SYNTHESIS OF METHIONINES SPECIFICALLY LABELLED WITH ^{2}H OR ^{13}C

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SUMMARY

Efficient methods for synthesising L-[methyl-^{13}C]methionine from [^{13}C]iodomethane and L-methionine, and L-[3,4-$^{13}C_2$] methionine from [$^{13}C_2$]ethene, are described; the preparation of [3,3-$^{2}H_2$]methionine and [2,3,3-$^{2}H_3$]methionine from methionine by an exchange method is detailed.

Key Words; Methionine, Carbon-13, Deuterium

INTRODUCTION

A large number of biosynthetic pathways start from L-methionine and utilise either its methyl group or the C1-C4 chain (in part or whole). Methyl-labelled methionines have been invaluable for identifying carbon atoms in natural products that originate from methionine *via* S-adenosylmethionine[1,2]. Examples of the utilisation of C1-C4 of methionine have been identified by means of ^{14}C-labelling in the biosynthesis of ethylene[3] (at C3 and C4), the polyamines spermine and spermidine[4] (C2-C4), and azetidine-2-carboxylic acid[5] (C1-C4).

This paper describes an improved synthesis of methyl-labelled methionines (L-[*methyl*-$^{2}H_3$] and L-[*methyl*-^{13}C]), the first synthesis of a methionine labelled at *both* C3 and C4 (L-[3,4-$^{13}C_2$]methionine) and the preparation of methionines specifically labelled with deuterium at C2 and/or C3. These methionines were required for studies of the biosynthesis of ethylene[6] and spermidine[7].

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METHYL-LABELLED METHIONINES

In classical syntheses of methyl-labelled methionines, the thiolate anion of homocysteine, obtained by treating either homocysteine with sodamide in liquid ammonia[8] or S-benzylhomocysteine with sodium in ammonia[2,9], was reacted with a labelled iodomethane. A more convenient preparation of the thiolate anion of homocysteine is *via* reduction of methionine with sodium in ammonia[10] and this reaction has been exploited for the synthesis of L-[*methyl*-$^2H_3$]methionine from L-methionine[11]. In our hands this procedure gave only 50-60% of methionine contaminated with homocysteine [2-3% of the methionine; identified by t.l.c. and Ellman's reagent, 5,5'-dithio*bis*-(2-nitrobenzoic acid)[12]]. The main drawback of this method is that addition of ammonium chloride to the reaction, supposedly to prevent racemisation[10], leads to difficulties in effecting an efficient separation of methionine from the excess of this salt, and from sodium chloride, by crystallisation from aqueous ethanol.

We have found that the use of lithium in place of sodium, and the addition of 1 mol ammonium chloride per mol methionine, enables a high recovery of pure labelled L-methionine to be achieved by crystallisation from ethanol containing a small amount of water. Lithium chloride is soluble in ethanol (*ca*. 20 wt. % at 20°C) and is therefore easily removed. In some experiments, omission of ammonium chloride did not lead to racemisation. However, in other experiments partial racemisation was observed and could be prevented by adding ammonium chloride to neutralise the probable causative agent lithium amide. To avoid incomplete reaction of the thiolate anion of homocysteine with iodomethane and hence contamination of product methionine with homocysteine, aliquots of the reaction mixture should be periodically tested with Ellman's reagent[12]. After reducing

L-methionine with lithium in liquid ammonia, 1.02 mol equivalent of a labelled iodomethane was added and the reaction was allowed to proceed for 2 hours. Ellman's test was then positive for free thiol. After further addition(s) of iodomethane (two portions totalling 0.13 mol equiv., in one experiment) Ellman's test was negative and work-up gave pure labelled L-methionine. Using this procedure, L-[methyl-$^2H_3$]methionine (99 atom % ^2H) and L-[methyl-^{13}C]methionine (91 atom % ^{13}C) have been prepared in up to 83% yield from the appropriate labelled iodomethane. A further quantity of labelled methionine may be obtained by ion exchange chromatography of mother liquors.

