# Heterocyclic β-hydroxy-α-amino acids as substrates for a novel aldolase from *Streptomyces amakusaensis*; preparation of (2*R*,3*R*)-3-(2-thienyl)serine and (2*R*,3*R*)-3-(2-furyl)serine from racemic *threo* material

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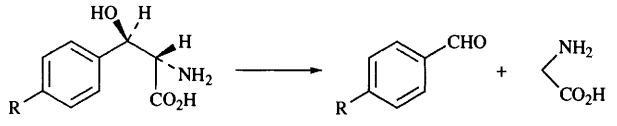
**A novel aldolase from *Streptomyces amakusaensis* which catalyses a reverse aldol reaction on (2*S*,3*R*)-β-hydroxy-α-amino acids is specific for aromatic compounds, but otherwise shows broad substrate tolerance. This is further illustrated by its ability to catalyse the cleavage of two aromatic heterocyclic compounds, namely (2*S*,3*S*)-3-(2-thienyl)serine 10 and (2*S*,3*S*)-3-(2-furyl)serine 12 at similar (but lower) rates to that of 3-phenylserine 2. This cleavage is stereospecific and leads to the preparation of optically pure (2*R*,3*R*)-3-(2-thienyl)serine 14 and (2*R*,3*R*)-3-(2-furyl)serine 15 from readily synthesised *threo* material. Evidence is presented that the aldolase is neither a serine hydroxymethyltransferase nor a threonine aldolase.**

In the course of a biosynthetic study[1] a quite novel aldolase was discovered in *Streptomyces amakusaensis* which catalyses a stereospecific reverse-aldol reaction on a broad range of aromatic (benzenoid) (2*S*,3*R*)-β-hydroxy-α-amino acids. Thus, for example, (2*S*,3*R*)-3-(4-hydroxyphenyl)serine **1** was efficiently cleaved by crude enzyme to give 4-hydroxybenzaldehyde **4** and glycine **6** (Scheme 1); other stereoisomers of **1** were found to be essentially inert to the enzyme.[2,3] Notably similar aldolase activity has been found in two other *Streptomyces* species.[4,5]



**1**; R = OH
**2**; R = H
**3**; R = H, racemate

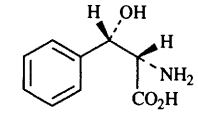
**4**; R = OH
**5**; R = H

**6**

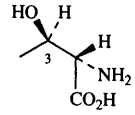
**Scheme 1**

We have now extensively purified the aldolase and checked the previously reported[2] high discrimination between *threo* and *erythro* stereochemistry. For accurate comparison of relative $V_{max}$ values and the recording of $K_M$ data, we have used a coupled enzyme system in which the aldehyde (as **5**, Scheme 1) generated in the aldolase reaction, was reduced by horse liver alcohol dehydrogenase in the presence of NADH; assay was by reduction in UV absorbance for NADH at 340 nm. With the purified enzyme we find a very large difference in relative rates for *threo*-3-phenylserine **3** and *erythro*-3-phenylserine **7** (respectively, $V_{max}$ = 100 and 9% and this is matched by a twelve-fold difference in yield measured by HPLC after 30 min). This confirms high stereoselectivity for the *threo*-3-phenylserine **3** as against the *erythro* isomer **7**. The stereochemistry observed is thus notably different from serine hydroxymethyltransferase (E.C. 2.1.2.1), which shows selectivity for the *S*-configuration at C-2 but poor *threo–erythro* discrimination.[6,7] From other evidence[2,3] we know that only the (2*S*,3*R*)-enantiomer of the *threo* compound is cleaved.

