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Stereochemical aspects of the metabolism of L- and D-amino acids

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Abstract. Syntheses of stereospecifically labelled samples of L- and D-amino acids and of enzyme inhibitors are reviewed, together with a discussion of the use of these compounds in elucidating the mechanism of action of enzymes involved in amino acid metabolism.

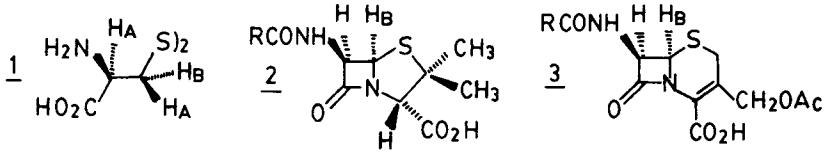
Keywords. Amino acids; stereochemistry; isotopes; metabolism; enzymes.

1. Introduction

L-Amino acids are important in all forms of life and a study of the biological reactions undergone by these compounds can be useful in medicine. D-Amino acids tend to be less common than L-amino acids but occur widely in bacteria. Enzymes which metabolise D-amino acids may, therefore, be important as targets for antibacterial drugs and an understanding of the mechanism of action of these enzymes is of importance for the design of such drugs.

Work in my research group on amino acid metabolism began in the 1970s with our interest in the role of L-cystine 1 in the biosynthesis of the β-lactam antibiotics penicillin 2 and cephalosporin 3. We were able to develop a synthesis of stereospecifically tritiated samples of L-cystine 1 $H_A = {}^3H$ and 1 $H_B = {}^3H$ (Morecombe and Young 1975; Young *et al* 1977) which we used to show that the cyclisation resulting in the β-lactam ring in penicillins 2 (Morecombe and Young 1975; Young *et al* 1977) and cephalosporins 3 (Huddleston *et al* 1978) occurred in nature with retention of stereochemistry.

The synthesis of the labelled samples of L-cystine was specific for this amino acid and was extremely time consuming so that, when we became interested in amino acid metabolism in general, a more general and facile synthesis was indicated. We



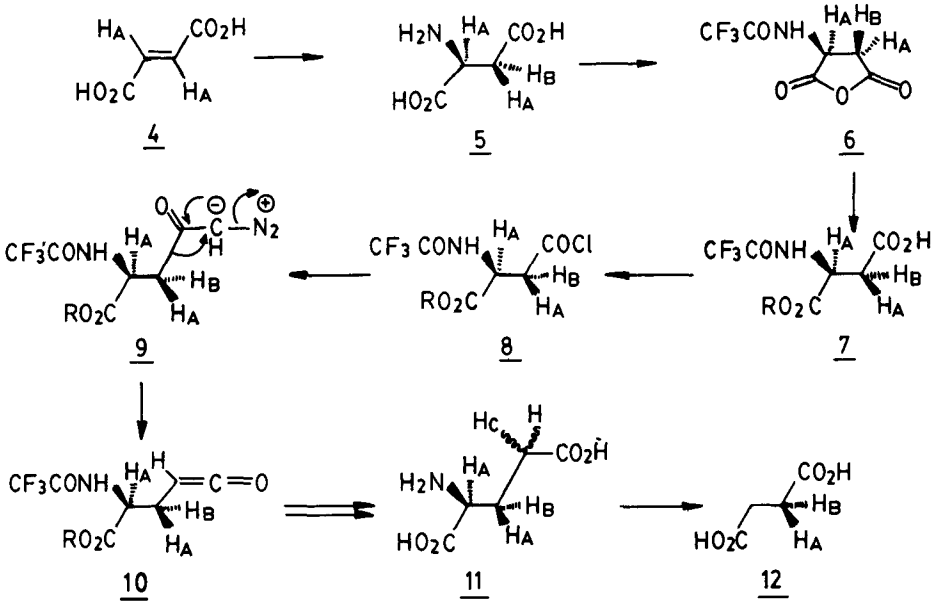
have achieved this using two chemico-enzymatic routes, one for L-amino acids and the other for D-amino acids and these methods and the use of the resultant labelled amino acids in elucidating the stereochemistry of their metabolic reactions are the subject of the present article.