Recently, a method for preparing L-[methyl-$^2H_3$]methionine, via base-catalysed exchange of dehydromethionine in methane[^2H]ol, was described[13]. L-[methyl-^{13}C]methionine prepared by the above method can be converted to L-[methyl-(^{13}C,$^2H_3$)]methionine by isotopic exchange[13].

METHIONINES LABELLED IN THE C1-C4 CHAIN

Methionine has been prepared via the reaction of 1-chloro-2-(methylthio)ethane with the carbanion (C2) of diethyl 2-acetamidomalonate[14]. The 1-chloro-2-(methylthio)ethane was prepared from 2-(methylthio)ethanol, but can also be obtained by reacting ethene with methanesulphenyl chloride[15]. We have combined these methods (cf. Scheme) to produce an efficient synthesis of rac.[3,4-$^{13}C_2$]methionine from [$^{13}C_2$]ethene. Resolution of this material, essentially as described[16], gave L-[3,4-$^{13}C_2$]methionine.

Tenenbaum et al.[17] reported exchange reactions of glycine, α- and β-alanine, α-aminobutyric acid and valine in deuterium oxide catalysed by Al(III) and pyridoxal. At pH 5.1 exchange of deuterium for hydrogen(s) at both C2 and C3 of the amino-acid

occurred at similar rates, whereas at pH 10.2 exchange at C2 was much faster than that at C3.

We have used Al(III)/pyridoxal in deuterium oxide to effect pH-dependent exchanges of methionine. Procedures are given in the Experimental Section for preparing rac.[2,3,3-$^{2}H_3$]methionine (exchange at pH 5.0) and rac.[3,3-$^{2}H_2$]methionine (exchange in $^{2}H_2O$ at pH 5.0 following by exchange in H_2O at pH 10.2). The rac.[2,3,3-$^{3}H_2$]methionine was resolved essentially by the method described[16].

Scheme

$$^{13}CH_2{=}^{13}CH_2 \xrightarrow{i} MeS^{13}CH_2{}^{13}CH_2Cl \xrightarrow{ii} MeS^{13}CH_2{}^{13}CH_2C(CO_2Et)_2NHAc \xrightarrow{iii} MeS^{13}CH_2{}^{13}CH_2\overset{+}{C}HNH_3CO_2^-$$

i $MeSCl/CH_2Cl_2$ ii $NaCNHAc(CO_2Et)_2/EtOH$

iii aq HCl, reflux.

EXPERIMENTAL

L-[Methyl-^{13}C]methionine

Using dry apparatus, liquid ammonia (ca. 200 cm^3) was distilled from a small quantity of sodium (ca. 0.25 g) on to (L)-methionine (4.46 g, 3 x 10^{-2} mol) over 1 h under dry nitrogen. During 20 min. flattened pieces of lithium (0.67 g, 9.6 x 10^{-2} mol) were added in several portions to the refluxing solution, which was maintained under an atmosphere of dry nitrogen. The resulting blue solution was stirred, and after 2 h the colour of the solution had been discharged. A small portion of the reaction mixture was

