Phytochemistry, 1969, Vol. 8, pp. 51 to 55. Pergamon Press. Printed in England

CONJUGATION OF D-β-METHIONINE INTO *N*-MALONYL-β-METHIONINE BY *NICOTIANA RUSTICA**

B. LADEŠIĆ and D. KEGLEVIĆ

Tracer Laboratory, Institute "Ruder Bošković", Zagreb, Yugoslavia

(*Received* 23 *July* 1968)

Abstract—Intact *Nicotiana rustica* plants were fed D-β-methionine-methyl-^{14}C and the rate of incorporation of radioactivity into the acidic fraction of methanolic root- and shoot-extracts was investigated. From the radioactive acidic fraction a thioether-positive peak and its sulphoxide were isolated and identified as *N*-malonyl-β-methionine and its sulphoxide. This was confirmed by comparison with synthetic *N*-malonyl-DL-, L-, and D-β-methionines and by crystallization of the radioactive thioether-positive spot to constant specific activity. Strong evidence was obtained for the D-configuration of the *N*-malonyl conjugate.

INTRODUCTION

OUR STUDIES on the metabolism of the optical isomers of α- and β-methionine† in *Nicotiana rustica* have shown[1] that, under identical experimental conditions, both D-isomers are metabolized by a pathway which is lacking with their enantiomorphs. After feeding the intact tobacco plant with D-α-methionine-methyl-^{14}C a significant portion of radioactivity in methanolic root- and shoot-extracts appeared in the acidic, non-amino acid fraction. A detailed study revealed[2] that under the conditions examined only a negligible part of D-α-methionine underwent oxidative deamination to α-keto-γ-methylthiobutyric acid, while the majority was conjugated with malonic acid into *N*-malonylmethionine.

In earlier experiments[1] with L- and D-β-methionine-methyl-^{14}C, the bulk of the activity absorbed by plant, after a 10-day metabolizing period, was recovered as the unchanged tracer and its sulphoxide. Nevertheless, with the D-β-isomer as the precursor, additional radioactivity appeared in the non-amino acid fraction isolated from the root extract; this activity proved to be associated with an acidic thioether-positive compound and with its sulphoxide. Some potential metabolites of D-β-methionine, i.e. β-keto-γ-methylthiobutyric acid, *S*-methyl-1-thiopropanone and *N*-acetyl-D-β-methionine were then synthesized[3] and compared with the acidic thioether-positive metabolite isolated from the plant; the results[3] excluded oxidative deamination and/or subsequent decarboxylation, as well as *N*-acetylation as possible metabolic routes for D-β-methionine in the tobacco plant.

In the present study, evidence is given that the tobacco plant metabolizes D-β-methionine, a structurally and sterically unnatural amino acid, into the corresponding *N*-malonyl conjugate.

---

* Part V in the series "Biochemical Studies in Tobacco Plants".

† When necessary for clarity methionine will be designated as α-methionine, and β-amino-γ-methylthiobutyric acid as β-methionine.

[1] B. LADEŠIĆ and D. KEGLEVIĆ, *Arch. Biochem. Biophys.* **111**, 653 (1965).

[2] D. KEGLEVIĆ, B. LADEŠIĆ and M. POKORNY, *Arch. Biochem. Biophys.* **124**, 443 (1968).

[3] B. LADEŠIĆ and D. KEGLEVIĆ, *Croat. Chem. Acta* **38**, 155 (1966).

RESULTS AND DISCUSSION

Methanolic root- and shoot-extracts were freed from amino acids by ion-exchange chromatography, and the acidic water effluent was extracted with ethyl acetate. In this way, the thioether-positive peak containing the bulk of the water effluent activity was separated from its sulphoxide, which remained in the water layer. In Table 1, the percentages of the absorbed radioactivity found in water effluents at various times are given: the data obtained strongly suggest the root as the place where the acidic, thioether-positive metabolite is formed. The results obtained in experiment X, in which the tracer was applied through the leaves, point to the same conclusion. Although 4 days after injection the leaves still contained 90 per cent of the original activity, there was no increase in the thioether-positive peak. On the other hand, in the root the percentage of the thioether-positive peak (1 per cent) was only slightly lower than that of the recovered β-methionine (1·7 per cent).