2. L-Glutamic acid and L-proline

Our first synthetic target was L-glutamic acid (11) labelled stereospecifically at C-3 with deuterium. Because of our interest in the overall stereochemistry of the reaction catalysed by the enzyme glutamate mutase (see § 3) we also required the synthesis to afford samples of L-glutamic acid labelled stereospecifically at C-3 with deuterium and non-stereospecifically at C-4 with tritium.

We chose as the starting point for our synthesis the amination of fumaric acid 4 by the enzyme aspartase (EC 4·3·1·1). This was known to yield aspartic acid 5 by anti-addition of ammonia (Young 1978). This enzyme is commercially available and we were able to use it to prepare large amount of (2*S*,3*R*)[3-$^2H_1$)-aspartic acid 5 $H_B = {}^2H$ from fumaric acid 4 and $N^2H_4O^2H$ and of (2*S*,3*S*)[2,3-$^2H_2$]-aspartic acid 5 $H_A = {}^2H$ from [2,3-$^2H_2$]-fumaric acid 4 $H_A = {}^2H$ and NH_4OH (Field and Young 1979, 1983). More recently we have used immobilised cells of *Escherischia coli* instead of aspartase and have obtained the labelled samples of aspartic acid in higher yield and improved isotopic purity (Dieterich and Young 1993)

Having obtained large quantities of stereospecifically labelled aspartic acid 5 by the use of an enzyme, the remaining problems in the synthesis (Field and Young 1979, 1983) were purely chemical and are outlined in scheme 1. Homologation of the β-carboxyl group required that the α-carboxyl be protected, and we were able to achieve this by formation of the trifluoroacetylanhydrides 6 using trifluoroacetic anhydride. Reaction with alcohols was reasonably regiospecific giving predominantly the α-esters 7 which could be purified. The labelled esters 7 were converted to the diazoketones 9 *via* the acid chlorides 8. The next stage was a photochemical Wolff rearrangement which should proceed *via* the ketene 10 so that, in ^3H_2O, this would be quenched to give protected glutamic acids with stereospecific deuterium labelling



Scheme 1.

at C-3 and non-stereospecific tritium labelling at C-4. The reaction was carried out separately in the presence of ^3H_2O and of unlabelled water and the products were deprotected to yield the appropriate labelled samples of L-glutamic acid 11.

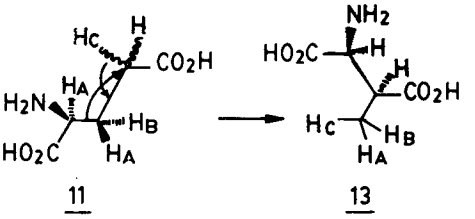
The ^1H NMR spectra of the deuterated samples of L-glutamic acid indicated that the Wolff rearrangement had been stereospecific. Although Wolff rearrangements are expected to occur with retention of stereochemistry at the migrating centre, this was the first example of such a rearrangement where the migrating group was a *primary chiral centre*. It was, therefore, necessary to confirm the absolute stereochemistry at C-3 and this was achieved by degradation of the deuteriated L-glutamic acids, 11 $H_A = {}^2H$ and 11 $H_B = {}^2H$ to the corresponding samples of labelled succinic acid 12 and comparing their ORD and CD spectra with those of authentic samples.

We have recently achieved a synthesis of samples of (2*S*, 3*S*) [3-$^2H_1$] and (2*S*, 3*R*) [2,3-$^2H_2$]-proline by controlled hydrolysis of the trifluoroacetamide group in the diazoketones 9 so that the aminoketones corresponding to 10 were trapped as pyroglutamate derivaties. These were then readily converted to the corresponding samples of proline (Dieterich and Young 1993).