removed and evaporated, and the residue in D_2O was examined by ^{1}H n.m.r. spectroscopy. The spectrum showed unreacted methionine (MeS at δ 2.1) and additional quantities of lithium were added (up to 1 mol equiv. was necessary) until examination by ^{1}H n.m.r. spectroscopy showed the absence of methionine. Ammonium chloride (1.6 g, 3 x 10^{-2} mol) was added followed by [^{13}C]iodomethane (4.4 g, 3.1 x 10^{-2} mol from B.O.C. Ltd. Prochem), 91 atom % ^{13}C. The reaction was boiled under reflux for 2 h, when testing a small aliquot of the solution with Ellman's reagent[12] showed the presence of free thiol. A further portion of labelled iodomethane (0.29 g, 2.0 x 10^{-3} mol) was added, and the solution was boiled under reflux for another hour. Another Ellman's test still showed the presence of free thiol, and so a further portion of labelled iodomethane (0.27 g, 1.9 x 10^{-3} mol) was added. After refluxing the solution for a further hour, no free thiol could be detected by Ellman's reagent. The solvent was removed by leaving the reaction vessel to warm up overnight under an atmosphere of nitrogen. The off-white residue was dissolved in water (100 cm^3) and was evaporated to dryness under reduced pressure (50°C, 20 mm Hg). This procedure was repeated twice. The solid residue was dissolved in water (200 cm^3) and the pH of the solution was reduced to 5.6 by the addition of 2 M hydrochloric acid. Evaporation to *ca.* 15 cm^3 gave white crystals, which redissolved on boiling, and after the addition of boiling ethanol (300 cm^3) the solution was stored at -20°C for 12 h. The crystalline solid which formed was collected at the pump, and was washed with ice-cold ethanol/water (5 cm^3), ethanol (2 x 5 cm^3), and with ether (2 x 5 cm^3). Drying gave (L)-[*methyl*-^{13}C]methionine (3.61 g, 81%), 91 atom %, as a white crystalline solid, m.p. 273-277°C, δ (D_2O) 2.1 (0.9 x 3 H, d, J ^{13}C-^{1}H 138.6 Hz, $^{13}CH_3S$; and 0.1 x 3 H, s, $^{12}CH_3S$), 2.28 (2 H, m),

2.7 (2 H, m), and 4.22 p.p.m. (1 H, t), pure by t.l.c. [Kieselgel F_{254}, 880 ammonia/ethanol (23:77), ninhydrin spray, R_f 0.45], no detectable free thiol (Ellman's test), ammonium ions (Nessler's reagent) or lithium ions (flame test), $[\alpha]_D^{22} = 21.7^\circ$ (c 0.084, 5 M HCl) (cf. authentic (L)-methionine used as starting material: $[\alpha]_D^{22} = 22.0^\circ$ (c 0.095, 5 M HCl)).

The mother liquor from the above recrystallisation (for an experiment carried out on twice the above scale) was evaporated to dryness under reduced pressure, and the residue was dissolved in 2.5 M hydrochloric acid (20 cm^3). This solution was chromatographed on a column of Dowex 50 x 8 ion exchange resin (500 g) using hydrochloric acid (2.5 M) as eluate. The progress of the column was monitored by optical rotation measurements on the neat eluant. Early fractions were coloured (iodine) and later fractions contained (L)-[methyl-^{13}C]methionine, constituting a symmetrical band. The fractions containing product were combined and evaporated to dryness under reduced pressure (60°C, 20 mm Hg) and the solid residue was redissolved in water (50 cm^3). The pH of this solution was adjusted to 5.7 by the addition of saturated lithium hydroxide solution. Evaporation and recrystallisation as described above gave pure (L)-[methyl-^{13}C]methionine, 91 atom %, (0.73 g, 8%), as a white crystalline solid, with the same optical rotation as material obtained by direct crystallisation.

Methanesulphenyl Chloride

Sulphuryl chloride (14.3 g, 0.1 mol) was added dropwise over 15 min. to stirred dimethyl disulphide (9.4 g, 0.1 mol) at -15°C. The reaction was allowed to warm to room temperature over 2 h, and the product was then fractionally distilled. The fraction boiling between 30 and 32°C at 110 mm Hg was collected, the receiver being

cooled at -78°C, to give methanesulphenyl chloride as a deep orange liquid (12 g, 70%) [δ 2.9 (s, $C\underline{H}_3SCl$), n.b. absence of peak at δ 2.40 (s) due to $(C\underline{H}_3S)_2$] that was stored at -20°C and was used within 2 days of preparation.

