CCA-422

577.1:547.466.2.07
Original Scientific Paper

Biochemical Studies in Tobacco Plants. III.* Synthesis and Behaviour of Potential Metabolites of D-β-Methionine in *Nicotiana rustica*

B. Ladešić and D. Keglević

Tracer Laboratory, Institute »Ruđer Bošković«, Zagreb, Croatia, Yugoslavia

Received April 29, 1966

In order to check whether β-keto-γ-methylthiobutyric acid (IV) was formed in *Nicotiana rustica* after administration of D-β-methionine-methyl-^{14}C, the Ba salt of IV was synthesized from S-methylthioacetyl malonate (I) as the starting compound. A comparison of IV and its decomposition products with the radioactive spots isolated from the plant material shows that the oxidative deamination of D-β-methionine is not a likely metabolic pathway. For the same reason N-acetyl-DL-β-methionine (V) and its sulphoxide VI were synthesized; comparison with plant material excluded also the N-acetylation of D-β-methionine as the metabolic route. 2,4-Dinitrophenyl-DL-, L- and D-β-methionine (VII) and the corresponding DL-sulphoxide VIII proved to be valuable derivatives for the characterization of β-methionine.

In order to investigate the metabolic fate of the optical and structural isomers of methionine in higher plants, we started, some years ago, some experiments[1,2] in which the optical isomers of α- and β-methionine-methyl-^{14}C were fed to the intact *Nicotiana rustica* plants. The fractionation of methanolic plant extracts on ion-exchange columns revealed that in the fraction containing organic acids, sugars and mineral salts, a considerably higher proportion of radioactivity appeared with D-α- as well as with D-β-methionine-methyl-^{14}C as the precursors, than when the corresponding enantiomorphs were administered to the plant under identical conditions.

In the case of D-β-methionine, the bulk of the activity in the fraction was found[2] to be associated with a thioether positive spot which, already under very mild conditions, underwent oxidation to the sulphoxide stage. In order to check whether this activity might be due to compound(s) arising from an oxidative deamination and/or to subsequent decarboxylation of D-β-methionine, some information about β-keto-γ-methylthiobutyric acid (IV) as a potential metabolite of this reaction was needed. First, it seemed to us that the conditions which would convert IV into the presumed decomposition products S-methyl-1-thiopropanone (II) and methylthioacetic acid should be known. Therefore, the synthesis of IV was undertaken and the conditions under which it undergoes decomposition were investigated.

---

* Part. II.: B. Ladešić and D. Keglević, *Arch. Biochem. Biophys.* **111** (1965) 653.

$CH_3SCH_2COCH(COOEt)_2$ (I) $CH_3SCH_2COCH_2COOEt$ (III) $CH_3SCH_2COCH_2COOH$ (IV)

$CH_3SCH_2COCH_3$ (II)

The thioketone II has already been prepared[3] from methylmercaptan and chloroacetone, while the β-keto ester III has been obtained[4] in a very poor yield from S-methylthioacetylchloride and sodium acetoacetate. We found Bowman's acidolysis of acylmalonic esters method[5] to be a much more suitable route for the synthesis of both compounds. Diethyl S-methylthioacetyl malonate (I) was prepared in high yield from S-methylthioacetylchloride and ethoxymagnesiummalonic ester. It was then subjected to complete and partial acidolysis respectively; in the first case, only the thioketone II and in the second II with the β-keto ester III were smoothly obtained. Careful hydrolysis of III and treatment of the hydrolysate with $BaCO_3$ gave the crystalline barium salt of the required β-keto acid IV.

As expected, the Ba salt of IV proved to be a labile compound decomposing slowly at room temperature even in the solid state. A comparison of its behaviour with the radioactive spots found in the non-amino acid fraction of methanolic plant extracts led to the following conclusions: 1) if formed in the plant as a metabolite of D-β-methionine, the β-keto acid IV would decompose into S-methyl-1-thiopropanone (II), methylthioacetic acid and some additional thioether-, sulphoxide- and carbonyl-positive compounds respectively, 2) the R_f values of the main radioactive spot do not coincide either with IV or with methylthioacetic acid, 3) on paper chromatograms the main decomposition product of IV in acidic medium, the thioketone II, cannot be detected because of its volatility. However, in methanolic plant extracts during the isolation procedure, no loss of radioactivity was observed. On the basis of these findings, it seems to us that the oxidative deamination is not likely to be a metabolic route of D-β-methionine in *Nicotiana rustica*.

