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PENICILLIN BIOSYNTHESIS: DEPENDENCE OF SUBSTRATE STEREOCHEMISTRY ON THE MODE OF SECOND RING CLOSURE

Jack E. Baldwin*, Robert M. Adlington, Christopher J. Schofield and Hong-Hoi Ting

The Dyson Perrins Laboratory and Oxford Centre for Molecular Sciences, University of Oxford, South Parks Road, Oxford, OX1 3QY, U.K.

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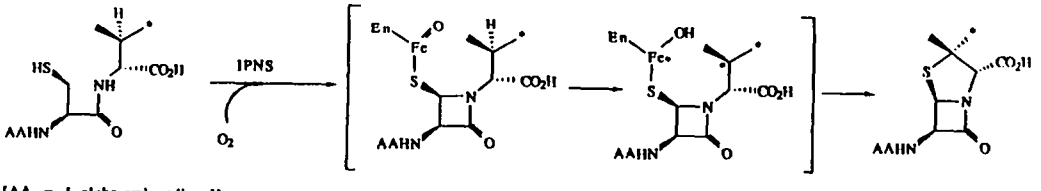
Abstract:
The stereochemical requirements for the conversion of tripeptides with unsaturated amino acids in the C-terminal position to bicyclic β-lactam products using isopenicillin N synthase was investigated using diastereomeric peptides containing 2R,3S-2-amino-3-methylpent-4-enoic acid and 2R,2R-2-amino-3-methylpent-4-enoic acid.+

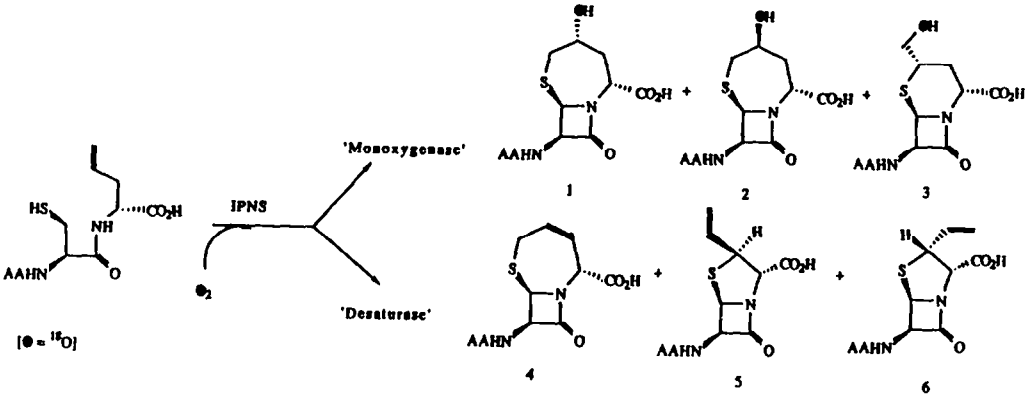
Mechanistic studies on the ferrous dependent oxygenase isopencillin N synthase (IPNS) have led us to propose the reaction sequence shown in Scheme 1, which proceeds *via* an enzyme-bound intermediate β-lactam-iron-oxene species or its equivalent.[1] This reactive entity can mediate the second ring closure with retention of stereochemistry by way of a rotationally restricted free radical equivalent or Fe-carbon bond at the 3-valinyl site.[2] Thus, replacement of the valine isopropyl group with an ethyl group, i.e. in the analogous α-aminobutyrate peptide, permitted rotation of the intermediate radical and resulted in both retention and inversion in the carbon-sulphur bond forming step, providing an epimeric mixture of norpenicillins.[1,3] Further characterisation of the reactivity of the proposed iron-oxene species was obtained through replacement of valine by the unsaturated amino acid allyl glycine, which gave both "desaturase" (-4H) as well as products containing a hydroxyl moiety, resulting from "monooxygenase" (-2H + O) pathways,[4] Scheme 2.



Scheme 1

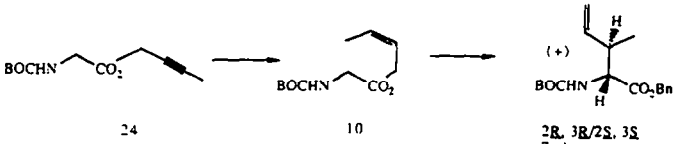
This work has been published in a preliminary form: J.E. Baldwin, R.M. Adlington, C.J. Schofield, and H.-H. Ting, J. Chem. Soc., Chem. Commun., 1989, 758.