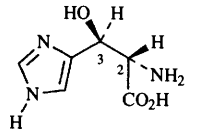
L-Threonine **8** (2*S*,3*R* configuration, as in **1**) has been examined, over the pH range 6–10, as a substrate for the *Streptomyces* aldolase and has been found to be very slowly



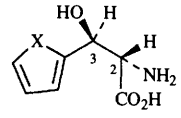
**7**; racemate



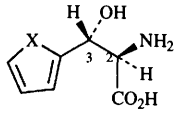
**8**



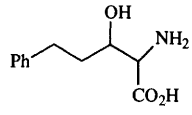
**9**



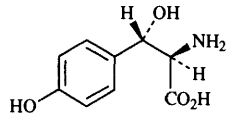
**10**; X = S
**11**; X = S, racemate
**12**; X = O
**13**; X = O, racemate



**14**; X = S
**15**; X = O



**16**



**17**

converted into reverse aldol product (0.06–0.25% in 2 h; *cf. threo*-3-phenylserine **3** where the conversion was 42% in 2 h under similar conditions). L-*allo*-Threonine (as **8**, 2*S*,3*S* configuration) was also a very poor enzyme substrate. We conclude that the *Streptomyces* enzyme is not a threonine aldolase (E.C. 4.2.1.5).

It is to be noted that the *Streptomyces* aldolase is inactivated on treatment with sodium borohydride[5] and that the activity of the enzyme, after purification and extensive dialysis, can be enhanced by the addition of pyridoxal 5-phosphate to incubation mixtures. From this we conclude that the aldolase is pyridoxal 5-phosphate-dependent, but rigorous co-factor identification awaits further results.

(2*S*,3*S*)-3-(Imidazol-4-yl)serine **9** has been found to be a satisfactory reverse aldol substrate with aldolase from *S. amakusaensis* and a poor substrate with enzyme from *S. tendae*.[4,5] We were therefore interested to examine other aromatic heterocyclic compounds, namely 3-(2-thienyl)serine **10** and 3-(2-furyl)serine **12**, as substrates for the *S. amakusaensis* enzyme. The racemic *threo* isomer **13** of 3-(2-furyl)serine was

prepared by a published method [8,9] and this was simply adapted for the synthesis of *threo*-3-(2-thienyl)serine **11**.[10,11] Examination of these compounds by $^1$H NMR spectroscopy confirmed that they were pure *threo* isomers. Both, when tested with the partially purified aldolase, were found to be good substrates undergoing cleavage to aldehyde plus glycine at a comparable rate (see below) to that of the racemate, **3**. Appropriate to anticipated stereospecificity a maximum conversion approaching 50% was observed, *i.e.* cleavage of only **10** and **12** in each of the racemates **11** and **13**, respectively. The evidence we have thus far obtained then indicates that only aromatic β-hydroxy-α-amino acids are substrates for the aldolase (*cf.* the aliphatic amino acids, threonine and *allo*-threonine, above), but significantly this aromaticity does not need to be benzenoid; heterocyclic rings are equally acceptable. It appears that the β-hydroxy group needs to be benzylic (or equivalent) since **16** is a poor substrate.[2] Furthermore, the reaction is stereospecific with both benzenoid (*cf.* refs. 2 and 3) and heterocyclic aromatic β-hydroxy-α-amino acids.

The preparation of homochiral β-hydroxy-α-amino acids is a worthwhile exercise since these compounds are found as constituents of a range of antibiotics,[12,13] *e.g.* vancomycin,[12] and are naturally occurring in their own right, *e.g.* (2*S*,3*S*)-3-(imidazol-4-yl)serine **9**.[14] The *S. amakusaensis* aldolase has been used to prepare, in good yield and with high enantiomeric purity, the (2*R*,3*S*)-isomers of representative benzenoid β-hydroxy-α-amino acids from racemic *threo* starting material.[3] The (2*S*,3*R*)-isomer undergoes aldolase-catalysed cleavage leaving the pure (2*R*,3*S*)-isomer behind. We have now used this procedure for the preparation of (2*R*,3*R*)-3-(2-thienyl)serine **14** and (2*R*,3*R*)-3-(2-furyl)serine **15** (the 3*R* configuration here corresponds to 3*S* in the benzenoid series) starting with the chemically synthesized *threo* racemates **11** and **13**, respectively. The reactions were readily carried out with partially purified enzyme on a useful scale and the products that were isolated in good yield were in excess of 95% optically pure. Optical purity was assessed by conversion of each compound into its *N*-camphanoyl derivative which was analysed by $^1$H NMR spectroscopy (the *N*-camphanoyl derivative of each racemic β-hydroxy-α-amino acid was used for comparison; clear differences in the chemical shifts of the diastereotopic α-protons were observed). The correctness of the configurations was deduced from the known stereospecificity of the aldolase[2] and this was supported by CD studies in which the absorption curves of **14** and **15** were found to be closely similar (see Fig. 1) to that of (2*R*,3*S*)-3-(4-hydroxyphenyl)serine **17** with established absolute configuration.[3] This was supported by comparison of the optical rotation of **15** with that obtained by others using a chemical method to prepare the enantiomer of **15**.[15] It follows that the enzyme catalyses the cleavage of β-hydroxy-α-amino acids with the same stereochemistry (and specificity) whatever the type of aromatic substituent.