3. Glutamate mutase and showdomycin biosynthesis

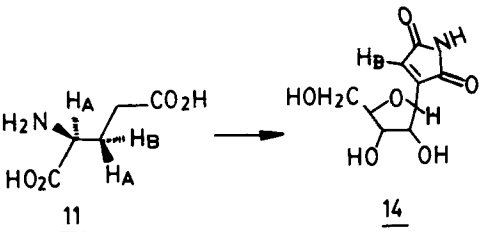
Our original reason for preparing samples of glutamic acid labelled stereospecifically at C-3 with deuterium and non-stereospecifically at C-4 with tritium was to investigate the stereochemistry of the rearrangement shown in scheme 2 which is catalysed by the enzyme glutamate mutase (EC 5.4.99.1). When the tritium migrates, then a methyl group will be created, the chirality of which can be assessed. We had purified the relevant enzyme from *Clostridium tetanomorphum* when it was reported (Hartrampf and Buckel 1984) that the rearrangement gives a racemic methyl group at the migrating terminus.

We were able to use our samples of stereospecifically deuteriated glutamic acid, 11 $H_A = {}^2H$ and 11 $H_B = {}^2H$ to assess the stereochemistry of hydrogen loss in formation of the maleimide ring in the biosynthesis of the nucleoside antibiotic showdomycin 14, shown in scheme 3. When the labelled samples of L-glutamate were



11 13

Scheme 2.



11 14

Scheme 3.

separately adminstered to growing liquid cultures of *Streptomyces showdoensis*, then only the product from feeding (2*S*, 3*S*) [3-$^2H_1$]-glutamic acid, 11 $H_A = {}^2H$ showed an absorption in the ^{2}H-NMR spectrum corresponding to the vinyl hydrogen (Buchanan *et al* 1984). Thus the antibiotic is formed in nature with loss of the 3-pro-*R* hydrogen and retention of the *3-pro-S* hydrogen of L-glutamic acid.

4. L-Serine and 2-aminoethanol

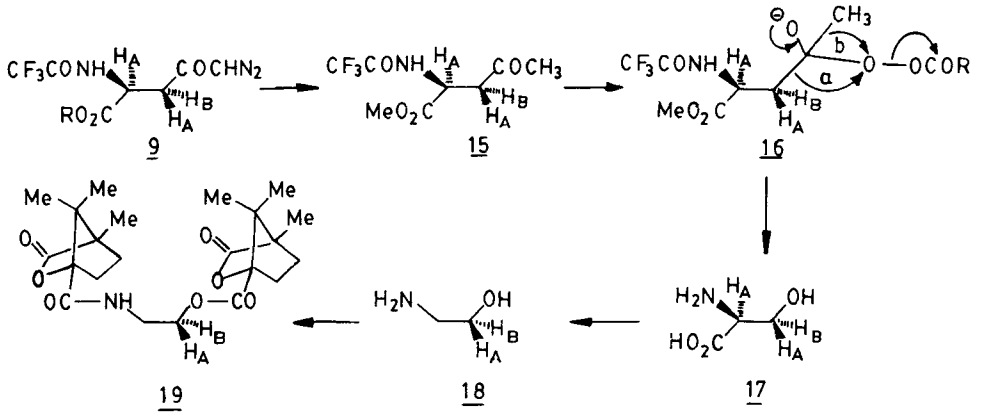
The amino acid L-serine 17 is involved in a plethora of metabolic processes and so we have prepared stereospecifically labelled samples of this amino acid by the modification outlined in scheme 4 of our synthesis of L-glutamic acid in scheme 1 (Gani and Young 1982, 1983b).

The labelled diazoketones 9 R = Me, $H_A = {}^2H$ and 9 R = Me, $H_B = {}^2H$ were reduced with HI to the corresponding methyl ketones 15 without loss of label. Baeyer–Villiger reaction using pertrifluoroacetic acid then gave the desired samples of labelled L-serine, 17 $H_A = {}^2H$ and 17 $H_B = {}^2H$ after deprotection. Although we obtained sufficient material for our metabolic studies, the yield in the Baeyer–Villiger reaction was low due to migration of the methyl group (b in 16) competing with the desired rearrangement (a in 16).

Because of our interest in a second vitamin B_{12} mediated rearrangement catalysed by the enzyme ethanolamine ammonia-lyase (see § 5) we decided to investigate the decarboxylation of serine 17 to 2-aminoethanol 18. This was achieved (Gani and Young 1982; Gani *et al* 1983b) by heating the labelled samples of L-serine with *p*-methoxyacetophenone and hydrolysis of the product. Since the labelled samples of 2-aminoethanol were enantiomers, it was not possible to assess chiral purity directly by NMR methods. However, when these were converted to the diastereoisomeric dicamphanoates 19, the ^{1}H NMR spectra indicated that decarboxylation had occurred without affecting the chirally labelled centre.

