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Use of inhibitors to study reactions catalyzed by enzymes requiring pyridoxal phosphate as coenzyme*

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Abstract: The stereochemistry of a variety of pyridoxal phosphate-mediated enzymic reactions has been studied using enzyme inhibitors that are stereospecifically labeled in the β-position with deuterium. A versatile synthesis has been developed to prepare a wide variety of stereospecifically labeled D- and L-amino acids and inhibitors. Investigation of the "turnover" of β-chloro-D-alanine and D- and L-serine-O-sulfate by D-amino acid aminotransferase and L-aspartate aminotransferase respectively has shown that reaction within the active site of the former enzyme occurs with retention of stereochemistry. Although L-aspartate aminotransferase is an enzyme of the α-family, when it was incubated with β-chloro-L-alanine in the presence of 2-mercaptoethanol, β-substitution occurred. This was shown to involve retention of stereochemistry, an outcome typical of reactions catalyzed by enzymes of the β-family that have little or no homology with enzymes of the α-family. Formation of the "Schnackerz intermediate" has been studied as has the D-amino acid oxidase catalyzed reaction of the naturally occurring inhibitor D-propargylglycine.

INTRODUCTION

Our interest in the metabolism of amino acids began several years ago when we investigated the ring closure step in the biosynthesis of penicillins and cephalosporins [1]. We subsequently investigated several other metabolic reactions involving L-amino acids [2]. Theories [3–5] to generalize and explain the chemical basis for the widely differing reactions involving amino acids catalyzed by enzymes requiring the coenzyme pyridoxal phosphate (PLP) were proposed at an early date and have largely been confirmed by subsequent events. Dunathan [6] proposed that the family of PLP-dependent enzymes evolved from a common ancestor protein but analysis of primary sequences has classified these into three groups of homologous proteins, the α, β and γ families [7]. Comparison of homology (Z values) indicates that the α- and γ-families may be distantly related but the β-family is not significantly related to either of these families. X-ray crystal structures [8,9] of a few PLP-mediated enzymes are available and these and site-specific mutagenesis studies have led to a greater understanding of the reactions.

As D-amino acids are rare in mammals but occur commonly in bacteria, the enzymes metabolizing them are potential targets for antibacterial drugs. Further, there are enzymes that catalyze reactions that seem to be identical in every respect except for the α-stereochemistry of the substrate. We therefore felt that investigation of the stereochemical consequences at other centers in the reaction might reveal

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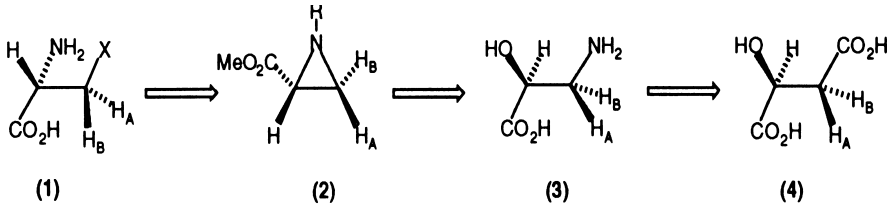
some interesting features. The stereochemistry of reactions of amino acids has been much studied over the years [10] and it has been shown that there are stereochemical differences in protonation at the 4´-position of the PLP intermediate as well as at the 2-position when D-amino acid aminotransferase and the L-aminotransferases are compared [11]. Our own interest lay in the stereochemistry of reactions at the β-center of the substrate, and since inhibitors of the relevant enzymes might not only be potential antibacterial compounds but also might allow us to trap stereospecifically labeled intermediates in the enzymic process, we decided to prepare labeled inhibitors and to study their interaction with enzymes catalysing reactions of both D- and L-amino acids.

A GENERAL SYNTHESIS OF D-AMINO ACIDS STEREOSPECIFICALLY LABELED IN THE BETA-POSITION

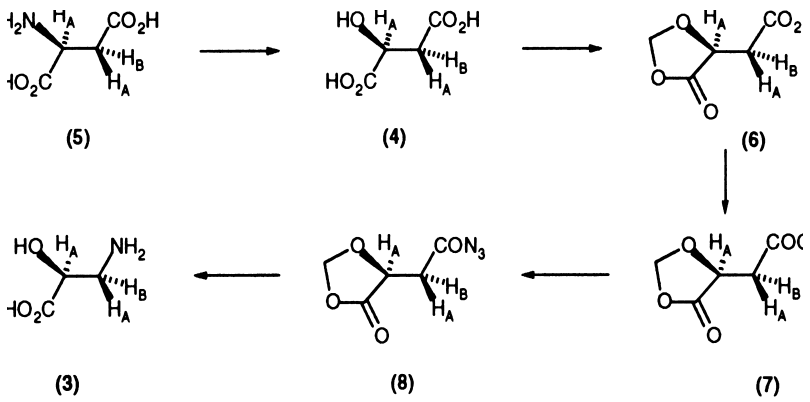
Although we had devised several syntheses of L-amino acids which were stereospecifically labeled in the β-position, these were not fully versatile. We therefore developed a more general synthesis of stereospecifically labeled samples of both L- and D-amino acids. Our retrosynthetic analysis for the D-series is shown in Scheme 1. We reasoned that, if we could synthesize a suitably labeled aziridine **(2)**, it would react with a variety of nucleophiles with inversion of configuration at the labeled β-center and allow us to prepare a variety of labeled D-amino acids **(1)** at will. To access the aziridines **(2)**, we realized that if stereospecifically labeled samples of isoserine **(3)** could be prepared, they might be cyclized with inversion of stereochemistry at the α-center yielding D-amino acid stereochemistry. The labeled samples of isoserine (**3**) were to be prepared by Curtius or Hofmann rearrangement methods using stereospecifically labeled samples of malic acid **(4)** which we were confident we could prepare using enzymatic methods.