The oxygenase products (1), (2) and (3) were attributed to [2 + 2] cycloaddition of the iron-oxene moiety to the double bond of the allyl residue,[5] the homocephem (4) to an ene-type reaction and the penams (5) and (6) to the normal desaturative closure.[6] In order to establish whether stereochemical effects were responsible for the multiple pathways shown in Scheme 2 we devised the following experiments. Since chain braching at the β-position of the C-terminal aminoacid apparently impeded rotation of this entity, vide supra, presumably a 3-methyl substituent in the allyl glycine unit would similarly restrict the active site conformations of the entity. Since such a substituent could exist in two distinct diastereotopic forms, each of which would present different groups to the reactive iron-oxene centre, then the products of these reactions might reveal the stereochemical requirements for the various possible reaction pathways.



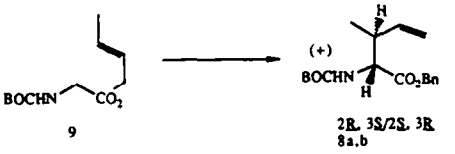
Scheme 2

Suitably protected forms of the desired 2R,3R/2S,3S (7a,b) and 2R,3S/2S,3R (8a,b) 3-methylallylglycines were prepared from the appropriate crotonyl esters of N-^{t}butoxycarbonyl glycines (10) and (9) using the Ireland-Claisen rearrangement essentially as reported by Bartlett (Schemes 3,4).[7]

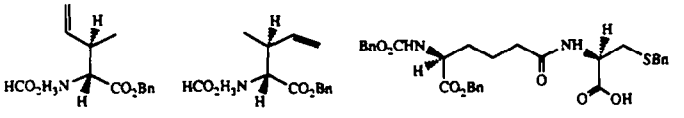


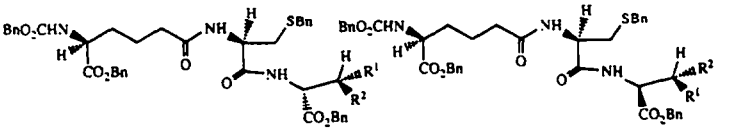
Scheme 3

These acids were esterified and purified. The esters were N-deprotected to give the formate salts (11) and (12) which were separately coupled[8] with protected L-α-aminoadipoyl-L-cysteine (13) to give the diastereomeric tripeptrides (14a,b) and (15a,b) respectively, which were separated by chromatography [i.e. (14a) from (14b) and (15a) and (15b)] (see experimental section for details).



Scheme 4

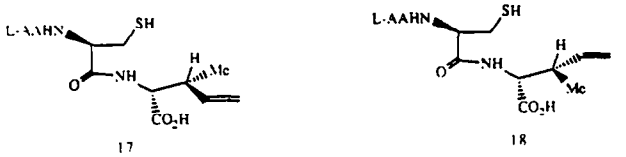




14a, R^1 = Me, R^2 = CH=CH$_2$
15a, R^1 = CH=CH$_2$, R^2 = Me
16a, R^1 = Me, R^2 = Et
16b, R^1 = Et, R^2 = Me

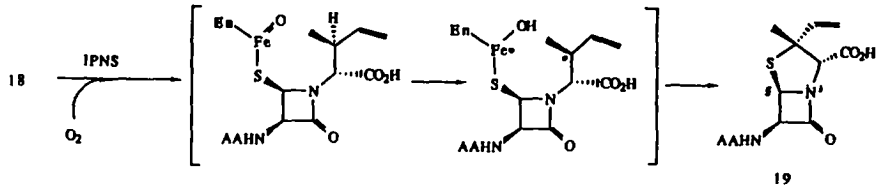
14b, R^1 = Me, R^2 = CH=CH$_2$
15b, R^1 = CH=CH$_2$, R^2 = Me

Confirmation of the stereochemistry of (14a) and (15a) followed from comparison, after reduction [H_2 (1 atm), $(Ph_3P)_3RhCl$] to suitably protected form of the isoleucinyl (16a) and allo-isoleucinyl (16b) tripeptides respectively.[9] (14a) was deprotected to (17) and (15a) to give (18) (Na,NH_3)[8].