5. Metabolic reactions of L-serine and 2-aminoethanol

Given our early interest in the biosynthesis of the β-lactam ring in penicillins and cephalosporins, and the fact that the antibiotic nocardicin A 20 was derived in nature



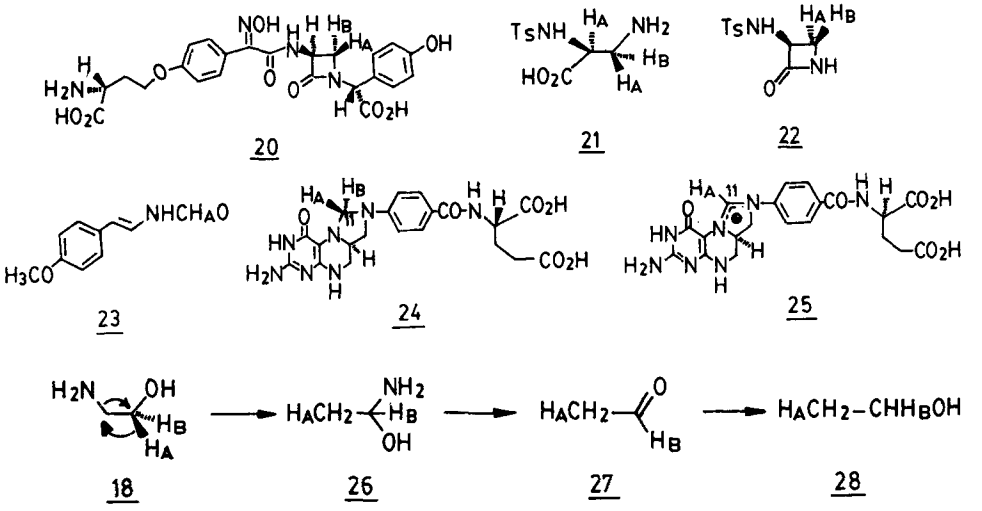
Scheme 4.

from L-serine, one of the first uses for our labelled samples of L-serine 17 was to assess the stereochemistry of β-lactam ring closure in the biosynthesis of nocardicin A. With this end in view, we used our stereospecifically labelled samples of aspartic acid 5 to synthesise the diamino-acid derivatives 21 $H_A = {}^2H$ and 21 $H_B = {}^2H$ by Hofmann rearrangement of the corresponding amides. These were converted to the labelled β-lactams 22 so that an NMR assay for the two protons H_A and H_B in the antibiotic 20 might be put on a firm footing (Gani and Young 1983b). At this point, however, the cyclisation was shown by another group (Townsend and Brown 1982; Townsend *et al* 1983) to occur with overall inversion of stereochemistry at C-3 of L-serine.

The biosynthesis of the metabolite tuberin 23 involves a tetrahydrofolic acid mediated one-carbon transfer to yield the N-formyl group. Carbon C_{11} of compound 25 is involved in this transfer and it is itself derived by the action of a dehydrogenase on compound 24. Carbon C_{11} in compound 24 is, in turn, derived from C-3 of L-serine and so we were able to use the biosynthesis of tuberin 23 as a 'black box' with which to verify the stereochemical outcome of the dehydrogenase step 24 → 25. Feeding the labelled samples of L-serine 17 $H_A = {}^2H$ and 17 $H_B = {}^2H$ gave samples of tuberin 23 in which the deuterium in the (3*S*) position was preferentially retained in the N-formyl group. This indicated, when taken with other work, that the dehydrogenase removed the *11-pro-R* hydrogen preferentially in compound 23 (Cable *et al* 1987).

Although not directly relevant to L-serine metabolism, we have used our samples of labelled L-serine 17 to assess the stereochemical outcome of the catabolism of the anti-cancer drug 5-fluorouracil (Gani and Young 1983a, 1985) and the samples of labelled L-aspartic acid 5 to assess the stereochemical outcome of the catabolism of the RNA base uracil (Gani and Young 1983a, 1985).