Scheme 1

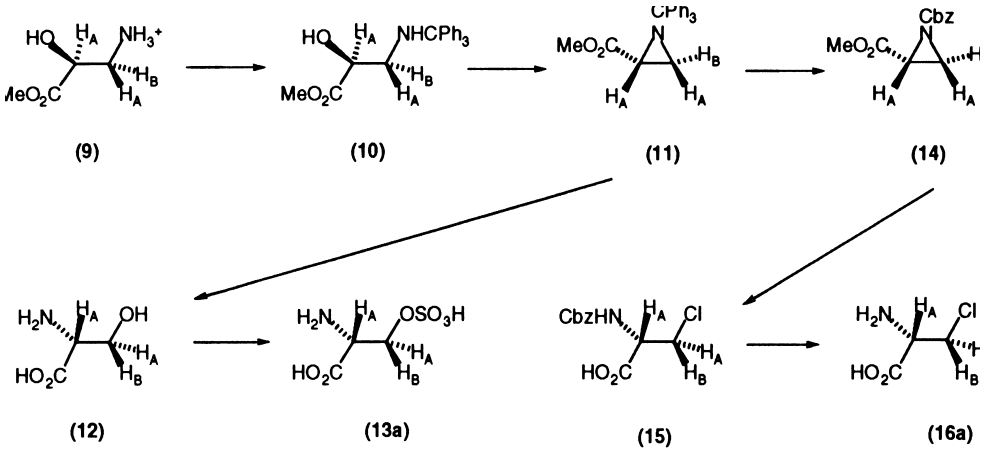
Initially we prepared samples of the stereospecifically labeled malic acids **(4, H_A = ^{2}H)** and **(4, H_B = ^{2}H)** by *anti* addition of water to the double bond of [2,3-$^2H_2$]-fumaric acid and of ^2H_2O to fumaric acid using the enzyme fumarase (EC 4.2.1.2) [12]. We later found it more efficient to prepare the labeled samples of malic acid (**4**) by nitrosation of the labeled aspartic acids **(5)** which were prepared by *anti* addition of ammonia to fumarate using the enzyme aspartase (EC 4.3.1.1) [12]. Protection of the α-carboxyl group of malic acid was achieved by conversion to the dioxolidinones **(6)** as in Scheme 2, thus allowing the β-carboxyl groups to be converted to the acylazides **(8)** *via* the acid chlorides (**7**). Curtius rearrangement was then effected to yield the desired samples of isoserine **(3, H_A = ^{2}H)** and **(3, H_B = ^{2}H)**, assuming retention of stereochemistry at the migrating primary labeled carbon atom which is usual in such rearrangements with a chiral migrating group.

The labeled samples of isoserine **(3)** were converted to the methyl esters **(9)** which were tritylated and converted to the aziridines **(11)** as in Scheme 3. The ring closure reaction was predicted to proceed with inversion of stereochemistry to give the configuration of a D-amino acid at the α-center. The aziridines **(11)** were then converted to the labeled D-serines **(12, H_A = ^{2}H)** and **(12, H_B = ^{2}H)** by reaction with perchloric acid.



Scheme 2

Stereochemistry was confirmed at the α-center by specific rotation and at the labeled β-center by comparison of the ^{1}H-NMR spectra with those of samples of stereospecifically labeled L-serine which we had prepared by an independent synthesis [13]. Our assumptions on the stereospecificity of the Curtius rearrangement step, the ring closure step and the nucleophilic substitution step had therefore been verified. The samples of D-serine (**12**) were readily converted to the corresponding samples of the labeled enzyme inhibitor D-serine-O-sulfate (**13**) (Scheme 3).



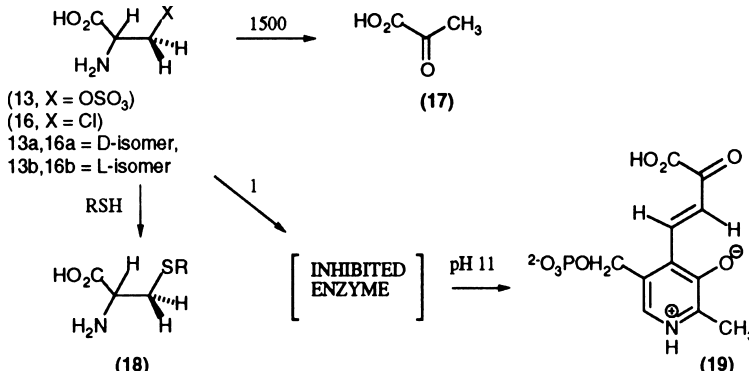
Scheme 3

The trityl aziridines (**11**) proved to be insufficiently electrophilic for preparation of a larger variety of D-amino acids by nucleophilic substitution and so the nitrogen was substituted with an electron withdrawing group. This was achieved by removal of the trityl group with CF_3CO_2H and reaction of the resultant unstable unprotected aziridine with benzyl chloroformate under Schotten–Baumann conditions. The resultant deuteriated aziridines (**14**) could then be converted to the chlorides (**16**) by reaction with $TiCl_4$ and deprotection using sulfuric acid.