The next finding[2], *i.e.* the fact that the radioactive spot after acid hydrolysis yielded radioactive methionine as one of the products in the hydrolysate, suggested N-acetylation as a feasible metabolic pathway. Therefore, N-acetyl-DL-β-methionine (V) and N-acetyl-DL-β-methionine sulphoxide (VI) were synthesized for comparison purposes. However, their R_f values as well as the relative stability of V towards oxidation to the sulphoxide stage were not consistent with the behaviour of the radioactive spot from non-amino acid fraction.

With the aim to characterize β-methionine arising from the radioactive spot by acid hydrolysis, a derivative of this amino acid with well defined characteristics was sought. Treatment of DL-, L- and D-β-methionine with fluorodinitrobenzene[6] gave the corresponding 2,4-dinitrophenylderivatives VII in high yields; they proved to have well defined melting points and remarkably high rotation values. The R_f values of VII and 2,4-dinitrophenyl-β-methionine sulphoxide (VIII) differ slightly but definitely in solvents *B* and *F* from the corresponding α-methionine derivatives. This property permits the mutual resolution of α- from β-methionine by paper chromatography.

EXPERIMENTAL

All melting points are uncorrected.

Paper chromatography

Descending one dimensional chromatography was performed on Whatman No. 1 paper in the following solvents: *A* = *n*-butanol-acetic acid-water (60:15:25), *B* = *iso*propanol-ammonia-water (10:1:1), *C* = phenol-water (80:20), *D* = methanol--water (95:5), *E* = *n*-butanol-methylethylketone-water (2:2:1), *F* = *t*.-amylalcohol--potassium hydrogenphthalate[7]. The spots were visualized with platinum reagent[8] for thioethers and sulphoxides, hydroiodic acid starch reagent[9] for sulphoxides, and 2,4-dinitrophenylhydrazine (0.5% in 2*N* HCl) for keto compounds.

Diethyl S-methylthioacetylmalonate (I)

Freshly prepared, dry ethoxymagnesiummalonic ester[10] (from 0.12 mole diethyl malonate, 0.12 g. atom magnesium turnings and 12.6 ml. absolute ethanol) was dissolved in 40 ml. of dry benzene, and placed in a three necked flask equipped with a dropping funnel, a stirrer and a condenser protected by a drying tube. Under stirring, a solution of S-methylthioacetylchloride[11] (12.7 g., 0.1 mole) in 15 ml. of dry benzene was dropped in at such a rate that rapid boiling was maintained; after the addition, the mixture was stirred and heated under reflux for 2 hours. The cooled syrupy mixture was decomposed with 25% sulphuric acid (135 ml.), the benzene layer separated, and the aqueous layer extracted several times with ether. The combined organic extracts were washed with a saturated NaCl solution and dried over Na_2SO_4. After the removal of the solvent the remaining oil was distilled: b.p. 102—104°/0.09 mm., yield: 20.4 g., 82% (calc'd. on S-methylthioacetyl-chloride). For analysis the oil was redistilled at 88—90°/0.03 mm. IR spectrum (neat): 1740 s (ester CO), 1660 s (chelated conjugated CO), 1620 s (conjugated C = C) $cm.^{-1}$.

Anal. $C_{10}H_{16}O_5S$ (248.30) calc'd: C 48.37; H 6.50; S 12.91%
found : C 48.23; H 6.48; S 13.33%

S-Methyl-1-thiopropanone (II)

A mixture of I (5 g., 20 mmole), propionic acid (11.8 g., 0.16 mmole) and 1 drop of conc. sulphuric acid was boiled under reflux until the vigorous evolution of CO_2 had ceased, whereupon 1.5 ml. of 4*N*-H_2SO_4 was added and the heating prolonged for one and half hour. The mixture was poured on to ice, neutralized to pH 6.5, extracted with ether, the extracts washed with sodium bicarbonate solution, and dried over Na_2SO_4. After the removal of the solvent, the remaining oil was distilled at 54—56°/20 mm., yield: 770 mg., 37%. For analysis it was redistilled at 97—99°/120 mm. (lit.[3]: 152.5—153°), colourless oil which darkens on standing.

