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Synthesis of L-β-Hydroxyaminoacids Using Serine Hydroxymethyltransferase

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Abstract The enzyme serine hydroxymethyltransferase (EC 2.1.2.1) has been used in the synthetic direction with a variety of aldehyde substrates to form carbon-carbon bonds with the creation of two chiral centres. A variety of β-hydroxyaminoacids has been prepared in reasonable yields but, although L-stereospecificity is observed at the α-centre, stereospecificity is not high at the β-centre when the reaction is conducted on the preparative scale.

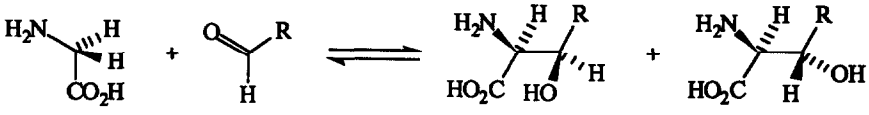
β-Hydroxyaminoacids constitute an important class of compounds. They are natural products in their own right and are components of more complex compounds, some of which have useful and interesting biological properties. Thus β-hydroxytyrosine is present in antibiotics such as vancomycin,[1] bouvardin,[2] orienticins[3] and hypeptin,[4] β-hydroxyleucine is present in lysobactin,[5] katanosins,[6] hypeptin,[4] azinothricin[7] and leucinostatin ;[8] 3-hydroxyhomotyrosine is present in echinocardin D;[9] β-hydroxyasparagine is present in katanosins[6] and hypeptin[4] and β-hydroxyproline and β-hydroxyaspartic acid are present in empedopeptin.[10] β-Hydroxyglutamic acid is active on cranial nerves and has been used in the synthesis of tricholomic acid [11] while β-hydroxyaspartic acid appears to play an important role in blood clotting proteins.[12] β-Hydroxyaminoacids have also been used in the synthesis of important natural products such as the β-lactam antibiotics.[13-15]

The importance of β-hydroxyaminoacids has led to considerable recent interest in stereospecific chemical synthesis of these compounds.[16] Enzyme catalysed reactions are being used more often in synthesis because of their well known stereospecificity,[17] although the use of enzymes which catayse the formation of carbon carbon bonds is comparatively rare. We were, therefore, interested to see if the enzyme serine hydroxymethyltransferase (EC 2.1.2.1), which catalyses an aldol reaction, might be applied to the synthesis of β-hydroxyaminoacids. Since two chiral centres can be created in the process, the products would be of great interest.

Serine hydroxymethyltransferase normally catalyses the reversible interconversion of glycine and serine[18] using the coenzymes pyridoxal phosphate and tetrahydrofolic acid. It has, however, also been shown to catalyse the cleavage of several other β-hydroxyaminoacids[19-22] in the absence of tetrahydrofolic acid and, although stereospecificity is not absolute, *erythro*- β-hydroxyaminoacids were shown to have lower K_M values and higher V_{max} values than the corresponding *threo*- isomers. The enzyme has been used in the synthetic direction, albeit on the radiochemical scale, to prepare L-allothreonine from glycine and acetaldehyde.[23,24] In this synthesis, 98% allothreonine was obtained, contaminated with 2% threonine.[23,24]

Because of its broad substrate specificity and apparent stereoselectivity, it seemed that, if the enzymic reaction could be used preparatively in the synthetic direction then a useful synthesis of biologically and synthetically important chiral β-hydroxyaminoacids could be developed. In view of the comparatively small number of carbon-carbon bond forming enzymes to have been used preparatively,[17] this would be an important addition to the developing field of the use of enzymes in chemical synthesis.

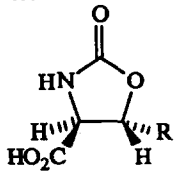
We therefore isolated serine hydroxymethyltransferase from pig liver by an adaptation of the method reported by Matthews *et al.* [25] Preliminary studies showed that the reaction could be driven in the synthetic direction using a ten molar excess of glycine (1) and so a variety of aldehydes (2) were incubated with the enzyme and ten equivalents of glycine (1) until thin layer chromatography indicated that reaction was complete. The reaction was then terminated by addition of 1M aqueous trichloroacetic acid, the precipated protein was removed by centrifugation, and the amino acids were separated by ion exchange chromatography. The successful reactions are shown in the table and indicate that a number of aldehydes participate in the reaction and that yields can be reasonable. The yield in the reaction of glycine (1) with imidazole-2-carboxaldehyde (2c) to give the β-hydroxyisohistidines (3c) + (4c) was particularly impressive, although the isomeric imidazole-4-carboxaldehyde (2g) did not give measurable yields of product. Reaction with indole-3-carboxaldehyde (2h) and 5-methoxyindole-3-carboxaldehyde (2i), which would have led to noradrenalin analogues of tryptophan and serotonin, was also unsuccessful.