ENZYME STUDIES 1

D- and L-Serine-O-sulfates (**13**) and D-and L-β-chloroalanines (**16**) are known to be inhibitor/substrates of D- and L-amino acid aminotransferases and so, having samples of the compounds of the D-series stereospecifically labeled in the β-position, we were in a position to study their turnover with D-amino

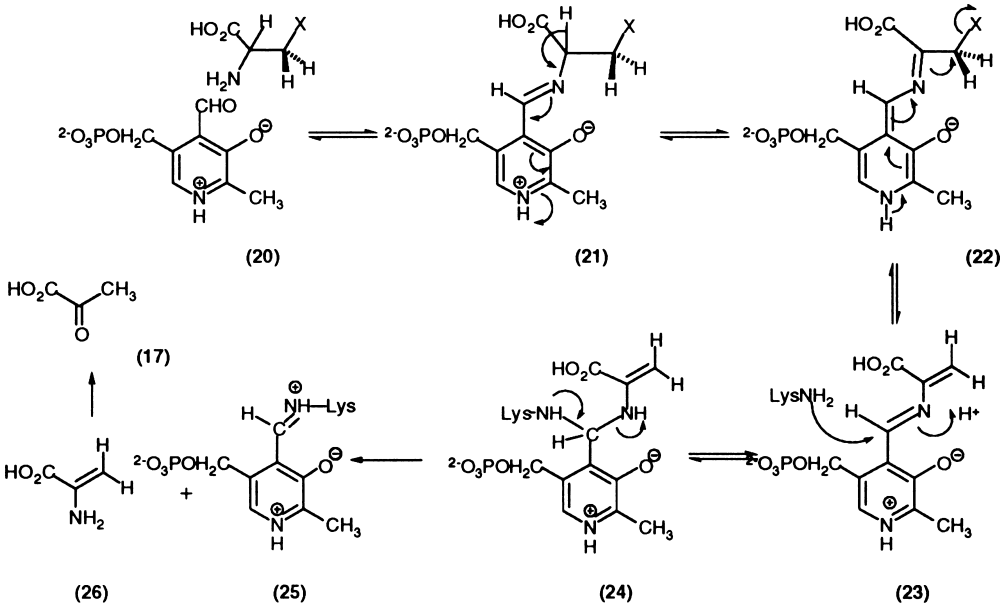
acid aminotransferase (EC 2.6.1.21). When these compounds act as substrates, the product is pyruvic acid (**17**), there being 1500 turnovers to one inhibition. The inhibitor complex, when treated with base, has been shown to yield the compound **(19)**, known as the "Schnackerz product" [14,15], and when the inhibitors are incubated with the enzymes in the presence of a thiol, a substituted cysteine is produced [16,17]. We now began our enzyme studies by investigating these three processes.



Scheme 4

Study of the turnover of D- and L- serine-O-sulfate and beta-chloro-D-alanine by amino acid aminotransferase and L-aspartate aminotransferase

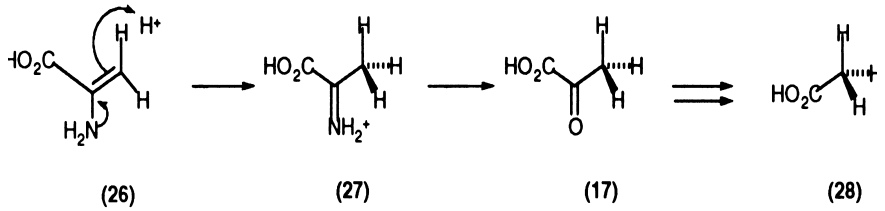
When β-chloroalanine **(16)** and serine-O-sulfate **(13)** are substrates for a variety of PLP enzymes, the product is pyruvic acid (**17**). It was suggested that the mechanism for the formation of pyruvate **(17)** was as shown in Scheme 5. Here, formation of the PLP imine (**21**) is followed by rearrangement to the



Scheme 5

extended enamine form (**22**). This allows elimination of the leaving group to yield the compound (**23**) which can be converted to the free enamine **(26)** as shown in Scheme 5. Protonation to an iminium species **(27)** (Scheme 6) then allows hydrolysis to pyruvate **(17)**.

As our first task was to discover the stereochemistry of the reaction of the compounds as substrates, we incubated the stereospecifically deuteriated samples of D-serine-O-sulfate (**13a**) and β-chloro-D-alanine (**16a**) with D-amino acid aminotransferase (EC 2.6.1.21) in the presence of ^3H_2O, lactate dehydrogenase (EC 1.1.1.27) and NADH. The dehydrogenase was present to reduce the pyruvate **(17)**



Scheme 6

Table 1 Stereochemical analysis of samples of lactate obtained on incubation of the labelled inhibitors D-serine-O-sulfate **(13a)** and β-chloro-D-alanine **(16a)** with D-amino acid aminotransferase (EC 2.6.1.21), lactate dehydrogenase (EC 1.1.1.27), and NADH.

Exp.	Substrate	F value[a]	e e
1	(2R,3S)-[3-$^2H_1$]-serine-O-sulfate	54.6	16% R
2	(2R,3R)-[2,3-$^2H_2$]-serine-O-sulfate	41.6	29% S
3	(2S,3S)-[3-$^2H_1$]-β-chloroalanine	54.3	15% R
4	(2S,3R)-[2,3-$^2H_2$]-β-chloroalanine	47.3	10% S

[a] The F value is the percentage tritium retention in fumarase in the assay.

formed to D-lactate *in situ* and thus prevent adventitious racemization through exchange. The samples of lactate were degraded to acetate (**28**), and the chirality was assessed by Professor H. G. Floss and his colleagues as summarized in Table 1.