Anal. C_4H_8OS (104.17) calc'd.: C 46.12; H 7.74; S 30.78%
found: C 46.36; H 7.83; S 31.07%

Semicarbazone White crystals from water; m.p. 154—155°.

Anal. $C_5H_{11}N_3OS$ (161.23) calc'd.: C 37.25; H 6.88; N 26.08; S 19.89%
found : C 37.08; H 6.76; N 26.03; S 19.96%

Ethyl β-keto-γ-S-methyl butyrate (III)

A mixture of I (7.59 g., 30 mmole), acetic acid (9.1 g., 0.15 mole) and 2 drops of conc. sulphuric acid were boiled under reflux for 3 hours. After cooling, 200 mg. of barium carbonate were added, the mixture shaken at room temp. for 3 hours, filtered and distilled at 20 mm. until the bath temp. reached 100°. The fraction (b.p. 27—50°) containing II was worked up, and II isolated in a 24% yield..

The liquid remained in the distillation flask was distilled *in vacuo:* 1.63 g. of III, b.p. 65—69°/0.09 mm., (31%) was obtained. For analysis it was redistilled at 68—70°/0.09 mm. (lit.[4]: 142—150°/29 mm.). IR spectrum (neat): 1725 s (ester CO), 1705 s (ketone CO), 1645 w (chelated conjugated CO), 1605 w (conjugated C=C) $cm.^{-1}$.

Anal. $C_7H_{12}O_3S$ (176.24) calc'd.: C 47.72; H 6.86; S 18.19%
found : C 47.39; H 6.95; S 18.68%

Semicarbazone White crystals from water; m.p. 118.5—119.5°.

Anal. $C_8H_{15}N_3O_3S$ (233.30) calc'd.: C 41.19; H 6.48; N 18.01; S 13.74%
found: C 41.37; H 6.44; N 18.11; S 14.10%

2.4 Dinitrophenylhydrazone Yellow needles from 80% ethanol; m.p. 97.5—98°.

Anal. $C_8H_{15}N_3O_3S$ (233.30) calc'd.: C 41.19; H 6.48; N 18.01; S 13.74%
found : C 44.03; H 4.61; N 15.69; S 8.87%

Barium salt of β-keto-γ-S-methyl butyric acid (IV)

To the ester III (353 mg., 2.0 mmole) 2.2 ml. of *N*-NaOH were added at 0° and the solution left to stand at 4° for one week. It was then extracted with ether, the water layer acidified with *N*-HCl to *p*H 3, and extracted several times with ether. After drying over Na_2SO_4 the solvent was removed *in vacuo* leaving IV as a reddish oil to which immediately 2 ml. water and solid barium carbonate (592 mg., 3 mmole) were added. After standing overnight the mixture was filtered, the filtrate evaporated *in vacuo*, the remaining crystaline residue (243 mg., 56%) was dissolved in a minimum amount of water and precipitated with absolute ethanol. Cream coloured crystals of the Ba salt of IV separated; for analysis they were redissolved in water and precipitated with ethanol. IR spectrum (KBr): 1590 vs (conjugate chelation, COO^-), 1390 s (COO^-), 1700 s (ketone CO), 717 w (CH_3-S-) cm^{-1}.

Anal. $C_5H_7O_3S$ Ba/2 (215.86) calc'd.: Ba 31.82; S 14.86%
found: Ba 31.82; S 14.81%

The crystals give a wine-red colour with $FeCl_3$ and evolve CO_2 with diluted hydrochloric acid. Addition of 2,4-dinitrophenylhydrazine in 2*N*-HCl to a water solution of Ba salt precipitated yellow crystals, identified as S-methyl-1-thiopropanone 2,4-dinitrophenylhydrazine[3], m.p. 105—106°. On paper chromatograms, the freshly prepared Ba salt revealed only one thioether- and carbonyl-positive spot; R_f values: *A*, 0.83; *C*, 0.76; *D*, 0.68; *E*, 0.35. In the analytically pure sample, after standing in a vacuum desiccator for about one month, $BaCO_3$ was detected, while on paper chromatograms several thioether- carbonyl- and sulphoxide- positive spots appeared. A very similar chromatographic pattern was obtained after passage of the freshly prepared Ba salt through the columns of Dowex 50-X4 in HN_4^+ and H^+ form. By paper chromatography, methylthioacetic acid but not S-methylthiopropanone (II), could be detected among the decomposition products. Experiments with pure II established that because of its volatility II cannot be detected by this technique.