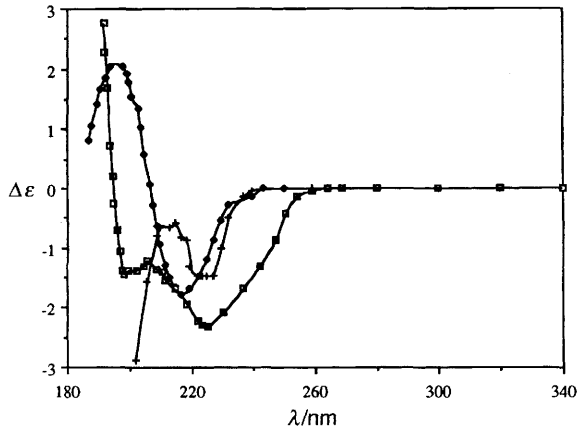



**Fig. 1** CD spectra of (2*R*,3*S*)-3-(4-hydroxyphenyl)serine **17** (+), (2*R*,3*R*)-3-(2-thienyl)serine **14** (◆) and (2*R*,3*R*)-3-(2-furyl)serine **15** (□)

The easy preparation of **15** in homochiral form is particularly valuable because β-hydroxymethylfurans find wide application as starting materials for organic synthesis.[16] Versatility is extended in the ready availability of **15** with an amino acid function and single stereochemistry at each of C-2 and C-3.

The natural substrate of the aldolase is obscure although 3-phenylserine **2** is an obvious candidate. In this regard we were interested to compare the relative $V_{max}$ and $K_M$ values for the racemic *threo* compounds, **3**, **11** and **13**. The latter two cannot reasonably be natural aldolase substrates but the binding ($K_M$ value) of each of the three compounds is similar (respectively, 4.52, 5.63 and 5.13 mM) and the relative rates were not enormously different (100, 61 and 26%). Further work is in hand on this problem.

## Experimental

Apparatus and general procedures were as published.[3] All $^1$H NMR spectra were obtained at 300 MHz on a General Electric Nicolet QE 300 spectrometer; *J* values are given in Hz. CD spectra were measured in aqueous solution (0.02 mg cm$^{-3}$) on a Jasco J720 spectropolarimeter. $[\alpha]_D$ Values are given in units of $10^{-1}$ deg cm$^2$ g$^{-1}$. Enzymes and coenzymes were purchased from Sigma.

### Purification and assay of *S. amakusaensis* aldolase

Aldolase was obtained from cultures of *S. amakusaensis* as previously reported[3] except that after sonication the protein was precipitated by acetone instead of ammonium sulfate. AR acetone was slowly added to the cell-free preparation produced after sonication in phosphate buffer at 4 °C to a final concentration of 10% v/v. After standing in ice for 30 min the precipitated protein was removed by centrifugation at 4 °C and 23 000*g* for 15 min. Acetone was added to the supernatant as above to a final concentration of 20% v/v. The precipitated protein was collected by centrifugation and resuspended in 20 mM phosphate buffer, pH 7.4, containing 0.1 mM dithiothreitol, 0.1 mM toluene-α-sulfonyl fluoride and 0.1 mM ethylenediaminetetraacetic acid (EDTA). This mixture was repeatedly dialysed against the same buffer at 4 °C. This acetone cut had the highest aldolase activity (approximately 1.0 units per mg protein, 2.5 units per cm$^3$ of enzyme solution) and was used to prepare **14** and **15**. 1 l of *S. amakusaensis* culture gave 200 cm$^3$ of sonicate and 20 cm$^3$ of final solution containing approximately 24 units of aldolase.