Having samples of stereospecifically labelled 2-aminoethanol 18 at our disposal, we were in a position to investigate the stereochemistry of the rearrangement 18 → 26 → 27 shown in scheme 5, mediated by coenzyme B_{12} and catalyzed by the enzyme ethanolamine ammonia-lyase. (EC 4.3.1.7). An experiment was conducted in which the labelled samples of ethanolamine 18 $H_A = {}^2H$ and 18 $H_B = {}^2H$ were



Scheme 5.

incubated with a mixture of this enzyme and the enzyme yeast alcohol dehydrogenase (EC 1.1.1.1) and NADH so that the acetaldehyde 27 formed was immediately reduced to ethanol (Gani *et al* 1982, 1983b). Using the ultra-violet spectral change on consumption of NADH, we were able to show that an isotope effect was manifest when the (1*S*)-hydrogen was replaced with deuterium. Further, ^2H NMR spectra of a derivative of the resultant ethanol showed, that the *1-pro-S* hydrogen had migrated to become part of the methyl group of the acetaldehyde.

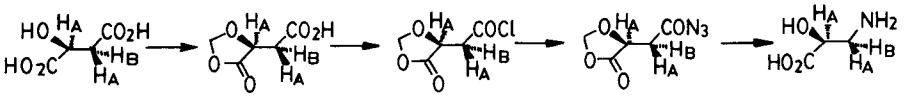
6. Synthesis of stereospecifically labelled D-amino acids

When we turned our attention to the metabolism of D-amino acids, we decided to attempt to find a synthesis of the labelled substrates which would be more versatile than that which we had used to prepare the L-isomers. Labelled aziridines such as 36 appealed to us as synthetic intermediates, as reaction with nucleophiles by an S_N2 process should lead to a wide variety of stereospecifically labelled D-amino acids.

The labelled malates 29 $H_A = {}^2H$ and 29 and $H_B = {}^2H$ were chosen as the starting compounds for the synthesis as they should be available by action of the enzyme fumarase (EC 4.2.1.2) on the fumaric acids 4 and 4 $H_A = {}^2H$ (Young 1978). This proved to be the case, although we now find it more convenient to access the labelled samples of malic acid by reaction of the labelled aspartic acids 5 $H_A = {}^2H$ and 5 $H_B = {}^2H$ with nitrous acid. The synthesis was then continued as outlined in scheme 6 (Axelsson *et al* 1991, 1994). Protection of the labelled samples of malic acid 29 was accomplished by formation of the formaldehyde adducts 30 which were converted to the azides 32 *via* the acid chlorides 31. Curtius rearrangement then proceeded with retention of stereochemistry at the primary migrating centre to give labelled samples of isoserine 33. These were converted to the methyl esters 34, tritylated and cyclised to yield the aziridines 36 as in scheme 7. Reaction of the aziridines 36 with perchloric acid gave the desired labelled samples of D-serine 37 $H_A = {}^2H$ and 37 $H_B = {}^2H$ by nucleophilic substitution with inversion, followed by deprotection. Since we had already prepared stereospecifically labelled samples of L-serine 17 (see § 4), comparison of specific rotation and ^1H NMR spectra verified the stereochemistry of labelling and showed that the assumptions that we had made on the setereospecificity of the various reactions in the synthesis had been correct.

