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SYNTHESIS OF (2R)- AND (2S)- [1-^{13}C]-2-AMINO-2-METHYLMALONIC ACID: CHIRAL SUBSTRATES FOR SERINE HYDROXYMETHYLTRANSFERASE

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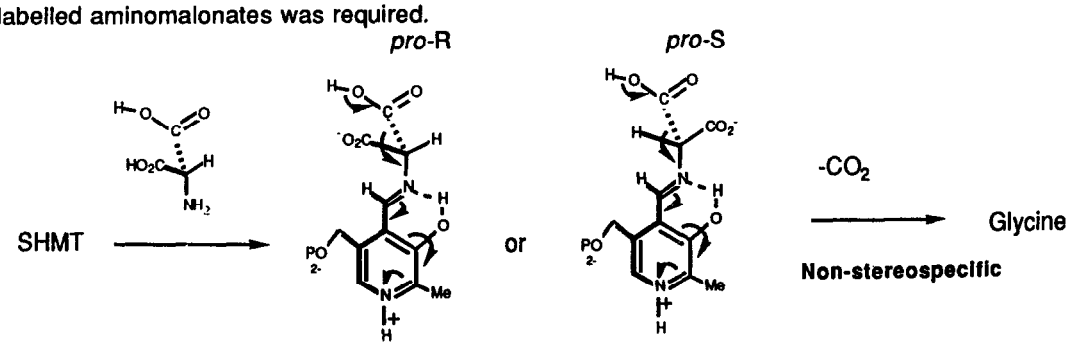
Abstract: (2R) - and (2S)-[1-^{13}C]-2-amino-2-methylmalonate, probes for the stereochemical course of the serine hydroxymethyltransferase reaction, have been synthesised from *bis*-lactim ethers derived from valine and alanine. Direct acylation of the anion with diethyl carbonate gave the N-ethoxycarbonyl derivative rather than the required ethyl ester. The ^{13}C-labelled carboxyl group was therefore introduced *via* treatment of the anion with [1-^{13}C]-acetyl chloride, followed by haloform oxidation. The resulting carboxylate salt was then esterified with methyl iodide, and the *bis*-lactim ether ring system was cleaved under acidic conditions to give the amino acid esters. Saponification and then separation by cation exchange chromatography gave the title compounds.

Aminomalonic acids have been shown to be substrates of several enzymes including serine hydroxymethyltransferase (SHMT)[1], aspartate β-decarboxylase[2] and α-dialkylamino acid transaminase[3]. In order to probe the stereochemical course and mechanism of the decarboxylation reaction catalysed by some of these enzymes, a synthesis of stereospecifically carboxyl group labelled aminomalonates was required.



Scheme 1

Syntheses of (2R)- and (2S)-[1-^{14}C]-aminomalonic acid had been reported,[2] starting from the appropriately labelled serine but, the utility of the compound in stereochemical studies is restricted because even under mild physiological conditions, the α-proton undergoes very rapid hydrogen exchange with the solvent, which leads to racemisation. Indeed, the interpretation of the reported

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non-stereospecific decarboxylation of aminomalonate by serine hydroxymethyltransferase, Scheme 1, called for special cognisance of α-proton exchange.[1] Since these complications prevent the unambiguous determination of the stereochemical courses for decarboxylation and subsequent reprotonation at C_α, the possibility of using stereospecifically labelled α-aminoalkylmalonic acids as probes, which could not undergo racemisation, was considered.