N-*Acetyl* -DL-β-*methionine* (V)

To a solution of DL-β-methionine[12] (522 mg., 3.5 mmole) in 1.75 ml. 2*N*-NaOH, acetic acid anhydride (0.91 ml.) and 2*N*-NaOH (4.55 ml.) were added alternatively at 0°, the solution left to stand at room temp. overnight, diluted with water to 100 ml. and passed through a column of Dowex 50-X4 in H^+ form. The column was washed with water until the effluent was no more acidic; it was then evaporated *in vacuo* and the remaining viscous oil triturated with absolute ether. After standing overnight at 0°, 629 mg., (94%) of V were obtained as white crystals with m.p. 74.5—76°. For analysis the substance was dissolved in absolute acetone and precipitated with petroleum ether, m.p. 76—77°. IR spectrum (KBr): 3300 s (NH), 1700 s, 1550 s (amide I and II), 1650 s (acid CO), 737 w (CH_3-S-) cm^{-1}.

Anal. $C_7H_{13}NO_3S$ (191.25) calc'd.: C 43.96; H 6.85; N 7.32; S 16.77%
found : C 43.84; H 7.02; N 7.33; S 16.41%

R_f values: *A:* 0.86, *B:* 0.45, *C:* 0.86.

N-*Acetyl*-DL-β-*methionine* sulphoxide (VI)

To a solution of V (191 mg., 1 mmole) in 1 ml. acetic acid, 2 ml. of 3% hydrogen peroxide was added and the mixture left to stand 24 hours at 0°. After evaporation *in vacuo* a viscous oil remained which was triturated with absolute ether. By standing overnight at 0°, 166 mg., (80%) of VI, m.p. 162.5—163.5° was obtained. For analysis

it was recrystallized from absolute ethanol, m.p. 166—167°. IR spectrum (KBr): 3030 s (NH), 1700 s, 1555 s (amide I and II), 1600 s (acid CO), 1010 s (S→0) cm^{-1}.

Anal. $C_7H_{13}NO_4S$ (207.26) calc'd.: C 40.57; H 6.32; N 6.76; S 15.47%
found : C 40.56; H 6.44; N 7.05; S 15.58%

R_f values: *A:* 0.56, *B:* 0.23, *C:* 0.85.

2,4-*Dinitrophenyl*-DL-β-*methionine* (VII)

To a solution of DL-β-methionine (298 mg., 2 mmole) and sodium bicarbonate (750 mg., 9 mmole) in 10 ml. water, a solution of 2,4-dinitrofluorobenzene (740 mg., 4 mmole) in 10 ml. of ethanol was added and the mixture shaken for 3 hours at room temperature. Ethanol was removed *in vacuo*, the excess of the reagent extracted with ether, the aqueous layer acidified with 6*N*-HCl to p_H 1, and extracted with ethyl acetate. After drying over Na_2SO_4, the solvent was removed *in vacuo*, the remaining oil disolved in acetone benzene (1 : 1) and precipitated with petroleum-ether; yellow crystals (484 mg., 77%) were obtained. For analysis they were recrystallized from ether-petrolether, m.p. 110—112°.

Anal. $C_{11}H_{13}N_3O_6S$ (315.31) calc'd.: C 41.90; H 4.15; N 13.32; S 10.17%
found : C 41.71; H 4.36; N 13.47; S 10.06%

R_f values: *A:* 0.94, *B:* 0.69, *F:* 0.82.

2,4-*Dinitrophenyl*-L-β-*methionine*

Prepared from L-β-methionine[13], $[\alpha]_D$ —23° (water) as described for VII. Yellow needles, m.p. 117—119°, $[\alpha]_D$—125° (c, 0.31, acetic acid), —190° (c, 0.33, *N*-NaOH).

Anal. found: C 41.88; H 4.27; N 13.26; S 9.86%

2,4-*Dinitrophenyl*-D-β-*methionine sulphoxide* (VII)

Prepared from D-β-methionine[14], $[\alpha]_D$ +24° (water) as described for VII. Yellow crystals, m.p. 116—118°, $[\alpha]_D$ +126° (c, 0.37, acetic acid), +191° (c, 0.33, *N*-NaOH).