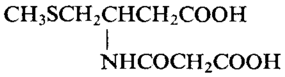
Table 1. Percentages of absorbed radioactivity in water effluent after administration of D-β-methionine-methyl-^{14}C (10–13 mg per plant)

Expt.*	Duration of expt. days	Radioactivity recovered in water effluent (%) Root	Shoot
I	4	4	3
II	4	13	4
III	4	4	3
IV	7	18	3
V	10	15	4
VI	10	20	3
VII	10	18	4
VIII	14	14	9
IX	28	12	10
X	4	1	4

* In Expts I–IX the tracer was fed in one dose through the root to the intact plant grown as a water culture; in Expt. X the tracer was applied by leaf-tip injection.

R_fs and electrophoretic mobilities of the thioether-positive peak were practically identical to those of *N*-malonyl-α-methionine. On acid hydrolysis, the thioether-positive peak yielded radioactive β-methionine; furthermore, when it was kept *in vacuo* at 140–150°, carbon dioxide was evolved and a new radioactive spot, identified as *N*-acetyl-β-methionine,[3] was formed (Fig. 1).

To obtain conclusive evidence of identity, *N*-malonyl-β-methionine was synthesized following essentially the reaction scheme elaborated[2] for the α-analogue. Starting with the methyl esters of DL-, L- and D-β-methionine hydrochlorides, the corresponding *N*-malonyl conjugates (I) were obtained in a three-step synthesis as viscous oils in good yields. They formed crystalline bis-dicyclohexylamine (DCHA) salts; the salt of the racemic compound crystallized considerably easier than those of the L- and D-isomers.



$$\begin{array}{l} CH_3SCH_2\underset{\displaystyle |}{C}HCH_2COOH \\ \qquad\qquad NHCOCH_2COOH \end{array}$$

N-Malonyl-β-methionine (I)

As expected, the conjugated (I) proved to be chromatographically and electrophoretically indistinguishable from the thioether-positive peak, and on admixture of the latter with the racemic compound, followed by addition of DCHA, the crystalline bis-DCHA salt of constant specific activity was obtained. Furthermore, when a sample of the racemate was oxidized with dilute H_2O_2 at room temperature, the resulting sulphoxide gave R values identical to those of the radioactive sulphoxide-positive peak (Fig. 1). This compound should be considered as an artifact formed during the isolation process rather than as a true metabolite of D-β-methionine in the plant.

Earlier results[1] indicate that *N*-malonyl-β-methionine has the D-configuration. Thus this conjugate was not detected in tobacco plants fed with L-β-methionine or with L-β-methionine-methyl-^{14}C; the latter isolated from the plant after a 14-day metabolizing period had the same optical rotation as the starting material. On the basis of these findings, the retention of configuration was also expected with the D-anomer. In fact, D-β-methionine-methyl-^{14}C administered in one dose to *Nicotiana rustica* and recovered from the plant after 10 days showed an optical rotation identical to that of an authentic sample.

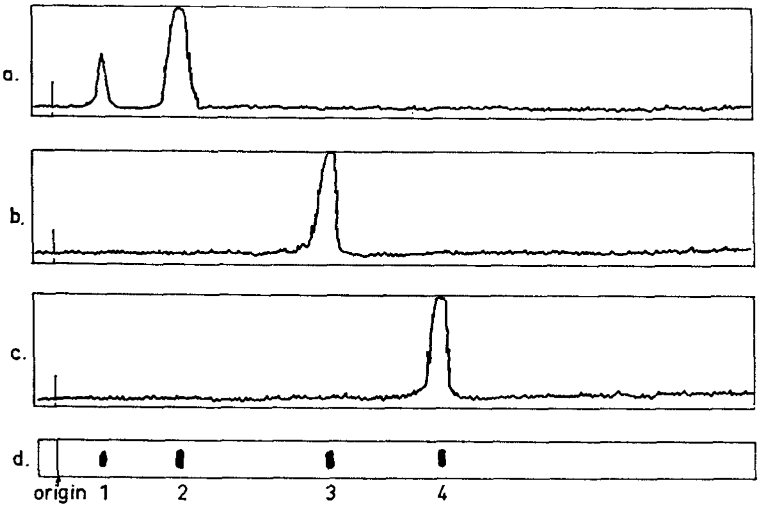


FIG. 1. IDENTIFICATION OF THE THIOETHER-POSITIVE PEAK BY PAPER CHROMATOGRAPHY IN ISOPROPANOL–AMMONIA–WATER (SOLVENT 2).