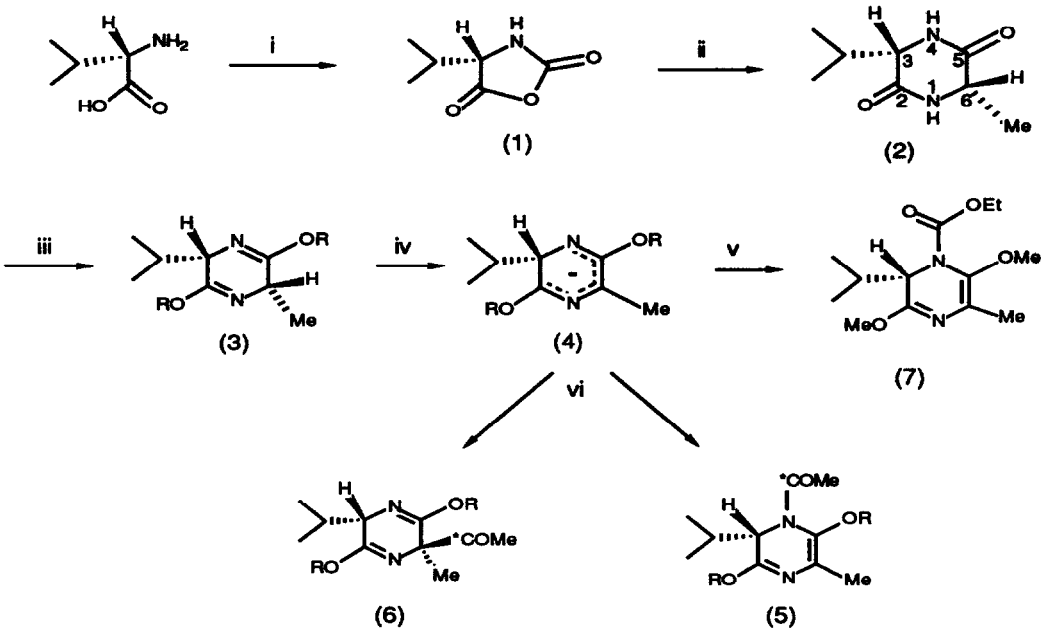
Thanassi and Fruton had reported that 2-amino-2-methylmalonic acid was an inhibitor for the decarboxylation of 2-aminomalonic acid by the cytosolic rat liver enzyme. However, the authors reported that the inhibitor was not a substrate, although under the standard conditions of the enzyme assay, and in the presence of pyridoxal phosphate, non-enzymic decarboxylation occurred.[4] Since L-α-methylserine was shown to be a slow retro-aldol substrate[5] for the enzyme, and differs from the methylmalonate only in that a carboxyl group is replaced by a hydroxymethyl group, it seemed to us as though Thanassi and Fruton's decarboxylation assay was not sensitive enough and, therefore, warranted further experimental scrutiny.

Accordingly, 2-amino-2-methylmalonic acid was prepared by the method of Bailey *et al.*[3] and was tested as a substrate for the cytosolic rabbit liver enzyme. At pH 7.5 significant enzymic decarboxylation occurred relative to control incubations containing pyridoxal phosphate but no enzyme, and alanine could be isolated. Thus, 2-amino-2-methylmalonic acid was, indeed, a substrate for SHMT,[6] albeit a slow one, and hence a synthesis of stereospecifically labelled α-aminoalkylmalonic acids was required.

The literature offers several routes to the synthesis of enantiomerically pure α-methyl serines which could be modified to our requirement. These include the hydroxymethylation of *bis*-lactim ethers,[7] the alkylation of optically active oxazolidines,[8] the use of D-galactosaldehyde as a chiral auxiliary[9], and the hydroxymethylation of methyl α-isocyanocarboxylate in the presence of a chiral (aminoalkyl) ferrocenylphosphine-gold complex[10]. Of these the *bis*-lactim ether methodology seemed most appropriate for the synthesis of 2-amino-2-methylmalonic acids.

Accordingly, the *cyclic*-diketopiperazine (**2**) was prepared from L-valine and L-alanine in 74% overall yield using a modification of the literature procedure[11] in which L-Val NCA was produced using diphosgene[12] instead of phosgene. Treatment with triethyloxonium tetrafluoroborate in dichloromethane at 20 °C for 48 h. gave the *bis*-ethyllactim ether (**3**) in 74% yield. The reagent was used in preference to the trimethyloxonium compound since it gave better yields in shorter reaction times.

Initially it was planned to introduce the [^{13}C]-labelled carboxylate group in one step through the reaction of the lithiated *bis*-lactim ether (**4**, R = Me) with either ethyl chloroformate or diethyl carbonate. Diethyl [^{13}C]-carbonate can be prepared from barium [^{13}C]-carbonate in good yield.[13] Reaction of the lithiated *bis*-lactim ether with ethyl chloroformate gave predominantly the N-acylated regioisomer (> 85 %), whereas the reaction with diethyl carbonate gave the urethane as the sole product[14].



i. $ClCO_2CCl_3$, charcoal, THF ; ii. AlaOMe.HCl, Et_3N, DCM, -80°C ;
iii. $[Et_3O]^+BF_4^-$, DCM, 48 h. or $[Me_3O]^+BF_4^-$, DCM, 72 h ; iv. *n*-BuLi, THF, -80°C ; v. $(EtO)_2CO$ or EtOCOCl ; vi. Me*COCl, 2 h, *C = ^{12}C or ^{13}C.