Enzyme activity was assayed as follows. Aldolase solution (0.5 cm$^3$) was added to a solution (1.0 cm$^3$) of racemic *threo*-3-(4-hydroxyphenyl)serine (3.8 mM) (as **1**) in sodium phosphate buffer (20 mM; pH 7.4) at 30 °C. The production of 4-hydroxybenzaldehyde **4** was measured[3] by HPLC at 281 nm, after 30 min incubation. One unit (U) of enzyme activity is defined as resulting in the formation of 1.0 μmol of **4** per h under these conditions.

More highly purified enzyme was obtained as follows. Ammonium sulfate addition (25–50% saturation) after sonication was used as a crude precipitation procedure. This fraction was then dialysed free of salt against an excess of 20 mM tricine buffer, pH 8.0 and then further purified by ion exchange chromatography using Q Sepharose 6FF resin, eluting the aldolase with a linear salt gradient (0–1 M NaCl in 20 mM tricine buffer, pH 8.0). Aldolase active fractions were then dialysed against an excess of 10 mM tricine buffer, pH 8.0, and finally purified by dye-ligand chromatography on Reactive Red 120 resin (Sigma) eluting with a step salt gradient (0–1 M NaCl in 10 mM tricine buffer, pH 8.0) to give a highly purified, active, aldolase sample (batch a: 56 μg protein per cm$^3$, 4.27 U cm$^{-3}$;

batch b: 23 μg protein per $cm^3$, 3.25 U $cm^{-3}$). These preparations were used to determine $K_M$ and relative $V_{max}$ values.

**Aldolase experiments with L-threonine 8 and L-*allo*-threonine**

Two methods were used to test the reaction of aldolase with these two amino acids. The first method used a Conway diffusion cell.[17] The inner well contained the following aqueous solutions: sodium phosphate buffer (2 $cm^3$, 0.15 M, pH 7.6), substrate (0.25 M, 0.25 $cm^3$), pyridoxal 5-phosphate (3 mM, 0.10 $cm^3$), aldolase (2.1 mg protein per $cm^3$, 0.5 U $mg^{-1}$, 0.15 $cm^3$); the outer well contained semicarbazide hydrochloride (6.7 mM, 3 $cm^3$). Acetaldehyde is the product of any reverse aldol reaction from threonine and *allo*-threonine and its formation as its semicarbazone was assayed after 15, 30, 60 and 120 min by UV examination (225 nm) of the contents of the outer well (after heating at 100 °C). Conversion was *ca.* 1% for threonine and *ca.* 2% for *allo*-threonine after 120 min.

The second method used a variation on a published procedure[7] which involved coupling the aldolase reaction with yeast alcohol dehydrogenase (YALD), the conversion of NADH (the cofactor for the latter enzyme) to NAD associated with the reduction of acetaldehyde was monitored by UV at 340 nm. The reaction mixture contained, at the appropriate pH values; potassium phosphate buffer (40 mM, 0.10 $cm^3$), YALD (62.5 U $cm^{-3}$, 0.10 $cm^3$), NADH (2.0 mM, 0.10 $cm^3$) and L-threonine (0.10 M, 0.50 $cm^{-3}$). To this was added the purified aldolase solution (3.25 U $cm^{-3}$, 0.20 $cm^3$) and the absorbance decrease was measured over 2 h at 30 °C: pH 6 (0.25% conversion), 7 (0.21%), 8 (0.18%), 9 (0.09%) and 10 (0.06%). Spiking the reaction mixture with acetaldehyde and observing the NADH to $NAD^+$ conversion confirmed that the method was working.