L-AAHN = L-α-aminoadipoyl

The peptide (18), with 2R,3S stereochemistry, gave upon incubation with a highly purified form of the IPNS enzyme[10] from Cephalosporium acremonium CO 728, the 2-α-vinyl penam (19), which was purified by reverse phase h.p.l.c., as the only observed β-lactam containing product [no other signals (J = ca 4 Hz) were observed in the region δ_H 4.8 - 6.0 in the crude incubation mixture]. Formulation as the 2-α-vinyl penam followed from nuclear Overhauser enhancement (n.O.e.) experiments in which irradiation of the 2β-methyl group, δ_H 1.59, gave enhancement of 2-H (12%) but not to 5-H, and irradiation of the C<u>H</u>=CH_2 proton, δ_H 5.91, gave enhancement of 5-H (3%). (19) showed the expected penicillinase sensitive antibacterial activity (ca 85%) at equivalent molar concentration to isopenicillin N) against Staphylococcus aureus N.C.T.C. 6571.

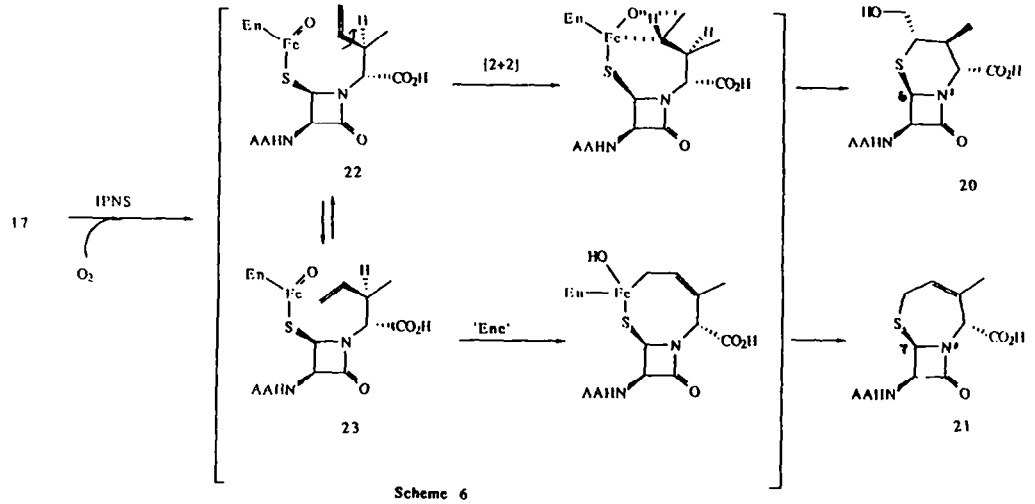


Scheme 5

The second tripeptide (17) (with 2R,3R stereochemistry) gave, upon similar incubation with IPNS and purification by h.p.l.c., the hydroxymethyl cepham (20) and the homoceph-3-em (21) without any detectable penam product [by ^1H n.m.r. or bioassay]. The α-stereochemistry of the hydroxymethyl group of (20) was consistent with n.O.e. experiments. Thus irradiation of the CH_2OH group, δ_H 3.68-3.84, gave n.O.e. to 6-H (8%) and 3-H (2%) whilst irradiation of 4-H, δ_H 2.73-2.81, gave no n.O.e. to 6-H. A β-C_3 methyl group was also consistent with the H_2, H_3 coupling constant whose magnitude (J < 1 Hz) has precendent in related cephams.[9] (20) showed no antibacterial activity versus S. aureus N.C.T.C. 6571 at a concentration of 100 μg ml^{-1}. The connectivity of (21) as S-CH_2-CH= was confirmed by proton decoupling experiments, and (21) showed no antibacterial activity against S. aureus N.C.T.C. 6571 at a concentration of 100 μg ml^{-1}.

The sharp contrast in product type between these two substrates and the allylglycine case, Scheme 2, is probably due to the restricted mobility of the 3-methyl allyl side chain in peptides (17) and (18) compared to that of the allyl glycine tripeptide case, reducing the number of possible geometrical relationships between the side chain and the putative iron-oxene reaction centre. Thus the conformational restriction around C_2-C_3, in the 3S

series (18) does not permit the [2 + 2] or the ene process since the vinyl group is remote for the reaction centre, Scheme 5. Consequently the "normal" desaturative process follows yielding a penam (19), with retention of configuration. In the 3R series (17), however, the vinyl function now is close to iron-oxene and the two conformations about the C_3 - C_4 bond, (22) and (23), permit the [2 + 2] and the ene reaction modes, respectively, Scheme 6, leading to the observed products (20) and (21). These results support the concept of a monocyclic intermediate, bonded directly to the iron-oxene centre, and provide further information concerning the geometrical relationships between these entities during catalytic turnover.