[1,2-$^{13}C_2$]-1-Chloro-2-(methylthio)ethane

A solution of methanesulphenyl chloride (6 g, 7.3 x 10^{-2} mol) in CH_2Cl_2 (50 cm^3) was placed in a 250 cm^3 flask, cooled to -25°C, and evacuated to ca. 2 mm Hg on a vacuum line. Dry [$^{13}C_2$]ethylene from B.O.C. Limited Prochem (2000 cm^3, 8.9 x 10^{-2} mol) was admitted, and the solution swirled. The admission of ethylene was continued until no further absorption occurred, and the solution was colourless (ca. 40 min.), whilst the temperature of the solution was kept below -25°C. Dichloromethane was distilled off at 20°C and 50 mm Hg to give [1,2-$^{13}C_2$]-1-chloro-2-(methylthio)ethane (ca. 7.6 g) as a colourless liquid, δ 2.15 (0.8 x 3 H, d, J ^{13}C-^1H 4 Hz), 2.8 (2 H, 2 x m, J ^{13}C-^1H 47 Hz), and 3.6 p.p.m. (2 H, 2 x m, J ^{13}C-^1H 48 Hz). ^{13}C n.m.r. ($C_6{}^2H_6$, TMS) δ 35.7 (d, J ^{13}C-^{13}C 37 Hz) and 42.1 p.p.m. (d, J ^{13}C-^{13}C 37 Hz), each doublet being astride a singlet of ca. 10% total signal intensity, arising from single labelled species.

Reaction between [1,2-$^{13}C_2$]-1-chloro-2-(methylthio)ethane and sodium diethyl acetamidomalonate

Using all dry apparatus, sodium (1.38 g, 6 x 10^{-2} mol) was dissolved in anhydrous ethanol (30 cm^3) by boiling the mixture under reflux with the exclusion of atmospheric moisture. Diethyl acetamidomalonate (12.15 g, 5.5 x 10^{-2} mol) was added and the mixture was boiled under reflux until a clear solution resulted. The product from the above reaction (ca. 7 x 10^{-2} mol [1,2-$^{13}C_2$]-1-chloro-2-(methylthio)ethane and dichloromethane) was added and the reaction was boiled under reflux, with the exclusion of

atmospheric moisture for 5 h. After cooling to 0°C, the precipitated sodium chloride was filtered off under suction, and washed with cold ethanol (3 x 5 cm^3). The combined filtrates were evaporated to dryness under reduced pressure to provide a brown oil, δ 1.25 (6 H, t), 2.08 (6 H, 3 x s), 2.4 (4 H, m), 4.3 (4 H, q), and 6.9 (1 H, s, broad) p.p.m. This product was dissolved in 2 M hydrochloric acid (60 cm^3) and the mixture was boiled under reflux for 6 h, with magnetic stirring. A further quantity of 2 M acid was added (60 cm^3) and the reaction was refluxed for a further 3 h. The orange solution which resulted was evaporated to dryness under reduced pressure (90°C, 15 mm Hg), to give an orange oil. This oil was taken up in water (30 cm^3) and the pH of the solution was adjusted to 7 by the addition of saturated aqueous lithium hydroxide. The solution was again evaporated to dryness under reduced pressure, and the solid residue was dissolved in boiling water (40 cm^3) and hot absolute ethanol was added (500 cm^3). The solution was stored at -20°C for 36 h, and the precipitate was then filtered off under suction and washed with cold ethanol (3 x 10 cm^3) and ether (3 x 10 cm^3). Air drying gave rac.[3,4-$^{13}C_2$]methionine (4.76 g, 3.2 x 10^{-2} mol) as faintly yellow crystals. Treatment with charcoal and recrystallisation from aqueous ethanol gave rac.[3,4-$^{13}C_2$]methionine as white crystals, 3.6 g, m.p. 274-276°C, 220 MHz ^{1}H n.m.r., δ (D_2O/DCl), 2.15 (3 H, d), 2.28 (0.9 x 2 H, 2 x m, J ^{13}C-^1H 135 Hz, and 0.1 x 2 H, t), and 4.29 p.p.m. (1 H, m), pure by t.l.c. (for system see above). The overall yield of this synthesis from [$^{13}C_2$]ethylene was 39%.