Scheme 2

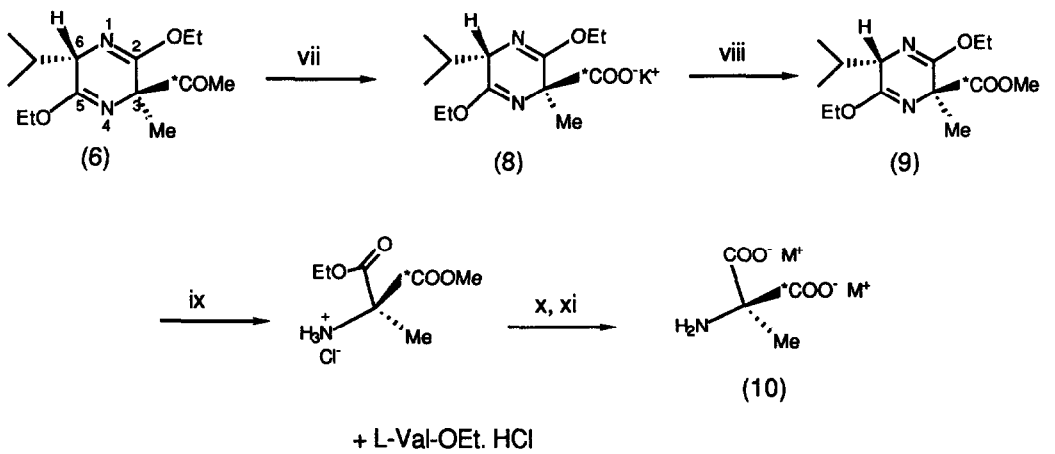
This result was surprising at the time, since N-acylation had not been reported in previous work on *bis*-lactim ethers. However, Scholkopf has recently reported a similar finding in which the reaction of methyl acetate with the lithiated *bis*-methyllactim ether (**4**, R = Me) gave the amide rather than the ketone.[15] As we were unable to modify the regioselectivity of the reaction in favour of the C-acylated product, through simple changes to the reaction conditions, alternative options were considered.

The use of the more regioselective acylating agent, methyl cyanoformate, Mander's reagent[16] was not pursued since the compound would have been difficult to prepare in ^{13}C-labelled form. However, the introduction of the carboxyl group in a masked form, as a methyl ketone which could be oxidised subsequently, seemed likely to succeed. Scholkopf had reported[15] that the reaction of acetyl chloride with the lithiated *bis*-methyllactim ether (**4**, R = Me) gave a mixture of C- and N-acylated products in the ratio *ca.* 11:1. The diastereomeric ratio of the C-acylated product, as determined by capillary g.l.c., was found to be (2S,5S):(2R, 5S) = 119:1.

In our laboratory the reaction conditions were optimised, to minimise the amount of N-acylation, such that only one regio- and diastereo- isomer (> 95% d.e.) of the acetylated compound was produced, as judged by ^1H- and ^{13}C-n.m.r. spectroscopy, Scheme 2.

In order to convert the methyl ketone (**6**) to the required carboxylate salt, haloform oxidation using bromine and sodium hydroxide as described by Vogel[17] was investigated first. The reaction produced a complex mixture of products, none of which were identifiable as the required compound. Under the more mild conditions described by Newman *et al.*[18] using potassium hypochlorite (3.5 equivalents, freshly prepared from calcium chlorite powder) the ketone was smoothly and completely converted to the required carboxylic acid salt (**8**), as judged by ^{1}H-n.m.r. spectroscopic analysis. Commercially available solutions of sodium hypochlorite were not as effective as potassium hypochlorite in the oxidation step and caused additional problems during the methyl esterification.

Attempts to neutralise the potassium salt (**8**) and extract the free acid into diethyl ether resulted in rapid spontaneous decarboxylation, hence prior protection of the carboxylic acid was required. Direct treatment of the unpurified potassium salt with methyl iodide gave the stable methyl ester (**9**) in 46% overall yield from the ketone (**6**), Scheme 3.



vii. KOCl, KOH, dioxane/H_2O ; *viii.* MeI, KOH, THF, 48 h. ; ix. 0.10 M HCl, 18 h. ; x. 2.0 M NaOH, 70°C, 15 min ; xi. Amberlite 120 H^+ ion exchange resin

Scheme 3

The esterified *bis*-lactim ether (**9**) was cleaved into its component amino acid ester hydrochlorides using typical literature conditions, 0.10 M hydrochloric acid at 20 °C for 48 h. The two amino acid ethyl ester hydrochlorides were saponified using 2.0 M NaOH, and the free acids were separated on Amberlite 120(H) cation exchange resin. These procedures gave the required 2-amino-2-methylmalonic acid (**10**) in 27% overall yield from the initial *bis*-lactim ether (**3**, R = Et).