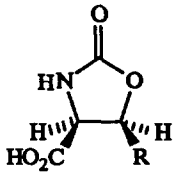
(1) (2) (3) (4)

In all cases, it was evident from the ^{1}H-nmr spectra that mixtures of the *erythro*- and *threo*- diastereoisomers were obtained. Since the coupling constant between the α- and β- protons is usually found to be slightly larger for the *threo*- than for the *erythro*- isomer,[26] we could assign the isomers on this basis. The chemical shifts did not correlate well with literature values[26,27] recorded in ^2H_2O, presumably because of pH differences and so an authentic sample of *threo*-DL-β-furylserine was prepared by the method of Inui.[28] The ^{1}H-nmr spectra of this and of an authentic sample of *threo*- β-phenylserine confirmed the stereochemical assignments. Further confirmation was obtained by preparation of the oxazolidinone derivatives (5d), (6, R=Me) and (5a) + (6a) from, respectively, authentic samples of *threo*- DL-β-phenylserine (3d) and L-allothreonine (4, R=Me) and from the enzymically prepared mixture of β-2-furylserines (3a)+(4a). The α,β-

coupling constant in the oxazolidinones was very much larger for the *erythro* isomer (6) (J *ca.* 9 Hz) than for the *threo* isomer (5) as expected.[26]

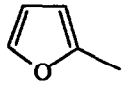
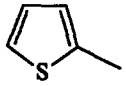
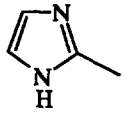
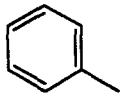
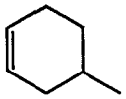


(5)



(6)

Table: Synthesis of β-hydroxyaminoacids using serine hydroxymethyltransferase

	R	Incubation Time (days)	Minimum Yield (%)	Ratio *threo* : *erythro* (3) : (4)
(a)		23	20	1 : 1
(b)		60	11	1.25 : 1
(c)		1 2 6 60	10 15 30 55	1 : 2 1 : 1.6 1 : 1.7 1.8 : 1
(d)		10 30	15 22	1.25 : 1 1.5 : 1
(e)	$CH_3(CH_2)_5$-	20	25	1.5 : 1
(f)		40	11	ratio undetermined

Since it is known that serine hydroxymethyltransferase does not cleave D-amino acids,[18] we expected that our synthetically prepared β-hydroxyamino acids would be members of the L-series. We confirmed this for the mixture of β-hydroxyisohistidines, (3c) + (4c) by reacting it separately with D-amino acid oxidase (EC 1.4.3.3) and then with L-amino acid oxidase (EC 1.4.3.2). The amount of amino acid present was estimated using the absorbance at 570 nm of a solution treated with ninhydrin. Whereas little oxidation was observed to have

occurred using the D-aminoacid oxidase, almost all of the amino acid was consumed using L-amino acid oxidase.

Our results show that, in spite of the earlier reports[23,24] that, on the radiochemical scale and with short incubation times, substantial bias towards *erythro*- β-hydroxyaminoacids was shown by serine hydroxymethyltransferase, on the preparative scale where reasonable yields required long incubation times, stereoselectivity was not of a high enough order to make the method synthetically useful. Longer incubation times seemed to favour the *threo* isomer. Since it is known that cleavage of the *erythro* isomer is favoured over cleavage of the *threo* isomer,[18] we attempted to alter the isomer ratio by reaction of mixtures of diastereoisomeric β-hydroxyaminoacids obtained from the enzymic reaction using conditions which would favour enzymatic cleavage. Mixtures of L-β-phenylserines **(3d)** + **(4d)**, and L-β-hydroxyisohistidines, **(3c)** + **(4c)** were treated in this way and, although marginal improvements in the ratio were observed, it was impossible to eliminate large enough quantities of the *erythro* isomer whilst maintaining reasonable product yields.

