactive determination as described above. Aliquots of the supernatant or water effluent were shaken with 0·2% 2,4-dinitrophenylhydrazine solution in 2 N HCl, the formed hydrazones were isolated by extraction procedure[1] and checked for radioactivity and chromatographic purity.

The same assays with synthetically prepared *N*-malonyl-D-methionine, as well as control experiments in which methionine and D-amino acid oxidase were omitted together and separately, were performed in parallel.

Syntheses

N-Ethoxymalonyl-D-methionine methyl ester was prepared from D-methionine methyl ester[8] and monoethyl malonate with dicyclohexylcarbodi-imide as already described for the DL-compound.[1] Viscous oil, yield

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90%, $[\alpha]_D$ +7·8° (*ca.* 2·0 in ethanol); for analysis it was distilled in a micro-tube at 145–150°/0·01 mm Hg (Al block). (Found: C, 47·41; H, 6·92; N, 5·25; S, 11·49. $C_{11}H_{19}NO_5S$ required: C, 47·64; H, 6·90; N, 5·05; S, 11·56%.)

N-*Malonyl*-D-*methionine* was obtained as described for the DL-compound;[1] viscous oil (yield 90%) which solidified on standing; $[\alpha]_D$ −17·2° (*ca.* 2·0 in ethanol). *Bis*-dicyclohexylamine salt: m.p. 142–143°, from ethanol–ether, $[\alpha]_D$ −17·5° (*ca.* 1·6 in ethanol). (Found: C, 64·41; H, 10·06; N, 7·20; S, 5·36. $C_{32}H_{59}N_3O_5S$ required: C, 64·28; H, 9·95; N, 7·03; S, 5·36%.) Ca-salt: white powder containing 1 mol. H_2O $[\alpha]_D$ −10·0° (*ca.* 1·7 in water). (Found: C, 32·78; H, 4·52; N, 4·80; Ca, 13·38. $C_8H_{11}NO_5SCa \times H_2O$ required: C, 32·98; H, 4·49; N, 4·81; Ca, 13·76%.)

N-*Malonyl*-D-*methionine sulphoxide, Ca-salt.* A solution of *N*-malonyl-D-methionine Ca-salt (136 mg) in 1 ml of water was oxidized with 0·05 ml of 30% H_2O_2 at room temp. for 3 hr. After evaporation the residue was crystallized from 60% EtOH; on standing at 0°, 109 mg (73%) of white crystals with $[\alpha]_D$ −20·0° (*ca.* 1·6 in water) separated. The compound crystallized with 1·5 H_2O. (Found: C, 30·45; H, 4·51; N, 4·56; Ca, 12·98. $C_8H_{11}NO_6SCa \times 1{\cdot}5\ H_2O$ required: C, 30·37; H, 4·46; N, 4·43; Ca, 12·66%.)

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