**Aldolase experiments with the substituted serines, 3, 7, 11 and 13**

The method used to determine the rate constants ($V_{max}$ and $K_M$) for compounds **3**, **7**, **11** and **13** was similar to that for L-threonine above, except that horse liver alcohol dehydrogenase (HLADH) was substituted for YALD and initial rates were determined at increasing substrate concentrations. The reaction mixture contained potassium phosphate buffer, pH 7.2 (1.0 M, 0.017 $cm^3$), HLADH (70.0 U $cm^{-3}$, 0.01 $cm^3$), NADH (2.0 mM, 0.10 $cm^3$) and substrate (concentrations: 0–50 mM). To this was added purified aldolase solution (either batch a: 4.27 U $cm^{-3}$, 0.20 $cm^3$ or batch b: 3.25 U $cm^{-3}$, 0.10 $cm^3$), the assay cuvette having a final volume of 1.0 $cm^3$. Batch a of protein: *threo*-phenylserine **3** (rel. $V_{max} = 100\%$; $K_M = 4.52 \pm 0.44$ mM; corresponds to the formation of 9.12 nmol product per min per μg protein), *threo*-3-(2-thienyl)serine **11** (rel. $V_{max} = 61\%$; $K_M = 5.63 \pm 0.99$ mM) and *threo*-3-(2-furyl)serine **13** (rel. $V_{max} = 26\%$; $K_M = 5.13 \pm 1.95$ mM). Batch b of protein: *threo*-3-phenylserine **3** (rel. $V_{max} = 100\%$; $K_M = 3.21 \pm 0.40$ mM; corresponds to the formation of 24.57 nmol product per min per μg protein) and *erythro*-3-phenylserine **7** (rel. $V_{max} = 9\%$; $K_M = 5.78 \pm 1.22$ mM).

All the above assays were performed at 30 °C and the decrease in $A_{340}$ was recorded (Kontron, Uvikon 930 spectrophotometer). In order to determine the steady-state kinetic parameters, data were fitted to the appropriate rate equations using the computer program Kaleidagraph (Abelbeck Software).

Racemic *threo*-3-phenylserine **3** and *erythro*-3-phenylserine **7** (both 3.68 mM overall, 0.40 $cm^3$) were incubated separately with purified aldolase (batch b, 0.20 $cm^3$) at 30 °C for 30 min. The reaction was terminated by the addition of 0.01 $cm^3$ of conc. HCl and the aldol product, benzaldehyde, was measured by HPLC at 248 nm.[3] A conversion of 24% was observed for the *threo*-compound, compared to 2% for the *erythro*-isomer.

Racemic *threo*-3-phenylserine **3** was prepared as previously described,[18] $\delta_H(D_2O)$ 7.48 (5 H, m), 5.31 (1 H, d, *J* 4.5), 3.92 (1 H, d, *J* 4.5). The *erythro*-isomer **7** was isolated by repeated recrystallization of the mother liquors obtained after recrystallization of the *threo*-isomer; $\delta_H(D_2O)$ 7.45 (5 H, m), 5.36 (1 H, d, *J* 4.1), 4.10 (1 H, d, *J* 4.1).

**Preparation of (2*R*,3*R*)-3-(2-thienyl)serine 14 and (2*R*,3*R*)-3-(2-furyl)serine 15**

A simple comparison of the rates of cleavage of **11** and **13** with that of racemic **1** was obtained with a solution (1.5 $cm^3$) of racemic samples of each compound (3.6 mM) in phosphate buffer with aldolase (0.9 U $cm^{-3}$) at 30 °C; the appropriate aldehyde was assayed by HPLC. Similar conversions of 30–45% in 4 h were observed.

For the preparation of optically pure (2*R*,3*R*)-3-(2-thienyl)-serine **14** and (2*R*,3*R*)-3-(2-furyl)serine **15** from racemic *threo* material the following procedure was followed. The appropriate amino acid (2 mmol) was dissolved in 20 mM sodium phosphate buffer (15 $cm^3$, pH 7.4). To this was added the partially purified (acetone cut) *S. amakusaensis* enzyme solution (15 $cm^3$). The reaction was incubated at 30 °C for 8–10 h and it was monitored for the aldehyde product by HPLC. A further quantity (10 $cm^3$) of enzyme solution was added to the incubation mixture and this was incubated for a further 14–16 h or until a 50% conversion to product aldehyde had been achieved.

The reaction was terminated by the addition of 10% aqueous trifluoroacetic acid (2 $cm^3$). The mixture was stirred for 1 min and then centrifuged at 38 000*g* for 30 min. The supernatant was evaporated *in vacuo*, the residue was dissolved in water (10 $cm^3$) and the pH was adjusted to 6.5–7.0 (1.5 M NaOH). The solution was chromatographed on Dowex 1-X8-400 ion exchange resin eluting first with water (200 $cm^3$) and then a stepwise gradient of aqueous acetic acid (0.1–1.0 M, in 50 $cm^3$ portions). Amino acid mixed with glycine was obtained in the early fractions (analysis by NMR spectroscopy and TLC on Kieselgel $60F_{254}$, using ethanol–water, 2:1 with ninhydrin for visualisation). Further purification was achieved by chromatography on 'flash' silica, eluting with ethanol–water, 2:1. The resolved amino acids were thus obtained pure; they could be recrystallised from water–ethanol.