Experimental

^{1}H-nmr spectra were recorded on a Bruker WM360 Fourier transform instrument; mass spectra on Kratos MS80 and MS25 instruments by Mr. A. Greenway and U.V. spectra on a Phillips PU8720 spectrophotometer. T.l.c. was conducted using Merck precoated cellulose plates. The aldehydes **(2a)** to **(2f)** and **(2h)** and **(2i)** were purchased from The Aldrich Chemical Company and **(2g)** was prepared by oxidation of the corresponding alcohol.[29] Unless otherwise stated, other compounds were obtained from commercial sources.

Purification of Serine Hydroxymethyltransferase

(a) Assay: Yeast alcohol dehydrogenase (EC 1.1.1.1, Sigma, 0.25 mg) and NADH (1.0 mg) were dissolved in 0.04 M potassium phosphate buffer (10 ml, pH 7.4). A solution of serine hydroxymethyltransferase (0.01 ml) was added to this mixture (2.2 ml) together with a solution of L-allothreonine (1 mg) in distilled water (0.1 ml). The decrease in absorbance at λ_{max} 340 nm, due to conversion of NADH to NAD, was recorded at 2 min. intervals. One unit of enzyme is that amount of enzyme required to produce a change of 0.001 absorbance units per minute.

(b) Protein concentration was derived from the absorbance at 280 nm and 260 nm as already described.[30]

(c) Purification Pig liver was obtained from a freshly slaughtered animal (Ringer Slaughter House, Ringmer, East Sussex) and immediately frozen in solid CO_2. The frozen liver (400 gm) was cut into small pieces and homogenised for *ca.* 3 min. in a blender using a 0.05 M potassium phosphate buffer (1 litre, pH 7.4) containing 0.1 M NaF, 14 mM DL-serine, 10 mM EDTA, 0.1 mM pyridoxal phosphate, 0.1 mM folic acid and 2 mM 2-mercaptoethanol. The homogenate was centrifuged for 10 min. at 20,000 g and the supernatant was heated in a water bath with constant stirring. When the temperature reached 70 °C, the solution was cooled to 10 °C in an ice bath and centrifuged at 20,000 g for 10 min.

Ammonium sulphate (312 g) was added to the supernatant solution (*ca.*800 ml) to bring it to 60% saturation and, after 10 min. at room temperature, was centrifuged at 20,000 g for 10 min. The pellet obtained was dissolved in a 0.05 M potassium phosphate buffer (150 ml, pH 7.4) containing 1 mM DL-serine, 1 mM EDTA,

0.1 mM pyridoxal phosphate, 0.1 mM folic acid and 2 mM 2-mercaptoethanol and dialysed three times against a 20 fold excess of the same buffer over a period of 24 hours. The solution (200 ml) contained 8.75 g protein and assayed as a total of 172,800 units of enzyme.

DEAE-Sephadex A-50 (20 g, Aldrich) was prepared by leaving for 30 min. at room temperature with 0.5 M hydrochloric acid (700 ml), washing free of acid with distilled water, leaving for 30 min. at room temperature with 0.5 M sodium hydroxide (700 ml) and washing free of base with distilled water. The resin was finally equilibrated in a 0.05 M potassium phosphate buffer (*ca.* 200 ml, pH 7.4) containing 1 mM DL-serine, 1mM EDTA, 0.1 mM pyridoxal phosphate and 2 mM 2-mercaptoethanol and loaded onto a column (5 x 25 cm) in this buffer. The dialysed enzyme was applied to the column and eluted in the same buffer. Further elution with a 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM DL-serine, 1 mM EDTA, 0.1 mM pyridoxal phosphate and 2 mM 2-mercaptoethanol removed red proteins before the enzyme was eluted using a 0.3 M potassium phosphate buffer containing 1 mM DL-serine, 10 mM EDTA, 0.1 mM pyridoxal-phosphate and 2 mM 2-mercaptoethanol. The active fractions (500 ml) contained 1.6 g protein and assayed as a total of 92,500 units of enzyme.

Ammonium sulphate (234 g) was added to this solution to bring it to 60% saturation and, after 10 min. at room temperature, the mixture was centrifuged at 20,000 g for 10 min. The precipate was dissolved in a 0.05 M potassium phosphate buffer (60 ml, pH 7.4) containing 1 mM DL-serine, 1 mM EDTA, 0.1 mM pyridoxal phosphate and 2 mM 2-mercaptoethanol and dialysed against a 20 fold excess of the same buffer for 24 hours. The solution (75 ml) contained 1.3 g protein and assayed as 82,500 units of enzyme.