The chiral [1-^{13}C]-2-amino-2-methylmalonates have been used to demonstrate that the

decarboxylation reaction catalysed by serine hydroxymethyltransferase is stereospecific and occurs with retention of configuration at C-2.[19]

Full experimental details on the stereochemical determinations and the kinetic properties of these substrates with various mammalian and bacterial serine hydroxymethyltransferase enzymes will be reported elsewhere.

Experimental:

Melting points were determined using an electrothermal melting point apparatus and are uncorrected. Specific rotations were determined on an Optical activity Ltd. AA-100 polarimeter using a 5 cm pathlength cell. I.r. spectra were recorded using a Perkin Elmer 298 infra-red spectrophotometer. N.m.r. spectra were recorded on a Jeol JNM-GX270 instrument. Chloroform, TMS or the sodium salt of 3-(trimethylsilyl)propionic-2,2,3,3-$^2H_4$, (TMS(Na)) were used as reference standards for ^{1}H-n.m.r. (270 MHz), and chloroform was used as a standard for ^{13}C-n.m.r. (67.9 MHz). Mass spectra were obtained using an AEI-MS30 or VG-770 spectrometer. Microanalysis facilities were provided on a service basis by University College, London.

L-Valine N-carboxyanhydride ((2S)-4-isopropyl-oxazolidine-2,5 dione),(I). L-Valine (12.9 g, 0.11 mol) and activated charcoal (0.5 g) were suspended in anhydrous THF (100 ml), and to the suspension was added trichloromethyl chloroformate (10.0 ml, 0.083 mol). The temperature of the suspension was gradually raised to 60 °C, and was maintained at 60 °C for 1 h. Excess phosgene was then removed by purging the suspension with nitrogen and the suspension was then filtered through Celite. The yellow coloured filtrate was concentrated *in vacuo* (<40 °C) and the addition of pentane (*ca.* 500 ml) gave crystals of L-valine NCA which were recrystallised twice from diethyl ether/pentane (14.32 g, 91%), m.p. 70-71°C (lit.,[12] 71°C); (Found: C, 50.36; H, 6.41; N, 9.74. Calc. for $C_6H_9NO_3$: C, 50.27; H, 6.34; N, 9.79%); $[\alpha]_D$ - 43.1° (c 2.5 , acetone), (lit.,[12] $[\alpha]_D$ - 42.8° (c 2.48 in acetone); ν_{max} ($CHCl_3$) 1815 and 1755 cm^{-1}; δ_H (C^2HCl_3) 1.05 (6H, 2d, *J* 6 Hz, 4-CH_3), 2.26 (1H, s br, 3-CH), 4.23 (1H, s, 2-CH), 7.12 (1H, s br, 2-NH).

D-Valine N-carboxy anhydride ((2R)-4-isopropyl-oxazolidine-2,5-dione). The D-antipode was prepared in a similar manner, starting from D-valine, in 93% yield and all spectral parameters were similar to those for the L-enantiomer; m.p. 69 - 70 °C; (Found: C, 50.18; H, 6.43; N, 9.8%; Calc. for $C_6H_9NO_3$: C, 50.27; H, 6.34; N, 9.79%); $[\alpha]_D$ +43.7° (c 2.5, acetone).

***cyclo*-(L-Val-L-Ala),(2).** A solution of L-Val-NCA (8.0 g, 0.056 mol) in dry THF (100 ml) was added dropwise to a stirred solution of L-alanine methyl ester hydrochloride (7.81 g, 0.056 mol) and

triethylamine (12.7 g, 0.126 mol), in dry dichloromethane (70 ml) at -80 °C. The resulting suspension was stirred for 3 h. at -70 °C and then allowed to warm to room temperature. The precipitated triethylamine hydrochloride was removed by filtration, and the filtrate was concentrated *in vacuo*. The crude residue, which contained a mixture of L-Val-L-Ala-OMe and *cyclo*-(L-Val-L-Ala), was refluxed in toluene (250 ml) for 12 h. Upon cooling, the cyclised product precipitated and this was filtered, washed with cold ether and then recrystallised from water and dried to give colourless crystals (7.82 g, 82%), m.p. 236 °C; (Found: C, 56.48; H, 8.17; N, 16.39%; Calc. for $C_8H_{14}N_2O_2$; C, 56.45; H, 8.29; N, 16.46%); *m/z* (CI - NH_3) (Found $[M + H]^+$, 171.1128. $C_8H_{15}N_2O_2$ requires 171.1134); $[\alpha]_D$ -43.2° (c 0.5, H_2O); ν_{max} (nujol) 3320 (NH), 3200-3000 br (CH), 1670 cm^{-1} (C=O); δ_H (^2H_2O; ref. H^2HO) 0.69 and 0.81 (3H, d, *J* 7 Hz, 3-CH($C\underline{H}_3$)$_2$), 1.26 (3H, d, *J* 7 Hz, 6-CH_3), 2.03 (1H, doublet of septets [dsp], *J* 3 and 7 Hz, 3-C$\underline{H}$(CH_3)$_2$), 3.77 (1H, d, *J* 3 Hz, 3-H), 3.96 (1H, q, *J* 7Hz, 6-H); δ_C (^2H_2O; ref. MeOH) 14.01 (CH_3), 16.23 (CH_3), 18.20 (CH_3), 29.97 (CH), 48.37 (CH), 57.96 (CH), 167.46 (C=O), 169.56 (C=O).