Scheme 6

Acknowledgement: We thank S.E.R.C. and Pfizer PLC, Sandwich, Kent, U.K. for support to C.J.S.

General Experimental

Standard experiment procedure as previously reported was followed[11]. Proton magnetic resonance spectra were recorded at 500 MHz on a Bruker AM-500 spectrometer (samples in $CDCl_3$ were referenced to residual $C\underline{H}Cl_3$, δ = 7.27 p.p.m., and internal sodium (2,2,3,3-$^2H_4$)-3-trimethylsilylpropionate = 0.00 p.p.m. for aqueous samples. Coupling constants, J, were measured to the nearest 0.5 Hz. Carbon magnetic resonance spectra were recorded at 50.3 MHz on a Varian Gemini 200 spectrometer. Infra-red spectra were recorded on a Perkin Elmer 681 spectrophotometer. Mass spectra were recorded on V.G. Micromass 30F spectrometer (Desorption Chemical Ionisation (D.C.I.)] or a V.G. Micromass ZAB-IF spectrometer (Fast Atom Bombardment (F.A.B.)]. Percentage intensities are recorded in parenthesis.

H.p.l.c. was performed using a Waters 510 gradient controller, two Waters M-6000A pumps, a Rheodyne 7125 injector, a Waters 441 absorbance detector (λ_{max} = 220 nm) and a column packed with Hypersil 5μ ODS (octadecylsilane) (250 x 4.6 mm internal diameter).

Preparation of partially purified IPNS

IPNS was prepared from C. acremonium CO 728 essentially as previously described[10]. One International Unit (I.U.) is defined as the amount of enzyme required to form 1.0 μmol of isopenicillin N from tripeptide per minute at pH 7.5 and 30°C with the relevant cofactors.

N-(t-Butoxycarbonyl)glycine-E-but-2-enyl ester (9)

To a solution of E-crotyl alcohol (2.68 g, 36.0 mmol), N-(t-butoxycarbonyl)glycine (6.30 g, 36.0 mmol) and dicyclohexylcarbodiimide (7.42 g, 36.0 mmol) in ether (70 ml) at 0°C was added 4-(dimethylamino)pyridine (ca 25 mg). The reaction was allowed to rise to 20°C (over 30 min), was stirred for 3 hours at 20°C and stored for 12 hours at 0°C. The precipitated dicyclohexylurea was removed by filtration. The filtrate was washed (2N hydrochloric acid, saturated sodium bicarbonate and brine), dried (sodium sulphate) and evaporated to dryness. The residue was distilled (bulb to bulb, 150°C, 0.2 mbar) to give (9), identical by ^{1}H n.m.r. to the literature material (5.96 g, 73% c.f. literature[7] 77%).

N-(t-Butoxycarbonyl)glycine but-2-ynyl ester (24)

Propargyl alcohol (1.12 g, 20 mmol) was coupled with N-(t-butoxycarbonyl)glycine (1.75 g 10 mmol) as for the preparation of (9) to give, after chromatography (flash silica, ethyl acetate/petrol) the glycine ester (24) Yield (1.84 g, 81%). tlc (50% ethyl acetate/hexane)Rf 0.5; υ_{max} ($CHCl_3$) 1715s(CO), 1640w, 1512m, 1365m, 1168s, 1055m, 955m; δ_H 1.45 (9H, s, t-Bu), 1.71-1.87 (3H, m, Me), 3.95 (2H, d, J 5 Hz, NCH_2), 4.7-7.73 (2H, m, CH_2O), 5.01-5.04 (1H, bm, NH); m/e (NH_3, DCI) 228 (11, MH^+) 171 (100, MH^+-C_4H_9).