Resolution of rac.[3,4-$^{13}C_2$]methionine
(cf. procedure of ref. 16)

Rac.[3,4-$^{13}C_2$]methionine (0.91 g, 6 x 10^{-3} mol) and ammonium 1-α-bromocamphor-π-sulphonate (Aldrich, 1.98 g, 6 x 10^{-3} mol) were

dissolved in pre-warmed M hydrochloric acid (6 cm^3). The solution was allowed to cool to room temperature and soon deposited crystals (ca. 1.2 g) of the bromocamphorsulphonate of L-[3,4,-$^{13}C_2$]methionine. These were filtered off and were dissolved in water (3 cm^3). The amino-acid was precipitated by addition of conc. ammonia to pH 5.9 followed by addition of hot methanol (25 cm^3). The methionine was collected (263 mg, 58% of available L-isomer) and was recrystallised by dissolving in hot water (10 cm^3), reducing the volume to a minimum and adding hot methanol (30 cm^3). After standing the resulting mixture overnight at 0°C, the crystals of L-[3,4-$^{13}C_2$]methionine were filtered off, washed with cold methanol and dried *in vacuo*: 240 mg (54%), $[\alpha]_D^{20}$ + 21.5 (c 0.009 in 1 M HCl); ^{1}H n.m.r. spectrum in D_2O/DCl identical to that of rac. material (see above).

Rac.[2,3,3-$^{2}H_3$]methionine

A solution of Al(III) in $^{2}H_2O$ was prepared by heating hydrated aluminium sulphate [$Al_2(SO_4)_3.16H_2O$, 0.775 g, 1.23 x 10^{-3} mol] at 250°C to a constant weight and then cooling and adding $^{2}H_2O$ (2 cm^3). The resulting solution was dried to constant weight and the residue was dissolved in $^{2}H_2O$ (5 cm^3) to give a 0.25 M Al(III) solution.

Rac.-methionine (4.36 g, 3 x 10^{-2} mol) was suspended in $^{2}H_2O$ (5 cm^3) and heated with stirring for 30 min. The mixture was pumped to dryness and the residue was dissolved with warming in $^{2}H_2O$ (30 cm^3). To this solution was added Al(III) in $^{2}H_2O$ (3 cm^3, 7.5 x 10^{-4} mol) and pyridoxal hydrochloride (0.6 g, 3 x 10^{-3} mol). The pH of the resulting solution was adjusted to 5.0 ($p^{2}H$ 5.4) by addition of 40% Na$O^{2}H$ in $^{2}H_2O$. The reaction mixture was refluxed with stirring under nitrogen for 24 h. After cooling the reaction mixture to 0°C addition of pre-cooled

methanol (100 cm^3) gave a precipitate of slightly yellow crystals. These were filtered off, washed with cold methanol and were dissolved in water. To the aqueous solution was added decolourising charcoal and the mixture was stirred with warming for 1 h. The mixture was filtered through Celite and the volume of the filtrate was reduced to ca. 8 cm^3 before addition of hot ethanol (40 cm^3). After cooling to -20°C overnight the crystals of rac.[2,3,3-$^2H_2$]methionine were collected and dried: 3.1 g, 71%, δ (D_2O) 2.13 (3 H, s) and 2.63 p.p.m. (2 H, s). For mass spectrometric analysis the N-trifluoroacetyl butyl ester was prepared[18] and showed the following peaks and relative intensities for the molecular ion cluster: m/z 301 (0), 302 (19), 303 (21), and 304 (100), denoting 14% $^2H_1$, 14% $^2H_2$, 72% $^2H_3$ and an overall deuterium content of 86%. Resolution of rac.[2,3,3-$^2H_3$]methionine was carried out as described for [3,4-$^{13}C_2$]methionine and gave 58% of the available L-[2,3,3-$^2H_3$]methionine, $[\alpha]_D$ + 22.7 (c 0.022 in 1 M HCl).