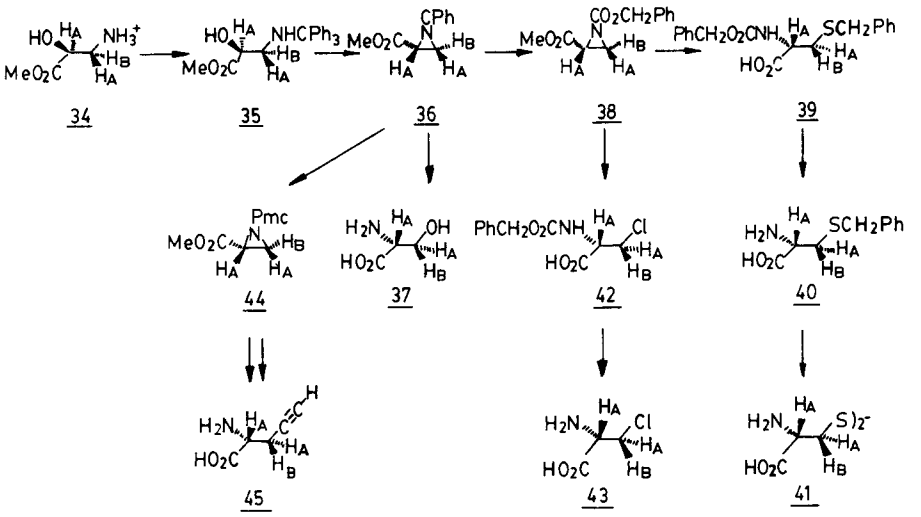
To prepare labelled samples of D-cystine 41, we needed to replace the trityl group of the aziridines 36 with a more electron withdrawing group. This was achieved by deprotection using trifluoroacetic acid and preparation of the urethanes 38 *in situ*. Reaction of these with benzylmercaptan and BF_3 gave the labelled protected D-amino acids 39 which were deprotected in two stages to yield labelled samples of D-cystine 41.

Labelled samples of the enzyme inhibitor D-β-chloroalanine 43 were also prepared by reaction of the urethanes 38 with $TiCl_4$ and deprotection.

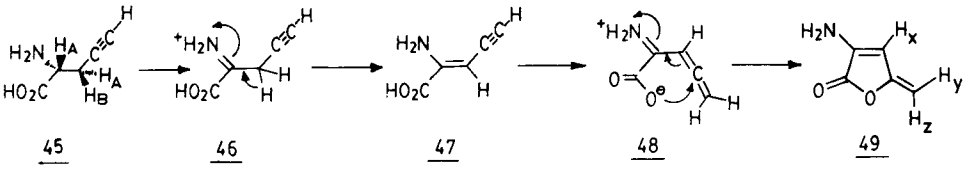


29 30 31 32 33

Scheme 6.



Scheme 7.



Scheme 8.

It has always been difficult to achieve clean nucleophilic substitution of 2-carboxy-aziridines with carbon nucleophiles but recently we have been successful in overcoming this problem by conversion of the tritylaziridines 36 to the 2,2,5,7,8- pentamethyl-chromanyl derivatives 44, saponification to the free acids and reaction with lithium trimethylsilylacetylide to give, on deprotection, labelled samples of the enzyme inhibitor/substrate D-propargylglycine 45 $H_A = {}^2H$ and 45 $H_B = {}^2H$ (Church and Young 1994).

7. Metabolism of D-amino acids and related compounds

We are currently studying the involvement of D-β-chloroalanine 42 and the O-sulphate of D-serine as enzyme inhibitors and are using our samples of D-cyst(e)ine in further studies. It is therefore too early to report on this work. We have, however, just completed an investigation of the role of D-proparylglycine 45 as a substrate for D-amino acid oxidase (Church and Young 1994).

This inhibitor/substrate is converted by D-amino acid oxidase (EC 1.4.3.3) to the lactone 49 in a process which involves loss of one of the hydrogens at C-3. The mechanism outlined in scheme 8 has been suggested to account for this reaction. We have incubated our samples of labelled propargylglycine 45 $H_A = {}^2H$ and 45 $H_B = {}^2H$ separately with D-amino acid oxidase and have obtained two samples of the lactone 49 each of which had ≈ 80–90% deuterium at H_X. This was in line with the operation of an isotope effect rather than with stereospecific reaction and so we would conclude that, after the first oxidation step to yield 46, the process is non-enzyme catalysed.

8. Conclusions

In this article I have summarised our ongoing work in using stereospecifically labelled amino acids to unravel the complexities of enzyme catalysed reactions. This is currently mainly involved with enzymes metabolising D-amino acids and it will be of some interest in future to compare the stereochemical consequences of reactions of two different enzymes which catalyse the same reaction, one using a D-amino acid as substrate and the other using an L-amino acid. The use of stereospecifically labelled inhibitors may be important in presenting an idea of what is happening at intermediate stages in these enzyme catalysed reactions.

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