N-(t-Butoxycarbonyl)glycine-Z-but-2-enylester (10)

A solution of glycine ester (24) (950 mg, 4.19 mmol) and quinoline (250 mg, 1.95 mmol) in methanol (20 ml) was hydrogenated over 5% palladium on barium sulphate until 90 ml of hydrogen was taken up (30 min). The catalyst was removed (celite) after which the solvent was removed in vacuo. The residue was dissolved in ether (25 ml), washed (2N hydrochloric acid, brine), dried (sodium sulphate) and evaporated to dryness. The residue was chromatographed (flash silica, ethyl acetate/hexane) to give (10) (921 mg, 96%) as an oil. tlc (50% ethyl acetate/petrol) Rf 0.5 υ_{max} ($CHCl_3$) 2990m, 1740s (CO), 1695s (CON), 1643w, 1512m, 1366m, 1167s, 1055m, 906m; δ_H 1.28 (9H, s, t-Bu), 1.58 (3H, d, J 5 Hz, CHMe), 3.77 (2H, d, J 6 Hz, NCH_2CO), 4.58 (2H, d, J 5 Hz, OCH_2), 5.34-5.47 (1H, m, C=CH), 5.50-5.61 (1H, m, C=CH), 5.62-5.74 (1H, bm, NH); m/e (NH_3DCI) 230 (8, MH^+), 173 (100, MH^+-C_4H_9).

(2R,3S and 2S,3R) (N-t-Butoxycarbonyl)-2-amino-3-methylpent-4-enoic acid benzyl ester (8a,b)

To a solution of E-glycine ester (9) (1.00 g, 4.37 mmol) in tetrahydrofuran (12 ml) at -78°C, was added a solution of lithium bis(trimethylsilyl)amide in tetrahydrofuran (9.6 ml of 1.0 molar solution) over 2 min. After stirring for 10 min, trimethylsilyl chloride (949 mg, 8.7 mmol) was added over 10 min and the solution was allowed to rise to 20°C (over ca 30 min). The reaction mixture was then heated to 55-60°C for 2 hours, cooled to 5°C and diluted with 10 ml of methanol. After stirring for 5 min, the mixture was evaporated to dryness. The residue was dissolved in 25 ml ether and extracted with 2N NaOH (4 x 10 ml); the combined aqueous layers were then acidified (5N HCl) and extracted with ethyl acetate (4 x 25 ml). The combined organic extracts were dried (sodium sulphate), filtered

and evaporated to dryness. The residue was then dissolved in dimethylformamide (10 ml) containing benzyl bromide (747 mg, 4.4 mmol), sodium bicarbonate (367 mg, 4.4 mmol) and sodium iodide (1 crystal). The reaction mixture was then stirred for 15 hours, after which the solvent was removed in vacuo, and the residue washed (2N hydrochloric acid, saturated sodium bicarbonate, and brine), and evaporated to dryness. The residue was chromatographed (flash silica, ethyl acetate/hexane) to give oil (531 mg, 38%). The diastereomeric ratio of the 2R,3S/2S,3R pair (8a,b) to the 2R,3R/2S,3S pair (7a,b) was judged by ^{1}H n.m.r. (300 MHz, integration of the CHMe resonances) to be >7:1).

Data for the 2R,3S/2S,3R (8a,b) pair: δ_H (300 MHz, $CDCl_3$) 1.01 (3H, d, J 6 Hz, CHMe), 1.44 (9H, s, t-Bu), 2.58-2.71 (1H, m, CHMe), 4.32-4.40 (1H, m, NCHCO), 5.01-5.27 (5H, m, C=CH_2, NH, CH_2Ph), 5.25-5.37 (1H, m, CH=C), 7.33-7.41 (5H, m, Ph); υ_{max} ($CHCl_3$) 3005m, 1730s(CO), 1710s(CO), 1600w, 1502m, 1205s, 1160m; m/e (NH_3, D.C.I.) 320 (100, MH^+), 91 (56).

(2R,3R and 2S,3S) (N-t-Butoxycarbonyl)-2-amino-3-methylpent-4-enoic benzyl ester (7a,b)

An analogues procedure to that used for the preparation of (8a,b) applied to Z-glycine ester (10) (478 mg, 2.08 mmol) gave after chromatography, a mixture of the 2R,3R/2S,3S (7a,b) and 2R,3S/2S,3R (8a,b) diastereomeric pairs with a ratio of ca 4:1. Yield (382 mg, 58%).