Anal. found: C 41.88; H 4.06; N 13.19; S 9.76%

2,4-*Dinitrophenyl*-DL-β-*methionine sulphoxide* (VIII)

To a solution of VII (158 mg., 0.5 mmole) in 2 ml. of acetic acid, 1 ml. of 3% hydrogen peroxide was added, and the solution left to stand at room temperature for 3 days. Acetic acid was removed *in vacuo* and the residue treated with absolute ethanol; on cooling 120 mg., (72% of VIII precipitated. After three recrystallisations from 50% methanol, the substance had m.p. 115—117°.

Anal. $C_{11}H_{13}H_3O_7S$ (331.31) calc'd.: C 39.88; H 3.95; N 12.68; S 9.68%
found : C 39.73; H 3.72; N 12.66; S 9.27%

R_f values: *A:* 0.84, *B:* 0.41, *F:* 0.40.

Acknowledgment. The authors are indebted to mr. O. Hadžija and Miss N. Horvatić for microanalyses.

REFERENCES

1. B Ladešić, Z. Devidé, N. Pravdić, and D. Keglević, *Arch. Biochem. Biophys.* **97** (1962) 556.
2. B. Ladešić and D. Keglević, *Arch. Biochem. Biophys.* **111** (1965) 653.
3. C. K. Bradsher, F. C. Brown, and R. J. Grantham, *J. Am. Chem. Soc.* **76** (1954) 114.
4. M. Jackman, A. J. Bergman, and S. Archer, *J. Am. Chem. Soc.* **70** (1948) 497.
5. R. E. Bowman, *J. Chem. Soc.* **1950**, 322.
6. K. R. Rao and H. A. Sober, *J. Am. Chem. Soc.* **76** (1954) 1328.
7. S. Blackburn and A. G. Lowther, *Biochem. J.* **48** (1951) 126.

8. G. Toennis and J. J. Kolb, *Anal. Chem.* **23** (1951) 823.
9. J. F. Thompson, W. N. Arnold, and C. J. Morris, *Nature* **197** (1963) 380.
10. *Org. Syntheses.* Coll. Vol. 4, John Wiley and Sons, Inc, New York, 1963, p. 285.
11. A. Mooradian, C. J. Cavallito, A. J. Bergman, E. J. Lawson, and C. M. Suter, *J. Am. Chem. Soc.* **71** (1949) 3372.
12. L. Birkofer und I. Hartwig, *Chem. Ber.* **87** (1954) 1189.
13. K. Balenović, D. Fleš, and I. Jambrešić, *Croat. Chem. Acta* **28** (1956) 303.
14. D. Keglević and B. Ladešić, *Croat. Chem. Acta* **31** (1959) 57.

IZVOD

Biokemijske studije u duhanu. III. Sinteza i svojstva potencijalnih metabolita D-β-metionina u duhanu *(Nicotiana rustica)*

B. Ladešić i D. Keglević

U želji da se ispita prelazi li D-β-metionin-metil-^{14}C u duhanu oksidativnom deaminacijom u β-keto-γ-metiltiomaslačnu kiselinu (IV), sintetizirana je Ba sol IV. S-Metiltioacetilmalonat I podvrgnut je parcijalnoj acidolizi, a dobiveni β-keto ester III hidroliziran je u Ba sol IV. Uspoređivanjem IV i njegovih razgradnih produkata s radioaktivnim mrljama kromatograma iz biljnog materijala, došlo se do zaključka da taj metabolički put nije vjerojatan. U svrhu identifikacije radioaktivnih metabolita, sintetizirani su N-acetil-DL-β-metionin (V) i njegov sulfoksid VI. Uspoređivanje s biljnim materijalom isključilo je i *N*-acetilaciju D-β-metionina kao mogući metabolički put. 2,4-Dinitrofenil derivati DL-, L- i D-β-metionina (VII) te DL-β-metionin sulfoksida VIII pokazali su se vrlo pogodni za karakterizaciju β-metionina.

TRACER LABORATORIJ
INSTITUT »RUĐER BOŠKOVIĆ«
ZAGREB

Primljeno 29. travnja 1966.