***cyclo*-(D-Val-D-Ala).** The enantiomer of (**2**) was prepared in a similar manner, using D-valine N-carboxyanhydride and D-alanine methyl ester hydrochloride as the starting materials, in 79% yield. All spectral parameters were similar to those for the enantiomer; m.p. 238 °C; (Found: C, 56.46; H, 8.29; N, 16.49%; Calc. for $C_8H_{14}N_2O_2$; C, 56.45; H, 8.29; N, 16.46%); *m/z* (CI - NH_3) (Found $[M + H]^+$, 171.1128. $C_8H_{15}N_2O_2$ requires 171.1134); $[\alpha]_D$ +42.6° (c 0.5, H_2O).

(3S,6S)-2,5-Diethoxy-3-isopropyl-6-methyl-3,6-dihydropyrazine,(3). A suspension of *cyclo*-(L-Val-L-Ala) (2.0 g, 11.0 mmol) and triethyloxonium tetrafluoroborate (6.27 g, 33.0 mmol) in anhydrous dichloromethane (200 ml) under nitrogen was vigorously stirred at room temperature for 48 h. (solution occurred after *ca.* 3 h.). Additional portions of triethyloxonium tetrafluoroborate (3.13 g, 16.5 mmol) were added after 12 and 24 h. To the resulting solution was added potassium phosphate buffer (100 ml, 100 mM, pH 7.0) and the aqueous layer was separated and extracted with dichloromethane (3 x 50 ml). The combined organic extracts were dried ($MgSO_4$), and the solution was concentrated *in vacuo* to give an oil which was purified by distillation to give (**3**) as a colourless oil (1.85 g, 74%), b.p. 64 °C/1.0 mmHg; (Found: M^+ 226.1682. $C_{12}H_{22}O_2N_2$ requires 226.1681); $[\alpha]_D$ + 58.8° (c 1.0, $CHCl_3$); ν_{max} ($CHCl_3$) 1695 cm^{-1}; δ_H (C^2HCl_3; ref. TMS) 0.75 and 1.05 (3H, d, *J* 7 Hz, 3-CH($C\underline{H}_3$)$_2$), 1.26 (6H, t, *J* 7 Hz, 2,5-$OCH_2C\underline{H}_3$), 1.36 (3H, d, *J* 7 Hz, 6-CH_3), 2.19 (1H, dsp, *J* 3 and 7 Hz, 3-C$\underline{H}$(CH_3)$_2$), 3.91 (1H, t, *J* 3 Hz, 3-H), 3.98-4.20 (5H, m, 2,5-$OC\underline{H}_2CH_3$ and 6-H) ; δ_C (C^2HCl_3) 14.25, 14.29 (2,5-$OCH_2\underline{C}H_3$), 17.35,19.49, 21.62 (all CH_3),

31.25, 51.51 (both CH), 60.30, 60.38 (2,5-O<u>C</u>H_2CH_3), 61.06 (CH), 162.60, 163.94 (O-C=N); *m/z* (EI) 226 (M^+, 17%), 183 ([M-CH$(CH_3)_2$]$^+$, 100%).

(3R,6R)-2,5-Diethoxy-3-isopropyl-6-methyl-3,6-dihydropyrazine. The (3R,6R)-enantiomer was prepared in a similar manner to (**3**), but from *cyclo*-(D-Val-D-Ala) in 76% yield and all spectral parameters were similar to those for the enantiomer. (Found: C, 63.92; H, 9.84; N, 12.30%; $C_{12}H_{22}O_2N_2$ requires C, 63.68; H, 9.80; N, 12.28 %); $[\alpha]_D$ -59.1° (c 1.0, $CHCl_3$); *m/z* (EI) (Found M^+ 226.1682. $C_{12}H_{22}N_2O_2$ requires 226.1681).