Enzymic Synthesis of the β-Hydroxyaminoacids - General

The aldehyde **(2)** (1 mmol) and glycine (10 mmol) were dissolved in 0.01 M potassium phosphate buffer (160 ml, pH 7.4) containing 1 mM EDTA. Serine hydroxymethyltransferase solution (1 ml, *ca.* 1000 units) was added and the mixture was incubated at 30 °C for the time period indicated in the table . The progress of the reaction was monitored on cellulose t.l.c. plates, eluting with n-butanol : acetone : water : diethylamine (1 : 1 : 1 : 0.1) and spraying with ninhydrin followed by heating to 100 °C. The reaction was terminated by addition of 10% aqueous trichloroacetic acid (1 ml) and centrifugation at 800 g for 20 min. The supernatant solution was freeze dried, dissolved in a minimum (*ca.* 10 ml) of distilled water and chromatographed on Dowex 1-X8-200 ion exchange resin (Dowex-1-chloride) which had been washed of all fines, left in 0.5 M HCl for 30-60 min. at room temperature, washed free of acid with distilled water, left for 30-60 min. in 0.5 M NaOH at room temperature and washed free of base with distilled water. The column was first eluted with distilled water (*ca.* 100 ml) and then with a gradient of 0.1 M to 0.5 M aqueous acetic acid. Unreacted glycine eluted first, followed by the β-hydroxyaminoacids. Yields and integrated ratios of *erythro : threo* isomers are shown in the table.

β-2-Furylserines (3a) + (4a): δ (^2H_2O) 3.89 (d, J = 4.5, α-H *threo* isomer), 3.97 (d, J = 4.5, α-H, *erythro* isomer), 5.10 (J = 4.4 β-H, *threo* isomer); 6.28 (2H, m, 3' and 4'H) and 7.35 (1H, m, 5'H); m/e (positive FAB; water / glycerol) 172 $(M+1)^+$.

β-2-Thienylserines (3b) + (4b): δ (^2H_2O) 3.87 (d, J = 3.51, α-H, *erythro* isomer); 3.97 (d, J = 3.84, α-H, *threo* isomer), 5.39 (d, J = 3.83 β-H, *threo* isomer), 5.46 (d, J = 3.5, β-H, *erythro* isomer), 6.92 (2H, m, 3' and 4'H), and 7.27 (1H, brd, 5'H); m/e (positive FAB; water / glycerol) 188 $(M+1)^+$.

β-Hydroxyisohistidines (3c) + (4c): δ (^2H_2O) 3.98 (d, J = 4.1, α-H, *threo* isomer), 4.03 (d, J = 3.4, α-H, *erythro* isomer), 5.33 (1H, m, β-H, both isomers) and 7.07-7.23 (3H, aromatics); m/e (positive FAB, water / glycerol) 172 $(M+1)^+$.

β-Phenylserines (3d) + (4d): δ (^2H_2O) 3.75 (d, J = 4.3, α-H, *threo* isomer), 3.93 (d, J = 4.1, α-H, *erythro* isomer), 5.14 (d, J = 4.3, β-H, *threo* isomer), 5.19 (d, J = 4.1, β-H, *erythro* isomer), and 7.21-7.31 (5H, m, aromatics), m/e (positive FAB, glycerol / water) 182 $(M+1).^+$

β-Hexylserines (3e) + (4e): δ (^2H_2O) 0.75 (3H, brd, CH_3), 1.17-1.38 (10H, $(CH_2)_5$), 3.03 (d, J = 4.3, α-H, *erythro* isomer), 3.22 (d, J = 4.5, α-H, *threo* isomer) and 3.68-3.71 (1H, m, β-H, both isomers); m/e (positive CI, NH_3) 190 $(M+1)^+$.

β-Cyclohex-4-enylserines (3f) and (4f): δ (^2H_2O) 1.15-2.15 (m, CH_2), 3.79-3.85 (α,β- H) and 5.64 (2H, m, olefinic); m/e (positive FAB, glycerol / water) 186 $(M+1)^+$.