(*a*) Scannogram of the water effluent. (*b*) Scannogram of the hydrolysis product of the thioether-positive peak. (*c*) Scannogram of the decarboxylation product of the thioether-positive peak. (*d*) Markers: 1, *N*-Malonyl-DL-β-methionine sulphoxide; 2, *N*-malonyl-D-β-methionine; 3, D-β-methionine; 4, *N*-acetyl-D-β-methionine.

The D-configuration of *N*-malonyl-α-methionine, which has been recently established (unpublished results), represents strong, although indirect, evidence for the D-configuration of the β-conjugate. *N*-Malonyl-α-methionine was subjected to acid hydrolysis, and the resulting amino acid was quantitatively deaminated by D-amino-oxidase. However, as D-β-methionine is not a substrate of D-amino-oxidase, this method is of no value in the present case.

When compared with D-α-methionine, the conjugation of D-β-methionine proceeds at a considerably slower rate and to a smaller extent. Nevertheless, the results obtained suggest malonic acid conjugation as a plausible detoxication pathway for D-amino acids in higher plants. This process presumably takes place to a greater or less degree with all D-amino acids, no matter whether their enantiomorphs are normal constituents of the plant or not.

EXPERIMENTAL

Materials and Methods

D-β-Methionine-methyl-^{14}C, 99·8 μc/mmole, $[\alpha]_D + 22{\cdot}5°$ (*c* 1·2 in water) was prepared as already described.[4] All evaporations were carried out in a water bath at 40°. The growing of *Nicotiana rustica* L. plants, the administration of the labelled compound, and extraction of the homogenized plant material with methanol was performed as already described.[4, 1] After removing the methanol, the remaining aqueous solution was neutralized to pH 7 and placed on a column of Dowex 50-X8 in H^+ form. The acidic, radioactive water effluent was concentrated *in vacuo*, adjusted to pH 2 with 2 N HCl and the radioactive thioether-positive peak was extracted with ethyl acetate, while the corresponding sulphoxide remained in the water layer.

Paper chromatography was carried out on Whatman No. 1 and 3 MM paper in solvent systems (all by vol.): (1) *n*-butanol–acetic acid–water (60:15:25); (2) *iso*-propanol–ammonia–water (10:1:1); and (3) *tert*-butanol–methyl ethyl ketone–ammonia–water (40:30:10:20). The spots were visualized with platinum reagent for thioethers and sulphoxides, and with the HI–starch reagent[5] for sulphoxides. Paper electrophoresis was performed on Whatman No. 1 paper at room temperature with a voltage gradient of 12 V per cm in pyridine–acetic acid pH 6·5.

Radioactivity was assayed as follows. Aliquots of liquid samples were plated in duplicate on stainless-steel planchets (1 in^2), dried under an i.r. lamp and counted as infinite thin specimens in a gas-flow or mica-window GM counter; after corrections the counts were compared with the precursor prepared in the same way. Solid samples were counted at infinite thickness and, after corrections, compared with the ^{14}C-polyethylene standards (Amersham, England). Chromatograms were scanned for radioactivity with an automatic GM mica-window scanner or with a Nuclear Chicago Actigraph II.

In the identification of the thioether-positive peak, acid hydrolysis was performed in 6 N HCl in a sealed Carius tube at 105° for 13 hr. Decarboxylation was performed in an evacuated microdistillation tube with a side-arm containing barium hydroxide; the tube was held in an Al block at 140–150° for 0·5 hr.