Rac.[3,3-$^2H_2$]methionine

To rac.[2,3,3-$^2H_3$]methionine (1.0 g, 6.58 x 10^{-3} mol) in H_2O (30 cm^3) was added aluminium sulphate solution (0.66 cm^3 of a 0.25 M solution in water) and pyridoxal hydrochloride (0.136 g, 6.8 x 10^{-4} mol). The pH of the solution was adjusted to 10.2 by the addition of 5 M aq. lithium hydroxide* solution. The rate of exchange of deuterium at C-2 was monitored by ^1H n.m.r. analysis. After 18 h at 37°C, the reaction mixture was concentrated under reduced pressure to ca. 10 cm^3. Hydrochloric acid (5 M) was added until the pH fell to 5.3, and the precipitated solid was redissolved by heating. Boiling ethanol (50 cm^3) was added, and the solution was stored for 12 h at -20°C. The

*If sodium hydroxide is used for neutralisation the product methionine is contaminated with sodium chloride (flame test).

crystals which formed were filtered off, washed and recrystallised by dissolving in minimum warm water and adding excess of hot ethanol. The crystals were filtered off and washed with ethanol and then ether. Drying gave rac.[3,3-$^{2}H_{2}$]methionine (0.78 g, 78%) as a white crystalline solid, m.p. 275-277°C, δ (D_2O/DCl) 2.15 (3 H, s), 2.65 (2 H, br, s) and 4.05 p.p.m. (1 H, br, s), pure by t.l.c. (for system see above), negative flame test for lithium. The integrals in the ^{1}H n.m.r. spectrum gave a ^{2}H content of ≥ 94% ^{2}H at C-3 and ≤ 8% at C-2.

REFERENCES

1. For a review see Cantoni, G. L. "The Biochemistry of Adenosylmethionine" (Salvatore, F., Borck, E., Zappia, F., Williams-Ashman, H.G., and Schlenk, F., eds.), Columbia University Press, New York and Guildford, p.557, 1977
2. e.g. Battersby, A. R., Ihara, M., McDonald, E., Redfern, J. R. and Golding, B. T., J.C.S. Perkin I, 158 (1977)
3. Yang, S. F. "Recent Advances in Phytochemistry" (Runeckles, V. C., Sondheimer, E., and Walton, D. C., eds.) Academic Press, New York, Vol. 7, p.131, 1974
4. Tabor, C. W. and Tabor, H., Ann. Rev. Biochem., 45, 285 (1976)
5. Leete, E., Phytochemistry, 14, 183 (1975)
6. cf. Billington, D. C., Golding, B. T. and Primrose, S. B., Biochem. J., 182, 827 (1979)
7. cf. Billington, D. C., Golding, B.T. and Nassereddin, I. K., J.C.S. Chem. Comm., 90 (1980)
8. Stekol, J. A. in "Methods in Enzymology", (Colonick, J. P. and Kaplan, N. O., eds.) Academic Press, London and New York, Vol. IV p.762, 1957
9. Melville, D. B., Rachele, J. R. and Keller, E. B., J. Biol. Chem., 169, 419 (1947)
10. Hope, D. B. and Humphries, J. F., J. Chem. Soc., 869 (1964)
11. Dolphin, D. and Endo, K., Analyt. Biochem., 36, 338 (1970)
12. Deakin, H., Ord, M. G. and Stockton, L. A., Biochem. J., 89, 297 (1963); Ellman, G., Arch. Biochem. Biophys., 82, 70 (1959)
13. Billington, D. C. and Golding, B. T., Tetrahedron Letters, 2937 (1979)
14. Goldsmith, D. and Tichler, M., J. Am. Chem. Soc., 68, 144 (1946)

15. Kharasch, N. and Buess, C. M., J. Am. Chem. Soc., 71, 2724 (1949); Mueller, W. H. and Butler, P. E., J. Am. Chem. Soc., 90, 2075 (1968)

16. Wheeler, G. P. and Ingersoll, A. W., J. Am. Chem. Soc., 73, 4604 (1951)

17. Tenenbaum, S. W., Witherup, T. H. and Abbott, E. H., Biochim. Biophys. Acta, 362, 308 (1974)

18. Gehrke, C. W. and Shahrokhi, F., Analyt. Biochem., 15, 97 (1966)