Synthesis of (2*R*)- and (2*S*)-[1-^{13}C]-2-Amino-2-methylmalonic Acid, Probes for the Serine Hydroxymethyltransferase Reaction: Stereospecific Decarboxylation of the 2-*pro-R* Carboxy Group with the Retention of Configuration

Neil R. Thomas,[a] Verne Schirch,[b] and David Gani*[a]

[a] *Chemistry Department, The University, Southampton SO9 5NH, U.K.*
[b] *Department of Biochemistry, Virginia Commonwealth University, Richmond, Virginia, U.S.A.*

2-Amino-2-methylmalonic acid and both of its [1-^{13}C]-enantiomers were synthesised and were then used to probe the stereochemical course and mechanism of serine hydroxymethyltransferase catalysed reactions; the *pro-R* carboxy group of 2-amino-2-methylmalonic acid was decarboxylated and was replaced by a proton with retention of configuration at C-2 to give (2*R*)-alanine.

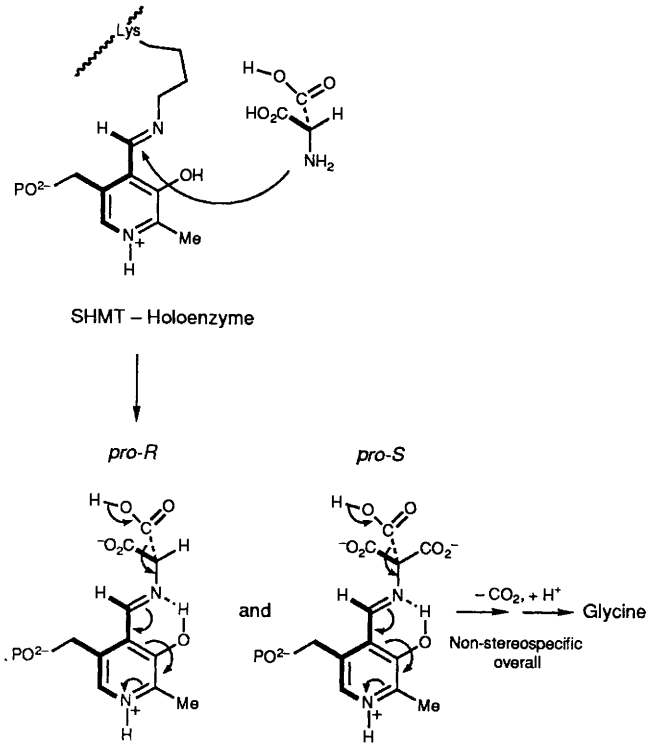
Serine hydroxymethyltransferase (SHMT) is a ubiquitous pyridoxal 5'-phosphate-dependent enzyme which catalyses the retro-aldol cleavage of L-serine to give glycine and formaldehyde.[1] The enzyme is unusual in that it shows a low regard for reaction-type specificity with α-amino acid substrates and is able to catalyse aldol/retro-aldol, transamination, and decarboxylation reactions with the appropriate substrates. In addition, the enzyme catalyses many of these reactions non-stereospecifically.[2,3]

In 1973 Palekar, Tate, and Meister showed that the decarboxylation of aminomalonic acid by SHMT in incubations conducted in tritiated water gave both (2*R*)- and (2*S*)-tritiated glycine.[4] Furthermore, studies with specific carboxy-labelled [^{14}C]aminomalonate confirmed that the enzyme decarboxylated the substrate in a nonstereospecific manner. The result was particularly interesting because it was the first, and remains the only, reported example of a non-stereospecific decarboxylation catalysed by a pyridoxal phosphate-dependent enzyme.[3]

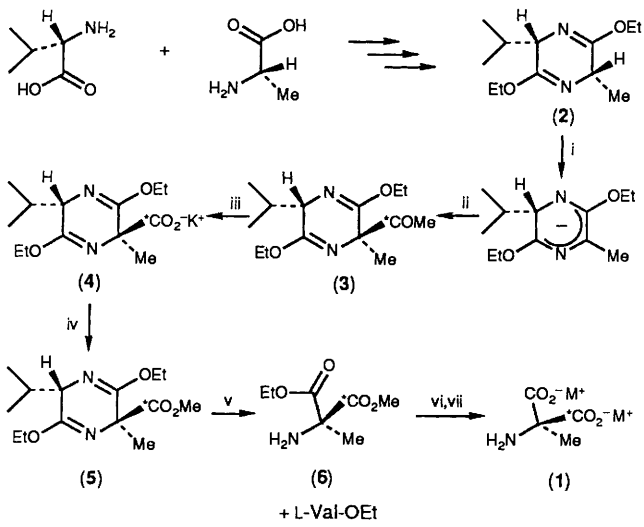
In order to explain the apparent lack of stereospecificity, two possible mechanistic courses can be invoked. In the first, the scheme originally proposed by Palekar *et al.*,[4] it can be envisaged that the aminomalonate substrate is able to bind in two conformations at the active site of the enzyme, such that each of the two carboxy groups can be positioned correctly for decarboxylation. If each conformer were equally populated and the decarboxylation and subsequent protonation steps occurred stereospecifically for each form, then the observed results would have been obtained, Scheme 1. Evidence in support of this mechanism is provided by the reports that both D-alanine[5] and L-alanine[6] and also L-phenylalanine[7] can bind to the enzyme and become involved in subsequent reactions.