Isolation of Unmetabolized D-β-*Methionine*

Ten *N. rustica* plants were fed in one dose with 100 mg D-β-methionine-methyl-^{14}C (11·1 μc/mmole, $[\alpha]_D + 22{\cdot}7°$, in water) through the root,[4] and left to metabolize 10 days. After homogenization, the methanolic shoot-extract was freed from inorganic salts by passing through a Dowex 50-X8 H^+ column, the amino acids were displaced with 2 N NH_4OH and then combined with the crude root-extract. After neutralization and concentration to about 10 ml, the material was absorbed on a Dowex 50-X8 NH_4^+ column (130 × 1·5 cm) and eluted gradually with water.[1] Fractions containing β-methionine were pooled, evaporated to dryness, and the residue was subjected to partition chromatography on a cellulose column (30 g, 63 × 1 cm) with butanol–acetic acid–water (60:15:25); the whole process was repeated with pure fractions once again. In this way practically pure β-methionine was obtained as a solid white residue; it was dissolved in hot ethanol, treated with charcoal and, by cooling and addition of some absolute ether, crystals (22 mg, $[\alpha]_D + 22{\cdot}0°$) of D-β-methionine deposited. A second crystallization gave 15 mg of material with $[\alpha]_D + 23{\cdot}0°$, spec. activity: 11·1 μc/mmole, m.p. 165–167° with no depression with an authentic sample.

Syntheses of N-*Malonyl*-β-*methionines*

DL-β-*Methionine methyl ester*. To a suspension of DL-β-methionine (450 mg, 3 mmoles) in methanol (4 ml), freshly distilled $SOCl_2$ (0.22 ml) was added with cooling, and the resulting clear solution was kept protected from moisture at 0° for 5 days. The solvent was evaporated *in vacuo*, the remaining oil triturated with dry ether and the crystalline hydrochloride (576 mg, 96 per cent) filtered off. After two recrystallizations from methanol–ether, it melted at 77–79°. (Found: C, 36·27; H, 7·00; N, 6·90; S, 15·55; Cl, 17·43. $C_6H_{14}NO_2S$ required: C, 36·10; H, 7·07; N, 7·01; S, 16·06; Cl, 17·75 per cent.) The base was liberated by treating the salt with the equimolar amount of triethylamine in methanol; after addition of abs. ether, triethylamine hydrochloride was filtered off, and the solvent evaporated *in vacuo*. A sample of the remaining oil was distilled at 65–75°/0·25 mm Hg (Al block). (Found: N, 8·76. $C_6H_{13}NO_2S$ required: C, 44·15; H, 8·03; N, 8·58; S, 19·64 per cent.) The D- and L-isomers were obtained similarly. The L-β-methionine methyl ester hydrochloride had m.p. 88–90°, $[\alpha]_D - 14{\cdot}3°$ (*c* 5·0 in water). (Found: C, 36·34; H, 6·93; N, 7·12 per cent); the free base had $[\alpha]_D - 0{\cdot}8°$ (*c* 5·0 in methanol). The D-β-methionine methyl ester hydrochloride had m.p. 88–90°, $[\alpha]_D + 14{\cdot}0°$ (*c* 5·0 in water). (Found: C, 36·24; H, 6·85; N, 6·91; S, 15·66 per cent); the free base boiled at

---

[4] B. LADEŠIĆ, Z. DEVIDÉ, N. PRAVDIĆ and D. KEGLEVIĆ, *Arch. Biochem. Biophys.* **97**, 556 (1962).

[5] J. F. THOMPSON, W. N. ARNOLD and C. J. MORRIS, *Nature* **197**, 380 (1963).

45–55°/0·1 mm Hg (Al block), $[\alpha]_D + 1{\cdot}0°$ (*c* 5·0 in methanol). (Found: C, 44·04; H, 7·87; N, 8·75; S, 19·74 per cent.)