The results showed that the reaction is stereoselective, with overall retention of stereochemistry when the leaving groups are replaced by hydrogen. The accompanying racemization may indicate that some protonation of the enamine **(26)** occurs outside of the active site. It was of interest to compare the stereospecificity of the above reaction with that of the corresponding L-aspartate aminotransferase (EC 2.6.1.1) catalyzed reaction. We therefore prepared stereospecifically deuteriated L-serine-O-sulfate **(13b)** from labeled L-serine. When these compounds were used as substrates for L-aspartate aminotransferase in ^3H_2O in the presence of lactate dehydrogenase and NADH, however, the resultant samples of lactate were shown by Professor Floss to be totally racemic. Protonation of the intermediate enamine **(26)** therefore appeared to have occurred entirely outside of the active site of the enzyme.

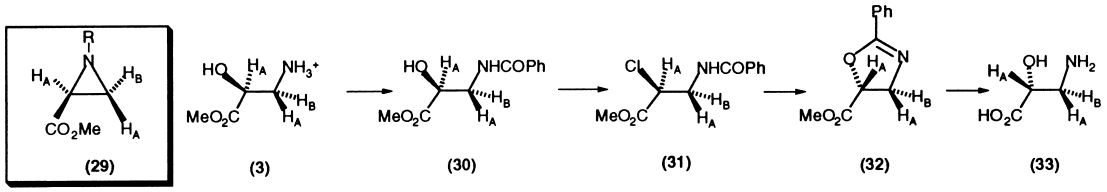
A GENERAL SYNTHESIS OF L-AMINO ACIDS STEREOSPECIFICALLY LABELED IN THE BETA-POSITION

Although we have synthesized several L-amino acids stereospecifically labeled in the β-position [2], the methodology was by no means as versatile as we would have liked. We therefore determined to develop

a versatile synthesis of stereospecifically labeled L-amino acids by adapting our synthesis of the D-series to prepare the protected labeled aziridines **(29)** of the L-series. The ring opening methodology which we had already developed might then be used to synthesize a very wide variety of stereospecifically labeled L-amino acids. The simplest solution to this problem would be to invert the stereochemistry at the α-center of some intermediate in our synthesis of the D-aziridines **(11)**. The key intermediates in this synthesis are the stereospecifically labeled samples of (2S)-isoserine **(3, H_B = ^{2}H)** and **(3, H_A = ^{2}H)**.

An appealing possibility was to use intramolecular nucleophilic substitution at the α-center by an amide on the adjacent nitrogen to achieve inversion, and so we prepared methyl N-benzoyl-(2S)-isoserinate **(30)** in 80% yield by reaction of the methyl ester **(3)** with benzoyl chloride under Schotten–Baumann conditions in aqueous sodium bicarbonate at 0 °C for 4 h. When the amide **(30)** was reacted with thionyl chloride in chloroform at temperatures less than 5 °C, the chloride **(31)** was obtained after 24 hours in 85% yield. Reaction for shorter times led to mixtures of the oxazoline **(32)** and the chloride **(31)**, implying that the chloride **(31)** was obtained *via* the oxazoline **(32)** by substitution by chloride anion. Heating the chloride **(31)** in toluene for 36 h gave the oxazoline **(32)** in 85% yield. The oxazoline **(32)** could also be obtained directly in good yield from the amide **(30)**, either by reaction with methanesulfonic anhydride or with trifluoromethanesulfonic anhydride at room temperature for 24 h in dichloromethane containing three equivalents of pyridine.

Having obtained the oxazoline **(32)**, the next step was hydrolysis to leave the new inverted stereochemical center at C-2 intact. This was achieved by heating the oxazoline to reflux in 1N aqueous hydrochloric acid in methanol. The resultant benzoyl amide had a rotation of opposite sign to that of the amide **(30)** and, on conversion to the Mosher's ester, was shown by ^{19}F- and ^{1}H- NMR spectroscopy to have an enantiomeric excess of >95%. Heating the oxazoline **(32)** to reflux with 5N aqueous hydrochloric acid for 5 hours gave (2R)-isoserine **(33)** in 70% yield. The optical purity was assessed by converting this to the benzoyl methyl ester by the route used to prepare the enantiomeric compound **(30)**. Conversion to the Mosher's ester by reaction with (R)(-)-α-methoxy-α-trifluoromethylphenylacetyl chloride followed by NMR studies showed an enantiomeric excess of >95%. Heating the oxazoline **(32)** for longer periods than 5 h was shown to reduce the enantiomeric excess slightly.