**(2*R*,3*R*)-3-(2-Thienyl)serine 14.** Yield from the aldolase reaction: 85% (before recrystallisation), mp 170–172 °C, $[\alpha]_D^{25}$ +55.1 (*c* 0.7, $H_2O$), $\delta_H(D_2O)$ 7.46 (1 H, d, *J* 4.5), 7.16 (1 H, d, *J* 4), 7.11 (1 H, dd, *J* 4, 4.5), 5.5 (1 H, d, *J* 4.5, 3-H), 3.99 (1 H, d, *J* 4.5, 2-H). Racemic material: mp 174–175 °C (lit.,[10] 183 °C); NMR spectrum identical to that of **14**; *m/z* (FAB) 188 ($M^+ + 1$), 170, 126 (Found: C, 41.1; H, 5.3; N, 6.7. Calc. for $C_7H_9NO_3S{\cdot}H_2O$: C, 41.0; H, 5.4; N, 6.8%).

**(2*R*,3*R*)-3-(2-Furyl)serine 15.** Yield from the aldolase reaction: 79% (before recrystallisation), $[\alpha]_D^{25}$ +72.6 (*c* 0.2, $H_2O$); $\delta_H(D_2O)$ 7.48 (1 H, apparent s), 6.41 (1 H, unresolved), 5.20 (1 H, d, *J* 4.5, 3-H) (*cf.* ref. 15 where different $\delta$ values are given for the furyl protons of the (2*S*,3*S*) material and $[\alpha]_D^{25}$ of −26.9). Racemic material: mp 200–202 °C (lit.,[11] 197 °C) NMR spectrum identical to that of **15**; *m/z* (FAB) 172 ($M^+ + 1$), 110 (Found: C, 49.1; H, 5.3; N, 8.1. Calc. for $C_7H_9NO_4$: C, 49.1; H, 5.3; N, 8.2%).

***N*-Camphanoyl derivatives of 11, 13, 14 and 15.** To a solution of the β-hydroxy-α-amino acid (0.18 mmol) and sodium hydroxide (0.36 mmol) in water (0.5 $cm^3$) was added a solution of (*S*)-(−)-camphanic chloride (Aldrich) (0.22 mmol) in toluene (0.5 $cm^3$) and the mixture was shaken intimately for 4 h at room temperature. The mixture was neutralized and washed with chloroform (2 × 10 $cm^3$) then acidified and extracted with chloroform (3 × 15 $cm^3$). The combined extracts were dried ($Na_2SO_4$) and evaporated to leave the *N*-camphanoyl derivative as a white solid.

The derivative of racemic 3-(2-thienyl)serine **11**: $\delta_H(CDCl_3)$ 4.97 (0.5 H, dd, *J* 2, 8.5, 2-H), 5.05 (0.5 H, dd, *J* 2, 8.5, 2-H), 5.74 (1 H, d, *J* 2, 3-H); (2*R*,3*R*) material **14** showed the dd at

4.97, the one at 5.05 being undetectable. The derivative of racemic 3-(2-furyl)serine **13**: $\delta_H(CDCl_3)$ 5.05 (0.5 H, dd, *J* 2, 8.5, 2-H), 5.09 (0.5 H, dd, *J* 2, 8.5, 2-H), 5.46 (1 H, d, *J* 2, 3-H), (2*R*,3*R*) material **15** showed the dd at 5.05, the one at 5.09 being undetectable. (The larger coupling in each case is to NH.)

## Acknowledgements

We thank Dr Alex Drake and the EPSRC service at University College, London for providing us with CD spectra, and Dr Alan Berry, Department of Biochemistry and Molecular Biology, University of Leeds for unstinting and invaluable advice. We are grateful to Sanofi and EPSRC for financial support in a CASE Studentship (to M. B.).

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*Paper* 6/03489F
*Received* 20*th May* 1996
*Accepted* 2*nd July* 1996