On the other hand, if the enzyme was able to catalyse the racemisation of the substrate prior to decarboxylation, the same observations might be expected. However, this latter scenario, prior racemisation, may have seemed unlikely to Palekar *et al.*[4] in view of their earlier finding that another PLP-dependent enzyme, aspartate β-decarboxylase, catalyses the stereospecific decarboxylation of aminomalonate.[8] In order to unravel the mechanistic and stereochemical ambiguities of many of the reactions catalysed by serine hydroxymethyltransferase, we sought a new substrate, preferably one which could not racemise.

Thanassi and Fruton had reported that 2-amino-2-methylmalonic acid was an inhibitor of 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, nonenzymic decarboxylation occurred.[9] Since L-α-methylserine was shown to be a slow retro-aldol substrate[10] for the enzyme, and differs from the methylmalonate only in that a carboxy group is replaced by a hydroxymethyl group, it



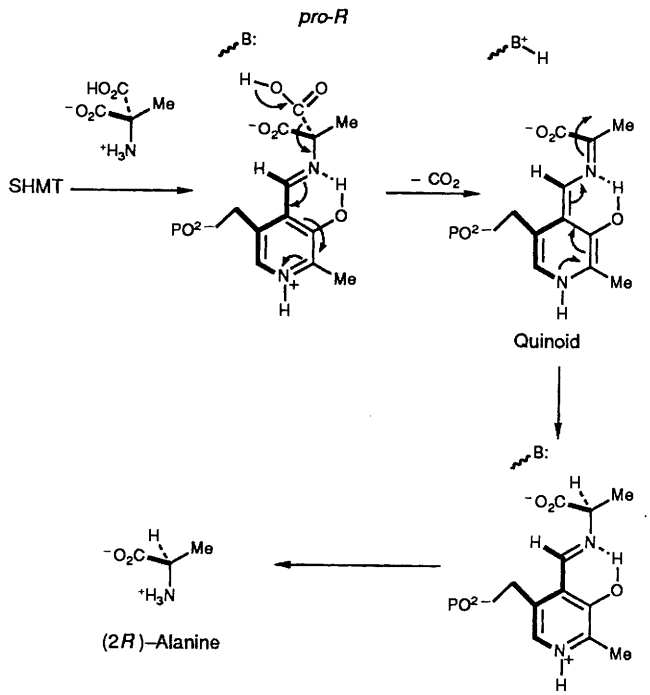
Scheme 1



Scheme 2. *Reagents and conditions:* i, Bu^{n}Li, −80 °C, tetrahydrofuran (THF); ii, Me*COCl, −80 °C, 2 h; iii, KOCl, NaOH, 4 h, aqueous dioxane; iv, MeI, THF, 12 h; v, 0.01 M HCl, 6 h; vi, 2 M NaOH, 60 °C, 10 min; vii, Amberlite IR-120(H$^+$).

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.*[11] 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 detected by TLC on cellulose after developing with ninhydrin. Thus, 2-amino-2-methylmalonic acid was,



Scheme 3

indeed, a substrate for SHMT, albeit a poor one.† By conducting the incubation on a larger scale, purification of the product was possible and sufficient quantities of alanine were obtained to allow characterisation by ^{1}H NMR spectroscopy.

The absolute stereochemistry of the alanine produced through decarboxylation of 2-amino-2-methylmalonic acid was determined by incubating aliquots of the reaction solution at various time intervals with enzyme cocktails containing either D-amino acid oxidase and lactate dehydrogenase or L-alanine dehydrogenase.[12] Analysis by these methods revealed that only (2*R*)-alanine was formed initially [note: SHMT is able to catalyse the racemisation of (2*R*)-alanine upon prolonged incubation.[13]] Hence, one stereochemical aspect of the decarboxylation had been solved, and whichever carboxy group was lost, the resulting quinoid was protonated from the *Si*-face at Cα to give (2*R*)-alanine.

In order to determine whether the cleavage of a unique carboxy group or the cleavage of both carboxy groups could give rise to a quinoid intermediate, we wished to incubate stereospecifically (carboxy group) labelled substrates with the enzyme. Accordingly, a synthesis of the enantiomers of [1-^{13}C]-2-amino-2-methylmalonic acid (**1**) was devised.