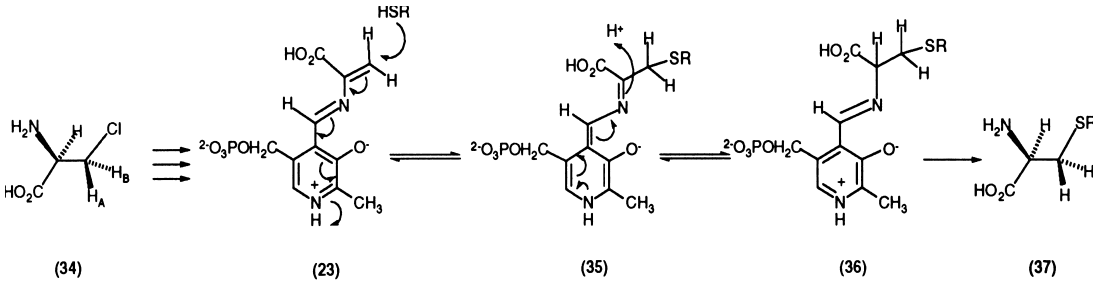
Scheme 7

Having succeeded in inverting the center C-2, we were able to use the reaction sequence to convert the labeled samples of methyl (2S)-isoserinate **(3, H_A = ^{2}H)** and **(3, H_B = ^{2}H)** to the corresponding samples of (2R)-isoserine **(33, H_A = ^{2}H)** and **(33, H_B = ^{2}H)**. The ^{1}H- and ^{2}H-NMR spectra of all pairs of diastereotopically labeled compounds in the reaction sequence confirmed the stereospecificity of the sequence and the inversion at C-2 was confirmed by comparison of the spectra of the labeled samples of (2S)- and (2R)-isoserine. Conversion of the labeled samples **(33)** of (2R)-isoserine to the labeled L-aziridines **(29, R = Ph_3C)** was carried out as shown in Scheme 3 for the D-isomers. The ^{1}H-NMR spectra of the aziridines **(29, R = Ph_3C)**, **(29, R = Ph_3C, H_A = ^{2}H)** and **(29, R = Ph_3C, H_B = ^{2}H)** were in keeping with the spectra of the diastereoisomerically labeled aziridines **(2)** from which we have synthesized a variety of stereospecifically labeled D-amino acids.

ENZYME STUDIES 2

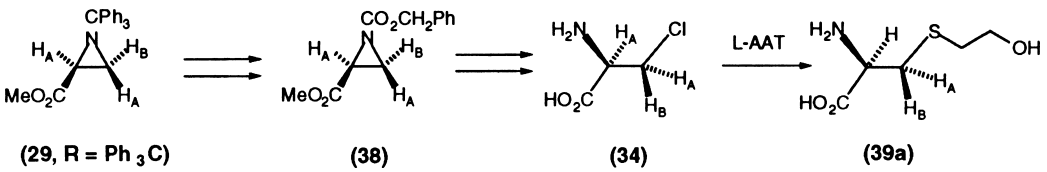
Study of the reaction of beta-chloroalanine with a thiol catalyzed by L-aspartate aminotransferase

L-Aspartate aminotransferase (EC 2.6.1.1) is an enzyme of the α-family, catalyzing transamination of L-aspartic acid to α-ketoglutaric acid to give oxaloacetate and L-glutamate. L-Serine-O-sulfate (**13b)** not only inhibits this enzyme and is converted to pyruvic acid **(17)** by it but is also converted to cysteine derivatives in the presence of thiols [16]. Further, inhibition of D-amino acid aminotransferase by β-bromo-D-alanine is reversed by addition of thiols, yielding cysteine derivatives [17]. Thus these enzymes have catalyzed a β-substitution reaction typical of an enzyme of the β-family, rather than the normal transamination reactions which are typical of α-family enzymes. This would suggest that the intermediate **(23)** in the inhibition process using β-chloro-L-alanine **(34)**, instead of proceeding as in Scheme 5**,** proceeds in the presence of a thiol as shown in Scheme 8 to give intermediate **(35)**, the precursor of the final cysteine derivative **(37)**.



Scheme 8

The change in role from catalyst for transamination to catalyst for β-substitution exhibited by aspartate aminotransferase on change of substrate from L-aspartate to β-chloro-L-alanine was intriguing. Thus, we decided to examine the overall stereochemistry of the β-substitution reaction in the presence of thiol to see whether it was in keeping with the retention generally shown by reactions catalyzed by the enzymes whose normal role this was [10]. We therefore prepared samples of β-chloro-L-alanine **(34)** labeled stereospecifically at C-3. This was achieved as in Scheme 9 by first converting the labeled samples of methyl (2S)-N-tritylaziridine-2-carboxylate **(29, R = Ph_3C, H_B = ^2H)** and **(29, R = Ph_3C, H_A = ^2H)** to the corresponding urethanes **(38)** by reaction with trifluoroacetic acid in chloroform to remove the trityl group, followed immediately by reaction with benzyl chloroformate under Schotten–Baumann conditions. β-Substitution with chloride ion was achieved by reaction with $TiCl_4$ in CH_2Cl_2-$CHCl_3$ as in the synthesis of the corresponding D-isomers. Deprotection in refluxing 4M H_2SO_4 then

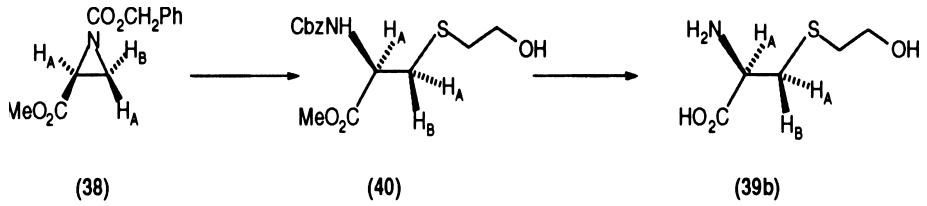


Scheme 9

gave the enzyme inhibitors **(34)**. The ^{1}H- and ^{2}H-NMR spectra indicated that the inhibitors **(34)** were unique diastereoisomers and, since substitution is accompanied by inversion of stereochemistry at the labeled β-position, these were assigned as (2R,3S)-[3-$^2H_1$]- and (2R,3R)-[2,3-$^2H_2$]-β-chloroalanine, **(34, H_B = ^{2}H)** and **(34, H_A = ^{2}H)** respectively.