Data for the 2S,3S/2R,3R (7a,b) pair: δ_H (500 MHz, $CDCl_3$) 1.08 (3H, d, J 6 Hz, CHMe), 1.45 (9H, s, t-Bu), 2.73-2.81 (1H, m, CHMe), 4.32-4.39 (1H, m, NCHCO), 4.93-5.24 (5H, m, CH=CH_2, NH,CH_2Ph), υ_{max} ($CHCl_3$) 3005m, 1732s(CO), 1710s(CO), 1600w, 1502m, 1205s, 1160m; m/e (NH_3, DCl) 320 (100, MH^+), 91 (56).

[(S)-(N-Benzyloxycarbonyl)-(α-benzyl)-δ-(α-aminodipoyl)]-[(S)-S-benzyl-cysteinyl]-[2R,3R]-2-amino-3-methylpent-4-enoic acid benzyl ester] (14)

Diprotected amino acid (7a,b) (2R,3R/2S,3S>2R,3S/2S,3R) (159 mg, 0.50 mmol) was dissolved in formic acid (1 ml). The solution was allowed to stand for 2 hours, after which it was evaporated to dryness, to give the crude formate salt (11) which was not isolated, dissolved in dichloromethane, washed (saturated sodium bicarbonate), dried (sodium sulphate) coupled [using EEDQ] with (13) to give an oil (179 mg, 46%). The product was a mixture of a 4 diastereomers of composition (as judged by ^{1}H n.m.r.): {(S,R,R,R), 4: (S,R,S,S), 4 [(14a,b)]: (S,R,R,S), 1: (S,R,S,R), 1, [(15a,b)}. The (S,R,R,R) (14a) and (S,R,R,S) (15a) isomers were separated from the (S,R,S,S) (14b) and (S,R,S,R) (15b) isomers by chromatography (preparative layer chromatography, 2 elutions with 40% ethyl acetate/hexane). The (S,R,R,R) (14a) and the (S,R,R,S) (15a) isomers were then separated by normal phase h.p.l.c. (silica column, mobile phase + 20% ethyl acetate/dichloromethane). The last quarter of the band eluting at 6 min was collected, concentrated and re-injected. This process was repeated three times, when the band eluting at 6 min was shown to contain only the (S,R,R,R) (14a) isomer and none of the (S,R,R,S) (15a) isomer (by ^{1}H n.m.r. 300 MHz). Data for the (S,R,R,R) (14a) isomer: m.p. 122-125°C; tlc (40% ethyl acetate/hexane) Rf 0.35; υ_{max} ($CHCl_3$) 3005m, 1735s (CO, shoulder), 1710bs (CO), 1602m, 1500m, 1500m, 1205m, 938m; δ_H (300 MHz, $CDCl_3$), 0.99 (3H, d, J 6.5 Hz, CHMe), 1.62-1.90 (4H, m, $CH_2CH_2CH_2CO$), 2.14-2.20 (3H, m, CH_2CO, CHMe), 2.61-2.81 (2H, m, CH_2S), 3.73 (2H, s, SCH_2Ph), 4.18-4.22, 4.42-4.45, 4.51-4.54 (3H, 3 x m, 3 x NCHCO), 5.00-5.16 (8H, m, 3 x OCH_2Ph, C=CH_2), 5.43-5.48 (1H, m, CH_2=CH), 6.15 (1H, d, J 8 Hz, NH), 6.72 (1H, d, J 8Hz, NH), 6.95 (1H, d, J 8.5, NH), 7.31-7.37 (20H, m, Ar); m/e (field desorption) 779 (M^+).

The protected tripeptide (S,R,R,R) (14a) (21 mg, 2.7 x 10^{-2} mmol) was hydrogenated with Wilkinson's catalyst (10 mg) in benzene for 20 min. The solution was filtered (celite) and evaporated to dryness. The ^{1}H NMR (300 MHz) of the crude reaction mixture was shown to contain new peaks corresponding to those of an authentic sample of the protected isoleucine tripeptide (16a).[9]

[(S)-(N-Benzyloxycarbonyl-(α-benzyl)-δ(α-aminoadipoyl)]-[(S)-S-benzyl-cysteinyl]-[(2R,3S)-2-amino-3-methylpent-4-enoic benzyl ester] (15a)