(3S,6S)-2,5-Diethoxy-6-isopropyl-3-acetyl-3-methyl-6-hydropyrazine,(6). To a pre-cooled solution of the dihydropyrazine (**3**, 2.85 g, 12.6 mmol) in anhydrous THF (25 ml) at -80 °C was added a solution of *n*-butyl lithium in hexane (5.3 ml of a 2.5 M soln, 13.8 mmol). The resulting solution was left stirring for 20 min. at -65 °C and was then cooled to -80 °C. A pre-cooled solution of acetyl chloride (1.0 g, 12.6 mmol) in THF (25 ml) at -80 °C was added dropwise and the resulting solution was stirred at -80 °C for 2 h. and then allowed to warm to room temperature. The solvent was removed *in vacuo* and the residue was dissolved in potassium phosphate buffer (25 ml, 100 mM, pH 7.0) and was then extracted with diethyl ether (3 x 25 ml). The combined organic phases were dried ($MgSO_4$) and concentrated *in vacuo* to give a yellow oil containing a mixture of C-(**6**) and N-acylated-(**5**) products. These were separated by slow column chromatography on silica (7:1 petroleum ether: ethyl acetate) to give the required C-acylated product (2.98 g, 87%), b.p. 53-54 °C at 0.1 mm Hg; $[\alpha]_D$ + 36.7 ° (c 1.0, $CHCl_3$); (Found: C, 62.69; H, 9.06; N, 10.37. Calc. for $C_{14}H_{24}N_2O_3$: C, 62.66; H, 9.01; N, 10.44%); *m/z* (CI - NH_3) (Found [M + H]$^+$ 269.1869. $C_{14}H_{25}N_2O_3$ requires 269.1865); ν_{max} ($CHCl_3$) 1735 (C=O), 1700 cm^{-1} (C=N); δ_H (C^2HCl_3; ref. TMS) 0.75 and 1.10 (3H, d, J 7 Hz, 6-CH(C<u>H</u>$_3$)$_2$), 1.24 and 1.27 (6H, 2t, J 7 Hz, 2,5-OCH$_2$C<u>H</u>$_3$), 1.44 (3H, s, 3-CH_3), 2.04 (3H, s, 3-$COCH_3$), 2.31 (1H, dsp, J 3 and 7 Hz, 6-C<u>H</u>$(CH_3)_2$), 4.04 (1H, d, J 3 Hz, 6-H), 4.06-4.20 (4H, m, 2,5-OC<u>H</u>$_2CH_3$) ; δ_C (C^2HCl_3) 14.14, 14.27 (2,5-OCH$_2$<u>C</u>H_3), 17.12,19.52, 22.86, 24.33 (all CH_3), 31.28, (CH), 60.50, 60.96 (2,5-O<u>C</u>H_2CH_3), 61.03 (CH), 161.36, 163.59 (O-C=N), 202.78 (C=O).

(3S,6S)-2,5-Diethoxy-6-isopropyl-3-[1′-^{13}C]-acetyl-3-methyl-6-hydropyrazine. This compound was prepared in a similar manner to (**6**), but using [1-^{13}C]-acetyl chloride. All spectral data was identical to that of (**6**) except, δ_H (C^2HCl_3; ref. TMS) 1.44 (3H, d, $J_{H\text{-}^{13}C}$ 4 Hz, 3-CH_3), 2.04 (3H, $J_{H\text{-}^{13}C}$ 6 Hz, 3-$COCH_3$), 4.04 ppm (1H, t, $J_{H\text{-}^{13}C}$ 2 Hz, 6-H); *m/z* (CI - NH_3)([M +H]$^+$ 270.1892; $^{12}C_{13}{}^{13}CH_{25}N_2O_3$ requires 270.1898); $[\alpha]_D$ +37.4° (c 1.0 in $CHCl_3$).

(3R,6R)-2,5-Diethoxy-6-isopropyl-3-[1′-^{13}C]-acetyl-3-methyl-6-hydropyrazine. The labelled (3R,6R)-isotopomer was prepared in a similar manner to (**6**), but starting from (3R,6R)-2,5-Diethoxy-3-Isopropyl-6-methyl-3,6-dihydropyrazine. All spectral data was identical to that of the labelled (3S,6S)-isomer. $[\alpha]_D$ - 35.5° (c 1.0, $CHCl_3$).