The enzyme L-aspartate aminotransferase (EC 2.6.1.1) was isolated by a standard purification protocol [18] from *E. coli* TY103 [19] transformed with plasmid pKDHE19/AspC. It had specific activity of 36.57 units/mg and appeared as one major band of molecular weight 43000 Da on SDS-PAGE. Incubation with (2R,3R)-[2,3-$^2H_2$]-β-chloroalanine **(34, H_A = ^{2}H)** in the presence of a variety of small thiols and under various conditions was now undertaken. The best results were obtained using mercaptoethanol at pH 8.4. This gave samples of labeled 3-(2-hydroxyethyl)-cysteine **(39)** in which the α-deuterium atom had exchanged as expected from the mechanism in Scheme 1. The β-deuterium atom also exchanged on prolonged incubation, an effect which had been previously noted in reactions with this enzyme [20,21]. However, when the incubation was stopped after three hours, a sample of (2R)-3-(2-hydroxyethyl)-cysteine **(39a)** was obtained with a ^{1}H-NMR spectrum, which showed that there was stereospecific labelling at C-3. (2R,3S)-[3-$^2H_1$]-β-Chloroalanine **(34, H_B = ^{2}H)** was therefore incubated for the same time when the ^{1}H-NMR spectrum of the product **(39a)** indicated that it was the C-3 epimer.

The trapping reaction was evidently stereospecific, and it was now necessary to determine the absolute stereochemistry of the overall reaction. This was achieved by an independent synthesis of samples of (2R)-3-(2-hydroxyethyl)-cysteine **(39)** labeled stereospecifically with deuterium at C-3 in an unambiguous manner. The synthesis is shown in Scheme 10, the labeled carbobenzyloxyaziridines **(38)** being reacted with mercaptoethanol containing a catalytic quantity of boron trifluoride etherate. Inversion of stereochemistry at the labeled atom, C-3, is expected, and ^{1}H- and ^{2}H-NMR spectra showed that the labeled products (**40, H_B = ^{2}H)** and **(40, H_A = ^{2}H)** were single diastereoisomers. Hydrolysis in refluxing 4M H_2SO_4 then gave the free amino acids **(39b)**.



(38) (40) (39b)

Scheme 10

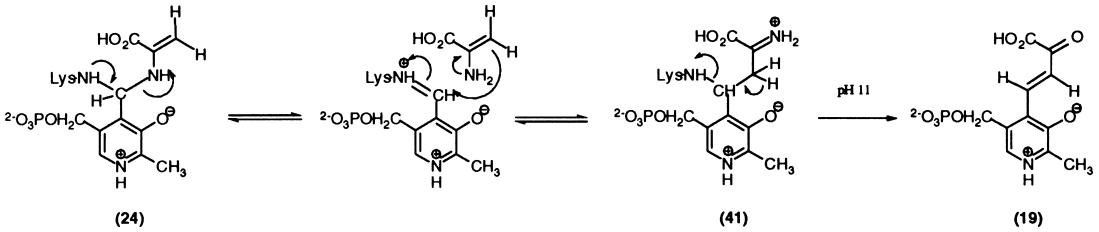
The ^{1}H-NMR spectra of the synthetic samples of (2R,3S)-[3-$^2H_1$]-3-(2-hydroxyethyl)-cysteine **(39b, H_B = ^{2}H)** and (2R,3R)-[2,3-$^2H_2$]-3-(2-hydroxyethyl)-cysteine **(39b, H_A = ^{2}H)** allowed the *3-pro-S* and *3-pro-R* protons in the spectrum of 3-(2-hydroxyethyl)-cysteine **(39)** to be assigned and therefore the absolute stereochemistry of the incubation products to be deduced. It was evident that the product from the incubation using (2R,3S)-[3-$^2H_1$]-3-β-chloroalanine **(34, H_B = ^{2}H)** was (2R,3S)-[3-$^2H_1$]-3-(2-hydroxyethyl)-cysteine **(39a, H_B = ^{2}H)** and that the product when (2R,3R)-[2,3-$^2H_2$]-3-β-chloroalanine **(34, H_B = ^{2}H)** was used was (2R,3R)-[3-$^2H_1$]-3-(2-hydroxyethyl)-cysteine **(39a, H_A = ^{2}H)**.

This implies that the β-replacement reaction, which in this case is catalyzed by an enzyme of the α-family, whose normal function is transamination, occurs with overall retention of stereochemistry. This is the general expectation [10] for the PLP-mediated enzymes of the β-family where β-replacement reactions are the norm and may imply a closer relationship between the families than homology [7] suggests. Although X-ray structures are available for aspartate aminotransferase [22] and other α-family enzymes, tryptophan synthase (EC 4.2.1.20) is still the only β-family enzyme the tertiary structure of which has been determined by X-ray crystallography [23]. It is of interest to note that, in the

presence of thiols, tryptophan synthase has been shown to catalyze transamination and the β-replacement of L-serine by mercaptoethanol [24]. Further, the Lys87Thr mutant of tryptophan synthase will not turn over the natural substrate L-serine in the absence of NH_4^+, but it will turn over β-chloro-L-alanine [25]. The stereochemistry has not been determined with this substrate.