The (S,R,R,S) (15a) isomer was prepared from diprotected amino acid (8a,b) (2R,3S/2S,3R>2R,3R/2S,3S) (127 mg, 0.42 mmol) using an analogous procedure to that used for the preparation of the (S,R,R,R) isomer (14a). The crude reaction mixture (156 mg, 50%) was a mixture of 4 diastereomers of composition [as judged by ^{1}H n.m.r. (300 MHz): {(S,R,R,S), 7: (S,R,S,R), 7 (15a,b): (S,R,R,R), 1: (S,R,S,S), 1 [14a,b]} The (S,R,R,S) (15a) and (S,R,R,R) (14a) isomers were separated from the (S,R,S,R) (15b) and (S,R,S,S) (14b) isomers by chromatography (preparative layer chromatography, 2 elutions with 40% ethyl acetate/hexane). The (S,R,R,S) (15a) isomer was then recrystallised until none of the (S,R,R,R) (14a) isomer could be identified in the ^{1}H n.m.r. (300 MHz). Data for (S,R,R,S) (15a) isomer: m.p. 116-118°C; tlc (40% ethyl acetate/petrol) Rf 0.35; υ_{max} ($CHCl_3$) 1730s (CO), 1700bs (CO), 1508m, 1378m, 1205m; δ_H (300 MHz, $CDCl_3$) 1.00 (3H, d, J 6.0 Hz, CHMe), 1.61-1.92 (4H, m $CH_2CH_2CH_2CO$), 2.07-2.25 (3H, m, CH_2CO, CHMe), 2.58-2.88 (2H, m, CH_2S), 3.75 (2H, s, SCH_2Ph), 4.36-4.48 (2H, m, 2 x NCHCO), 3.57-3.63 (1H, m, NCHCO), 4.99-5.21 (8H, m, $C=CH_2$, 3 x OCH_2Ph), 5.56-5.75 (2H, m, CH=C, NH), 6.35 (1H, d, J 8, NH), 6.91 (1H, d, J 8.5, NH), 7.31-7.37 (20H, m, Ar); m/e (field desorption) 779 (M^+).

The protected tripeptide (15a) (S,R,R,S) was shown to be reduced to the protected allo-isoleucine tripeptide (16b) in an analogous manner to that in which (14a) (S,R,R,R) was shown to correspond to the isoleucine tripeptide (16a).

(S)-δ-(α-Aminodipoyl)-(S)-cysteinyl-(2R,3R)-2-amino-3-methylpent-4-enoic acid (17)

The protected tripeptide (14a) (S,R,R,R) (85 mg, 0.11 mmol) was dissolved in tetrahydrofuran (3 ml) and NH_3 (l) (dried over sodium) (ca 10 ml) was added. Sodium was added in small pieced until a deep blue colouration persisted. The reaction was stirred for 10 minutes, after which ammonium sulphate was added, with shaking, until the blue colouration disappeared. The ammonia was evaporated under a stream of argon, the residue was suspended in water and the pH adjusted to 7.5, using 2N hydrochloric acid. Oxygen was then bubbled through the solution for 1 hour, when the solution was evaporated to dryness and purified by reverse phase h.p.l.c. (ODS column, mobile phase = 25% methanol/10 mmolar aqueous ammonium bicarbonate) to give the tripeptide (17) in the disulphide form (retention time = 9 min) (15 mg, 36%). δ_H (500 MHz, D_2O) 0.82 (3H, d, J 7 Hz, CHMe), 1.52-1.71 (4H, m, $CH_2CH_2CH_2CO$), 2.19-2.22 (2H, m, CH_2CO), 2.48-2.56 (1H, m, CHMe), 2.73-3.04 (2H, m, CH_2S), 3.48-3.54 (1H, m, NHCCO), 3.98 (1H, d, J 6.5 Hz, NCHCO), 4.87-4.96 (2H, m, $C=CH_2$), 5.49-5.58 (1H, m, CH=C). 1 x NCHCO obscured by HOD; m/e (positive argon FAB) 376 (MH^+).

(S)-δ-(α-Aminodipoyl)-(S)-cysteinyl-[(2R,3S)-2-amino-3-methylpent-4-enoic acid] (18)

The protected tripeptide (15a) (S,R,R,S) (43 mg, 5.5 x 10^{-2} mmol) was deprotected in an analogous fashion to that used for (14a), after which preparative electrophoresis (pH 3.5, 4 KV, 80 min), extracting the band 5-10

cm from the origin gave the tripeptide (18) in the disulphide form (16 mg, 77%).