(3S,6S)-2,5-Diethoxy-6-isopropyl-3-carboxymethyl-3-methyl-6-hydropyrazine,(9). To a pre-cooled solution of the acylated pyrazine (**6**, 0.28 g, 1.04 mmol) in dioxane (10 ml) at 4 °C was added a solution of potassium hypochlorite (3.17 ml, 5.22 mmol, 1.65 M) and the reaction was allowed to stir at 4 °C for 1 h. and then at room temperature. The solution was extracted with diethyl ether (10 ml), and the aqueous phase was concentrated *in vacuo* to give an off-white residue (a mixture of inorganic salts and the potassium salt (**8**) of the oxidised pyrazine) which was dried *in vacuo*. δ_H (^2H_2O; ref. H^2HO) 0.72 and 1.05 (3H, d, *J* 7 Hz, 6-CH($C\underline{H}_3$)$_2$), 1.38 (6H, 2t, *J* 7 Hz, 2,5-$OCH_2C\underline{H}_3$), 1.49 (3H, s, 3-CH_3), 2.38 (1H, dsp, *J* 3 and 7 Hz, 3-C$\underline{H}$(CH_3)$_2$), 4.06-4.32 (5H, m, 2,5-$OC\underline{H}_2CH_3$ and 6-H) ; δ_C (^2H_2O; ref. MeOH) 11.75, 11.82 (2,5-$OCH_2\underline{C}H_3$), 15.06, 17.08,22.39 (all CH_3), 29.53, (CH), 58.65 (CH), 59.63, 59.80 (2,5-OCH_2CH_3), 161.42, 162.29 (O-C=N), 174.87 (C=O).

The residue was suspended in anhydrous THF (25 ml) at 0 °C and methyl iodide (1.40 g, 10 mmol) was added dropwise. The mixture was stirred vigorously for 48 h. and then the solvent was removed *in vacuo*. The residue was dissolved in water (25 ml) and the solution was extracted with diethyl ether (4 x 25 ml). The combined organic phases were dried ($MgSO_4$) and concentrated *in vacuo* to give the methyl ester as a colourless oil (0.098 g, 33%). The aqueous layer was concentrated *in vacuo* to give an off-white residue which was dried, and the esterification procedure repeated to give a further (0.038g, 13%) of the ester (**9**), (Found: C, 59.03; H, 8.61; N, 9.71; $C_{14}H_{24}N_2O_4$ requires: C, 59.13; H, 8.51; N, 9.85%); $[\alpha]_D$ +7.2° (c 1.0, $CHCl_3$); ν_{max} ($CHCl_3$) 1692 (C=O), 1683 cm^{-1} sh (C=N); δ_H (C^2HCl_3; ref. TMS) 0.71 and 1.08 (3H, d, *J* 7 Hz, 6-CH($C\underline{H}_3$)$_2$), 1.23 and 1.25 (6H, t, *J* 7 Hz, 2,5-$OCH_2C\underline{H}_3$), 1.56 (3H, s, 3-CH_3), 2.31 (1H, dsp, *J* 3 and 7 Hz, 6-C$\underline{H}$(CH_3)$_2$), 3.70 (3H, s, 3-CO_2CH_3), 4.01 (1H, d, *J* 3Hz, 6-H), 4.06-4.22 (4H, m, 2,5-$OC\underline{H}_2CH_3$) ; δ_C (^2H_2O; ref. MeOH) 14.29, 17.07 (2,5-$OCH_2\underline{C}H_3$), 19.62, 24.44 (both CH_3), 31.11 (CH), 52.78 (CH_3) 61.02 (CH), 60.99 and 61.13 (2,5-$O\underline{C}H_2CH_3$), 62.90 (C), 160.60, 163.13 (O-C=N), 171.11 (C=O); *m/z* (EI) 284 (*M*$^+$, 3.1%), 256 ([*M* - Et]$^+$, 100%)).

(3S,6S)-2,5-Diethoxy-6-isopropyl-3-[1′-^{13}C]-carboxymethyl-3-methyl-6-hydropyrazine. The labelled isotopomer was prepared in a similar manner to compound (**9**) and the spectral data differed only as a result of introducing the ^{13}C-label; *m/z* (EI) (Found: *M*$^+$ 285.1752. $^{12}C_{13}{}^{13}CH_{24}N_2O_4$ requires 285.1769); $[\alpha]_D$ -6.9 °C (c 1.0 in $CHCl_3$); selected δ_H

(C^2HCl_3; ref. TMS) 1.56 (3H, d, $J_{H\text{-}^{13}C}$ 5 Hz, 3-CH_3), 3.69 (3H, $J_{H\text{-}^{13}C}$ 4 Hz, 3-$^{13}COOCH_3$), 4.01 ppm (1H, t, $J_{H\text{-}^{13}C}$ 2 Hz, 6-H).

(3R,6R)-2,5-Diethoxy-6-isopropyl-3-[1′-^{13}C]-carboxymethyl-3-methyl-6-hydropyrazine. The R-antipode was prepared in a similar manner to (**9**), but from (3R,6R)-2,5-diethoxy-6-isopropyl-3-[1′-^{13}C]-acetyl-3-methyl-6-hydropyrazine. Spectral data differed only as a result of introducing the ^{13}C-label and was identical to that for the labelled (3S,6S)-enantiomer. $[\alpha]_D$ -7.6 ° (c 1.0 , $CHCl_3$).