Study of the formation of the "Schnackerz Product"

A further process undergone on inhibition of PLP enzymes by the inhibitors β-chloroalanine **(16)** and serine-O-sulfate **(13)** was the formation of the so-called "Schnackerz compound" **(19)** when the inhibitor complex was treated with base to pH 11 [14,15]. The explanation given for this is that the first-formed inhibitor complex **(24)** undergoes fission followed by recombination as shown in Scheme 11 to give the inhibitor complex **(41)**. This then reacts as shown at pH 11 to yield the "Schnackerz compound" **(19)**.

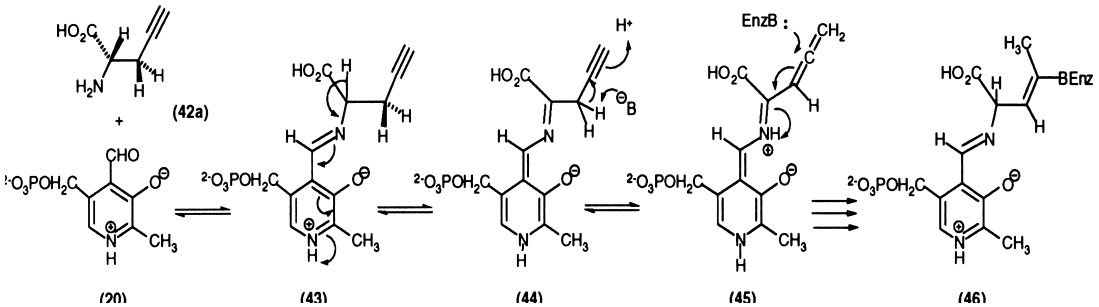


Scheme 11

It seemed that this process might involve stereospecific loss of one of the β-hydrogens of the inhibitor, and so we incubated a large amount of the enzyme L-glutamate decarboxylase (EC 4.1.1.15) with (2S,3R)-[3-$^2H_1$]-serine-O-sulfate and (2S,3S)-[2,3-$^2H_2$]-serine-O-sulfate respectively. In each case when the intermediate was treated with base to pH 11, the product **(19)** contained no label. It was evident that there had been exchange during the process. When we treated a sample of the compound **(19)** with base to pH 11 in ^2H_2O, no exchange was observed. However, when lysine was added at pH 11, then the product **(19)** was deuteriated in the position α- to the ketone. This suggests slow exchange of a lysine Michael adduct analogous to the inhibitor complex **(41)**.

L-Propargylglycine as an inhibitor

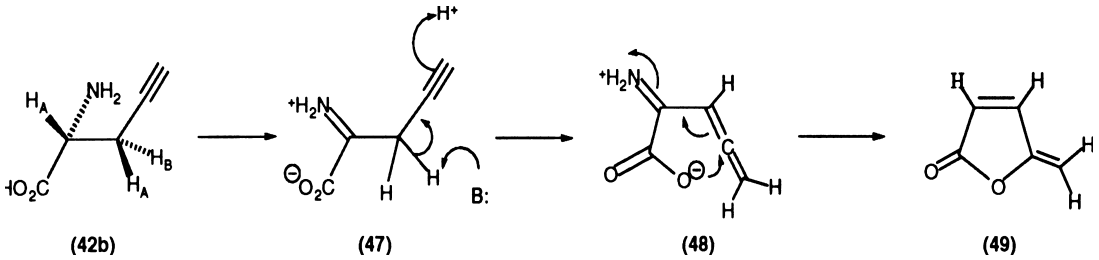
L-Propargylglycine **(42a)** inhibits PLP-mediated enzymes by a route thought to involve the mechanism indicated in Scheme 12, which involves enzymic loss of one β-hydrogen [26]. We were therefore interested



Scheme 12

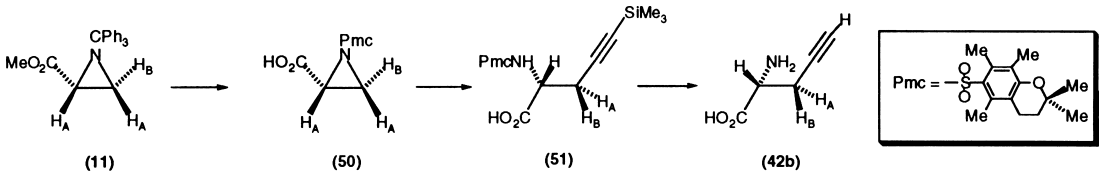
in synthesizing samples of this naturally occurring inhibitor which were stereospecifically labeled with deuterium in the β-position. Further, it has been noted that the enzymes cystathionine γ-synthase and methionine γ-lyase are reactivated in the presence of thiols [27].

The labeled aziridines of the D-series would also be of use for investigation of the mechanism of turnover of D-propargyglycine **(42b)** by D-amino acid oxidase (EC 1.4.3.3). When D-propargyglycine **(42b)** acts as a substrate, the product is the lactone **(49)** and the mechanism in Scheme 13 involving loss of a β-hydrogen has been suggested to account for its formation [28].



Scheme 13

Synthesis by our aziridine route would require cleavage of the ring with a carbon nucleophile. Although we have reacted N-tritylaziridines with perchloric acid to yield labeled samples of D-serine and have reacted N-carbobenzyloxyaziridines with heteronucleophiles such as thiols and chloride ion, problems of regioselectivity had been encountered using carbon nucleophiles in all other work to date [29]. We found that to achieve ring opening with carbon nucleophiles it was necessary to use the N-tosylaziridines and that regioselectivity problems were overcome by using aziridine free acids which, as the anions, direct attack away from the α-carbon atom. Problems encountered in removing the tosyl protecting group were overcome by using Ramage's 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) protecting group [30] instead of tosyl and the synthesis was completed as in Scheme 14. After nucleophilic attack on the Pmc derivative **(50)** to obtain the protected compounds **(51)**, deprotection using trifluoroacetic acid finally gave the required labeled D-propargyglycines **(42b, H_A = ^2H)** and **(42b, H_B = ^2H)**.