N-*Ethoxymalonyl*-DL-*β-methionine methyl ester.* To a solution of the above ester (298 mg, 1·82 mmoles) and monoethyl malonate (240 mg, 1·82 mmoles) in 4 ml tetrahydrofuran (THF), the equivalent amount of dicyclohexylcarbodi-imide (375 mg) in 1 ml THF was added, and the mixture was left to stand at room temperature for 3 days. Dicyclohexylurea (403 mg, 97 per cent) was filtered off, the solvent evaporated *in vacuo*, the remaining oil dissolved in ethyl acetate, the solution washed with 0·3 % H_2SO_4, water, aq. $NaHCO_3$ and water, and dried. After the removal of the solvent, the oil was dissolved in 1 ml of benzene and chromatographed on silica gel. After elution with benzene–ethyl acetate (1:1) and evaporation, the product was obtained as a colourless viscous oil (334 mg, 66 per cent). For analysis, it was distilled at 115–125°/0·007 mm Hg (Al block). (Found: C, 47·89; H, 6·95; N, 5·25; S, 11·56. $C_{11}H_{19}NO_5S$ required: C, 47·64; H, 6·90; N, 5·05; S, 11·56 per cent.) The *N*-ethoxymalonyl-L-β-methionine methyl ester was obtained as a viscous oil, $[\alpha]_D - 15{\cdot}5°$ (*c* 4·4 in ethanol). (Found: C, 47·92; H, 6·82; N, 5·08 per cent.) The *N*-ethoxymalonyl-D-β-methionine methyl ester was obtained as a viscous oil, $[\alpha]_D + 16{\cdot}0°$ (*c* 5·0 in ethanol). (Found: C, 47·31; H, 6·72; N, 5·26; S, 11·17 per cent.)

N-*Malonyl*-DL-*β-methionine.* The saponification of the ethyl ester (277 mg, 1 mmole) was performed in 4 ml acetone with 2 ml N NaOH at room temperature under shaking for 2 hr. Acetone was removed *in vacuo*, water added to the residue, the solution extracted with ethyl acetate, acidified with dilute HCl to pH 2 and then extracted repeatedly with ethyl acetate. The combined extracts were dried and evaporated *in vacuo*, leaving an oily product (197 mg, 84 per cent). A part of this (153 mg, 0·65 mmoles) was dissolved in 4 ml of water, the equivalent amount of $CaCO_3$ was added and the mixture was left to stand at room temperature for 3 days whereupon it was filtered and the Ca salt precipitated from the solution with ethanol (73 per cent yield). The salt was recrystallized from water–ethanol and obtained as a white powder containing 0·5 molecule of water. (Found: C, 33·67; H, 4·24; N, 5·13; Ca, 14·47. $C_8H_{11}NO_5SCa$, 0·5 H_2O required: C, 34·03; H, 4·28; N, 4·96; Ca, 14·20 per cent.) Another sample of the product (98 mg) was dissolved in ethanol (1 ml) and neutralized with ethanolic dicyclohexylamine (DCHA); by subsequent addition of dry ether at 0° during several days, the bis-DCHA-salt precipitated, m.p. 141–142°. (Found: C, 64·48; H, 9·81; N, 7·31; S, 5·06. $C_{32}H_{59}N_3O_5S$ required: C, 64·28; H, 9·95; N, 7·03; S, 5·36 per cent.) *N*-Malonyl-L-β-methionine was similarly obtained as a viscous oil in 80 per cent yield, $[\alpha]_D - 12{\cdot}5°$ (*c* 2·2 in ethanol). Bis-DCHA-salt: m.p. 123–125°, $[\alpha]_D - 12{\cdot}1°$ (*c* 2·0 in ethanol). (Found: C, 64·13; H, 10·02; N, 7·22; S, 5·69 per cent.) *N*-Malonyl-D-β-methionine was obtained as a viscous oil in 82 per cent yield, $[\alpha]_D + 12{\cdot}0°$ (*c* 2·0 in ethanol). R_fs: solvent (1), 0·75; solvent (2), 0·16; solvent (3), 0·46. Bis-DCHA-salt: m.p. 123–125°, $[\alpha]_D + 12{\cdot}7°$ (*c* 2·0 in ethanol). (Found: C, 64·49; H, 9·99; N, 6·84; S, 5·52 per cent.)

Acknowledgement—The authors are indebted to Dr. O. Hadžija for the microanalyses.