To synthesise the (*S*)-enantiomer, the Schollkopf bis-lactim ether (**2**) was prepared from L-valine and L-alanine according to the literature procedure.[14] The lithium enolate was acylated with [1-^{13}C]acetyl chloride to give the labelled acetyl bis-lactim ether (**3**). Oxidation with potassium hypochlorite in alkaline solution gave the salt (**4**) which was methylated with methyl iodide to give the methyl ester (**5**). Hydrolysis of the bis-lactim ether (**5**) under mild acidic conditions gave the 2-amino-2-methylmalonate diester (**6**) and ethyl L-valinate which were saponified with potassium hydroxide to give the chiral malonate salt (**1**) and L-valine. The 2-amino-2-methylmalonate compound was carefully purified by ion exchange chromatography and stored as the ammonium salt, Scheme 2.

† 2-Amino-2-methylmalonic acid is also a poor substrate for rabbit mitochondrial and *E. coli* serine hydroxymethyltransferase.

The (*R*)-enantiomer was prepared in a similar manner, starting from the bis-lactim ether derived from D-valine. All intermediates showed the expected spectral and analytical properties and the final products were deemed to be better than 95% enantiomerically pure as judged by analysis of the ^{1}H NMR spectra of the ester precursors (**5**).‡ Importantly, the ^{1}H NMR spectra of each of the 2-amino-methylmalonates indicated that no alanine was present after the purification.

The labelled chiral aminomalonates were each incubated with SHMT and pyridoxal phosphate at pH 7.5 and the resulting alanines were isolated. Examination of the products by ^{1}H and ^{13}C NMR spectroscopy indicated that the (2*R*)-2-amino-2-methylmalonate gave unlabelled alanine (δ_H 1.21, d, $J_{H\text{-}2,3}$ 6.8 Hz, in D_2O at pH 10) while the (2*S*)-enantiomer gave [1-^{13}C]alanine (δ_H 1.21, dd, $J_{H\text{-}2,3}$ 6.8, $J_{H\text{-}3,C\text{-}1}$ 4 Hz, in D_2O at pH 10). Thus, the *pro-R* carboxy group of the substrate was lost during the decarboxylation. Together with the finding that D-alanine is the decarboxylation product, it is evident that replacement of the *pro-R* carboxy group by a proton occurs with retention of configuration, Scheme 3.

Interestingly, the *pro-R* carboxy group of 2-amino-2-methylmalonic acid is expected to occupy the same position as the hydroxymethyl group of the physiological substrate L-serine at the active site of the enzyme, in complete accord with the Dunathan postulate.[15] These results indicate that Palekar, Tate, and Meister's results[4] are best rationalised in terms of the prior enzymic racemisation of 2-aminomalonic acid.

We thank the S.E.R.C. for a studentship to N. R. T. and the National Institute of Health for grant GM28143 to V. S.

Received, 27th November 1989; Com. 9/05064G

‡ The major impurity after the acylation step (step ii, scheme 2) is the N^1-acylated product and not C^5-acylated epimer of the desired compounds (**3**). Diastereoisomers of compound (**5**) are not detected by ^{1}H NMR spectroscopy.

References

1 R. L. Blakley, in 'Frontiers of Biology,' vol. 13, eds. A. Neuberger and E. D. Tatum, North-Holland, Amsterdam, 1969, p. 190.
2 L. Schirch, *Adv. Enzymol.*, 1982, **53**, 83.
3 D. Gani, 'Pyridoxal Dependent Systems,' in 'Comprehensive Medicinal Chemistry,' ed. P. G. Sammes, Pergamon Press, Oxford, 1989.
4 A. G. Palekar, S. S. Tate, and A. Meister, *J. Biol. Chem.*, 1973, **248**, 1158.
5 L. Schirch and W. T. Jenkins, *J. Biol. Chem.*, 1964, **239**, 3797.
6 J. Hansen and L. Davis, *Biochim. Biophys. Acta*, 1979, **568**, 321.
7 R. J. Ulevitch and R. G. Kallen, *Biochemistry*, 1977, **16**, 5350.
8 A. G. Palekar, S. S. Tate, and A. Meister, *Biochemistry*, 1971, **10**, 2180.
9 J. W. Thanassi and J. S. Fruton, *Biochemistry*, 1962, **1**, 975.
10 L. G. Schirch and M. Mason, *J. Biol. Chem.*, 1963, **238**, 1032.
11 G. B. Bailey, O. Chotamangsa, and K. Vuttivej, *Biochemistry*, 1970, **9**, 3243.
12 Modification of assay for alanine racemase activity; see B. Badet, D. Roise, and C. T. Walsh, *Biochemistry*, 1984, **23**, 5188, and ref. 13.
13 K. Shostack and V. Schirch, *Biochemistry*, 1988, **27**, 8007.
14 U. Schollkopf, U. Groth, K-O. Westphalen, and C. Deng, *Synthesis*, 1981, 969; U. Schollkopf, K-O. Westphalen, J. Schroder, and K. Korn, *Liebigs. Ann. Chem.*, 1988, 781.
15 H. C. Dunathan, *Proc. Natl. Acad. Sci. USA*, 1966, **55**, 713.