Scheme 14

These labeled inhibitor/substrates **(42b)** were incubated with D-amino acid oxidase, and the lactones **(49)** were isolated. Integration of ^{1}H-NMR spectra of these lactones showed that the carbon atom on the endocyclic double bond derived from the β-carbon of the inhibitor **(42b)** was 80–90% deuteriated in each case. These results indicate that removal of the proton at C-3 of the substrate is in line with the expected isotope effect and hence it is nonstereospecific. The result is therefore more in keeping with removal of this proton in a non-enzyme catalyzed step.

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REFERENCES

1. (a) D. J. Morecombe and D. W. Young. *J. Chem. Soc., Chem. Commun.* 198–199 (1975); (b) D. W. Young, D. J. Morecombe, P. K. Sen. *Eur. J. Biochem.* **75**, 133–147 (1977); (c) J. A. Huddleston, E. P. Abraham, D. W. Young, D. J. Morecombe, P. K. Sen. *Biochem. J.* **169**, 705–707 (1978).
2. See D. W. Young. In *Synthesis and Applications of Isotopically Labelled Compounds 1994*, J. Allen and R. Voges (eds.), Wiley, New York, 27–40 (1995) for references.
3. A. E. Braunstein and M. M. Shemyakin. *Biokhimya* **18**, 393–411 (1953).
4. D. E. Metzler, M. Ikawa, E. E. Snell, *J. Am. Chem. Soc*., **76**, 648–652 (1954).
5. H. C. Dunathan. *Proc. Natl. Acad. Sci. USA*, **55**, 712–716 (1966).
6. H. C. Dunathan and J. G. Voet. *Proc. Natl. Acad. Sci. USA*, **71**, 3888–3891 (1974).
7. F. W. Alexander, E. Sandmeier, P. K. Mehta, P. Christen. *Eur. J. Biochem*. **219**, 953–960 (1994).
8. R. A. John. *Biochim. Biophys. Acta* **1248**, 81–96 **(**1995).
9. P. Christen and D. E. Metzler (eds.). *Transaminases*, Chapter 3, Wiley-Interscience, pp 109–213 (1985).
10. D. W. Young. *Topics in Stereochemistry* **21**, 381–465 (1994).
11. T. Yoshimura, K. Nishimura, J. Ito, N. Esaki, H. Kagamiyama, J. M. Manning, K Soda. *J. Am. Chem. Soc*. **115**, 3897–3900 (1993).
12. D. W. Young. In *Isotopes in Organic Chemistry,* Vol 4, E. Buncel and C. C. Lee (eds.), Elsevier, Amsterdam, pp 177–294 (1978).
13. D. Gani and D. W. Young. *J. Chem. Soc., Perkin Trans. 1,* 2393–2398 (1983).
14. H. Ueno, J. J. Likos, D. E. Metzler. *Biochemistry* **21**, 4387–4393 (1982).
15. J. J. Likos, H. Ueno, R. W. Feldhaus, D. E. Metzler. *Biochemistry* **21**, 4377–4386 (1982).
16. D. Cavallini, G. Federici, F. Bossa, F. Granata. *Eur. J. Biochem*. **39**, 301–304 (1973).
17. T. S. Soper and J. M. Manning. *Biochemistry* **17**, 3377–3384 (1978).
18. M. Herold and K. Kirschner. *Biochemistry* **29**, 1907–1913 (1990).
19. T. Yano, S. Kuramitsu, S. Tanase, Y. Morino, K. Hiromi, H. Kagamiyama. *J. Biol. Chem*. **266**, 6079–6085 (1991).
20. U. Walter, H. Luthe, F. Gerhart, H.-D. Söling. *Eur. J. Biochem*. **59**, 395–403 (1975).
21. U. M. Babu and R. B. Johnston. *Biochemistry* **15**, 5671–5678 (1976).
22. See for example V. N. Malashkevich, M. D. Toney, J. N. Jansonius. *Biochemistry* **32**, 13451–13462 (1993).
23. T. R. Schneider, E. Gerhardt, M. Lee, P.-H. Liang, K. S. Anderson, I. Schlichting. *Biochemistry* **37**, 5394–5406 (1998).
24. E. W. Miles, M. Hatanaka, I. P. Crawford. *Biochemistry* **7**, 2742–2753 (1968).
25. Z. Lu, S. Nagata, P. McPhie, E.W. Miles. *J. Biol. Chem*. **268**, 8727–8734 (1993).
26. W. Washtien and R. H. Ables. *Biochemistry* **16**, 2485–2491 (1977).
27. M. Johnston, D. Jankowski, P. Marcotte, H. Tanaka, N. Esaki, K. Soda, C. Walsh. *Biochemistry*

18, 4690–4701 (1979).
28. P. Marcotte and C. Walsh. *Biochemistry* **17**, 5613–5620 (1978).
29. J. E. Baldwin, R. M. Adlington, I. A. O'Neil, C. Schofield, A. C. Spivey, J. B. Sweeney. *Tetrahedron*, **49,** 6309–6330 (1993).
30. R. Ramage, J. Green, A. J. Blake. *Tetrahedron*, **47**, 6353–6370 (1991).