2-Amino-2-methylmalonic acid, (10). A solution of the pyrazine (**9**) (1.0 g, 3.52 mmol) in 0.10 M HCl (74 ml, 7.39 mmol) and acetonitrile (500 ml) was stirred at room temperature for 18 h. The acetonitrile was removed *in vacuo* and the aqueous residue was extracted with diethyl ether (25 ml). The aqueous solution was adjusted to pH 11.0 using 2.0 M NaOH and then heated at 70 °C for 15 min. to effect saponification of the amino acid ester hydrochlorides. The resulting solution was cooled on ice, then concentrated *in vacuo* (<40 °C) to *ca.* 5 ml and the pH carefully adjusted to 7.0 using 1.0 M HCl. The free amino acids were then separated on a column of Amberlite 120(H) cation exchange resin (2.5 x 20 cm) where L-valine was retained on the column and the required compound was eluted by washing the column with water. The ninhydrin positive fractions were pooled and lyophilised. The resulting residue was recrystallised from aqueous acetone prior to storage at -30 °C. (0.32 g, 68%), m.p. 285-287 °C; (Found: C, 35.94; H, 5.34; N, 10.38. Calc. for $C_4H_7NO_4$: C, 36.09; H, 5.30; N, 10.52%); ν_{max} (nujol) 3000-2600 (OH, NH), 1730 (C=O), 1605 cm^{-1} (NH); δ_H (^2H_2O; TMS(Na)) 1.69 (3H, s, 2-CH_3); δ_C (^2H_2O; MeOH) 17.83 (3′-C), 61.94 (2-C), 169.04 (1,3-C); *m/z* (FAB - glycerol/water) 226 [*M* + H + glycerol]$^+$, 134 [*M* + H]$^+$.

(2S)-[1-^{13}C]-2-Amino-2-methylmalonic acid. This was synthesized from (3S,6S)-2,5-diethoxy-6-isopropyl-3-[1′-^{13}C]-carboxymethyl-3-methyl-6-hydropyrazine following the same procedure for the preparation of (**10**), δ_H (^2H_2O; TMS(Na)) 1.68 (3H, d, $J_{H\text{-}^{13}C}$ 4.25 Hz, 2-CH_3); *m/z* (FAB - glycerol/water) 227 [*M* + H + glycerol]$^+$, 135 [*M* + H]$^+$.

(2R)-[1-^{13}C]-2-Amino-2-methylmalonic acid. This was synthesized from (3R,6R)-2,5-diethoxy-6-isopropyl-3-[1′-^{13}C]-carboxymethyl-3-methyl-6-hydropyrazine following the same procedure for the preparation of (**10**). The product showed identical spectral data to that for the (2S)-antipode above.

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14. Spectral data for (**7**): $[\alpha]_D$ -85.3° (c 1.0, $CHCl_3$); $\nu_{max.}$ ($CHCl_3$) 2995 (C-H), 1720 (C=O), 1670 (C=N), 1635 cm^{-1} (C=C); δ_H (C^2HCl_3) 0.94 and 0.96 (3H, d, *J* 5 Hz, 6-$CH_2C\underline{H}_3$), 1.31 (3H, t, *J* 7 Hz, 1-$COOCH_2C\underline{H}_3$), 1.83 (1H, m, 6-H), 1.92 (3H, s, 3-CH_3), 3.66 and 3.76 (3H, s, 2,5-OCH_3), 4.25 (2H, q, *J* 5 Hz, 1-$CO_2C\underline{H}_2CH_3$), 4.34 ppm (1H, d, *J* 10 Hz, 6-H); δ_C (C^2HCl_3) 14.47 (3-$\underline{C}H_3$), 15.35 (1-$COOCH_2\underline{C}H_3$), 18.75 and 19.14 (6-$CH(\underline{C}H_3)_2$), 27. 36 (6-$\underline{C}H(CH_3)_2$), 53.48 (1-$CO_2\underline{C}H_2CH_3$), 59.17 and 59.94 (2,5-OCH_3), 62.44 (3-CH_3), 115.54 (3-C), 134.20 (2-C), 155.00 (1-$\underline{C}O_2CH_2CH_3$), 159.52 ppm (5-C); *m/z* (EI) 270 (M^+: 22%), 227 ([*M* - $CH(CH_3)_2$]$^+$:26 %), 183 ([*M* -$CH(CH_3)_2$ - CH_2CH_3 - CH_3]$^+$: 27 %).
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