Synthesis of the Oxazolidinones (5) and (6) - General:
A solution of the β-hydroxyaminoacid (15 mg) in 1 M aqueous KOH (5 ml) was cooled to 0-5 °C and treated with a 12.5% solution of phosgene in toluene (6 ml) with stirring. Stirring was continued for a further 1 hour at 0-5 °C and the aqueous layer was extracted with ethyl acetate. The combined organic layers were dried (Na_2SO_4) and the solvent was removed *in vacuo* to yield the oxazolidinone which was analysed by ^1H-nmr spectroscopy without further purification.

The Oxazolidinone (6, R = Me) was prepared from L-allothreonone δ ($[^2H_4]$-methanol): 1.28 (3H, d, J = 6.5), 4.48 (1H, d, J = 8.6, α-H) and 4.98 (1H, q, β-H). Other absorptions were present.

The Oxazolidinone (5d) was prepared from *threo*- DL-β-phenylserine; δ ($[^2H_4]$-methanol) 4.05 (1H, d, J = 4.8, α-H), 4.8 (1H, d, J = 4.8, β-H) and 7.1 (5H, m, aromatics). Other absorptions were present.

The Oxazolidinones (5a) + (6a) were prepared from the enzymatically synthesised mixture **(3a) + (4a)**. The major isomer had δ ($[^2H_4]$-methanol) 4.60 (d, J = 5.2, α-H) and 5.66 (d, J = 5.2, β-H) and the minor isomer had δ ($[^2H_4]$-methanol) 4.69 (d, J = 9.5, α-H). Other absorptions were present.

Treatment of β-Hydroxyisohistidines (3c) + (4c) with L-Amino Acid Oxidase:
The enzymatically prepared β-hydroxyisohistidines **(3c) + (4c)** (3 mg) were dissolved in TRIS.HCl buffer (1 ml, 0.1 mmol/ml, pH 8.5) and incubated with L-amino acid oxidase (Sigma, EC 1.4.3.2; 10 mg; 0.5 units/mg) at 30 °C for 9 days. The reaction was terminated by addition of 10% aqueous trichloroacetic acid (0.1 ml) and the precipitated protein was removed by centrifugation at 800 g for 20 min. The supernatant was freeze-dried and

dissolved in distilled water (1 ml). Samples (0.1 ml) were diluted to 2 ml with distilled water and a 0.2% w/v solution of ninhydrin in ethanol (1 ml) was added. The mixture was heated to near boiling for 15 min. and the absorbance at 570 nm was compared to a standard curve calibrated using known quantities of D-alanine. The estimated amount of amino acid present was 0.071 mg.

Treatment of β-Hydroxyisohistidines (3c) + (4c) with D-Amino Acid Oxidase:

The mixture of (3c) + (4c) (3 mg) was dissolved in TRIS.HCl buffer (1 ml, 0.1 mmol/ml, pH 8.5) and incubated with D-amino acid oxidase (Sigma, EC 1.4.3.3; 10 mg; 0.8 units/mg) at 30 °C for 9 days. The reaction was terminated by addition of 10% aqueous trichloroacetic acid (0.1 ml) and the precipitated protein was removed by centrifugation at 800 g for 20 min. The supernatant was freeze-dried and dissolved in distilled water (1 ml). Samples (0.1 ml) were diluted to 2 ml with distilled water and a 0.2% w/v solution of ninhydrin in ethanol (1 ml) was added. The mixture was heated to near boiling for 15 min. and the absorbance at 570 nm was compared to a standard curve calibrated using known quantities of L-alanine. The assay indicated that *ca.* 2.75 mg of amino acid were present.

Cleavage of the β-Phenylserines (3d) + (4d) using Serine Hydroxymethyltransferase:

Enzymatically prepared β-phenylserines (3d) + (4d) (25 mg; *threo : erythro* ratio = 1.5:1) were dissolved in HEPES buffer [pH 7.5, 2 ml of a solution made up from HEPES (130 mg), Na_2SO_4 (35.5 mg), EDTA (3.72 mg) in distilled water (10 ml)]. A solution of serine hydroxymethyltransferase (0.2 ml) was added and the mixture was incubated at 30 °C for 2 hours. The reaction was stopped by addition of 10% aqueous trichloroacetic acid (0.5 ml) and the precipitated protein was removed by centrifugation at 800 g for 20 min. The supernatant was freeze-dried and analysed using ^{1}H-nmr spectroscopy in ^2H_2O solution. The ratio of *threo : erythro* isomers was now 1.7 : 1. Incubation for 24 hours resulted in complete loss of amino acids. Similar results were obtained when the β-hydroxyisohistidines (3c) + (4c) were reacted.

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