δ_H (300 MHz) D_2O) 0.87 (3H, d J 7 Hz, CHMe), 1.51-1.74 (4H, m, $CH_2CH_2CH_2CO$), 2.08-2.26 (2H, m, CH_2CO), 2.55-2.57 (1H, m, CHMe), 2.77-3.03 (2H, m, CH_2S), 3.47-3.58 (1H, m, NCHCO), 4.14 (1H, d, J 5.5 Hz, NCHCO), 4.91-4.96 (2H, m, C=CH_2), 5.60-5.67 (1H, m, CH=C); m/e (positive argon FAB) 376 (MH^+).

Incubation of the tripeptide (S,R,R,R) (17) with the IPNS enzyme

The tripeptide (S,R,R,R) (17) (3 mg) was incubated with 20 I.U. of IPNS using the published procedure.[10] The ^1H n.m.r. (500 MHz) of the crude incubation mixture after protein precipitation indicated the presence of two beta-lactam products and no starting material (17). The crude product was purified by reverse phase h.p.l.c. (ODS column, mobile phase = 10 m molar ammonium bicarbonate, flow rate = 1 ml/min) to give:
Fraction (1) (retention time 2.8 min). Further purification by reverse phase HPLC (ODS column, 0.05% v/v formic acid in water, flow rate = 1 ml/min, retention time = 16.5 min).

(2R,3S,4S,6R,7R)-1-Aza-3-methyl-4-hydroxymethyl-7-[5S-5-amino-5-carboxypentanamido]-8-oxo-5-thiabicyclo[4.2.0]octane-2-carboxylic acid (20)

δ_H (D_2O, 500 MHz) 1.10 (3H, d, J 6 Hz, Me), 1.42-1.85 (4H, m, CH_2CH_2CO), 2.26-2.31 (2H, m, CH_2CO), 2.43-2.52 (1H, m, 3-H), 2.73-2.81 (1H, m, 4-H), 3.52-3.55 (1H, m, $CHCH_2CH_2$), 3.68 (1H, dd, J, 12, 6.5 Hz, CH_2OH), 3.84 (1H, dd, J 12, 8.5 Hz, CH_2OH), 3.86 (1H, d, <J 1 Hz, 2-H), 5.00, 5.24 (2H, ABq, J 4 Hz, 6-H, 7-H).

A 2D-COSY experiment[12] was consistent with the connectivity as indicated above. The stereochemistry of (20) was indicated to be as drawn by n.O.e experiments, thus irradiation at 3.68 - 3.84 (CH_2OH) gave NOE's at 6-H, 8%; 7-H, 2%; 3-H, 2%. Irradiation at 2.73-2.81 (4-H) gave no observed NOE to 6-H. m/e (positive argon FAB) 390 (MH^+). (20) showed no antibacterial activity against S. aureus NCTC 6751 at a concentration of 100 $\mu g\ ml^{-1}$.

Fraction (2) (retention time 5.1 min)
(2R,7R,8R)-1-Aza-3-methyl-8-[5S-5-amino-5-carboxypentanamido]-9-oxo-6-thiabicyclic[5.2.0] non-3-ene-2-carboxylic acid (21)

δ_H (D_2O, 500 MHz) 1.56-1.77 (4H, m, $CH_2CH_2CH_2CO$), 1.73 (3H, s, Me), 2.21-2.26 (2H, m, CH_2CO), 3.06 (1H, dd, J 15.5, 7 Hz, 5-H), 3.15 (1H, dd, J 15.5, 7 Hz, 5-H), 3.49-3.59 (1H, m, $CHCH_2CH_2$), 4.74 (1H, s, 2-H), 5.18, 5.40 (2H, ABq, J 4 Hz, 7-H, 8-H), 5.70 (1H, ca t, J Hz, 4-H).

A 2D-COSY experiment was consistent with the connectivity as indicated.[12] m/e (positive argon FAB) 372 (MH^+). (21) showed no antibacterial activity against S. aureus NCTC 6571 at a concentration of 100 $\mu g\ ml^{-1}$.

Incubation of the tripeptide (S,R,R,S) (18) with the IPNS enzyme

The tripeptide (S,R,R,S) (18) (3 mg) was incubated with the IPNS enzyme (25 I.U.) using the published procedure[10]. The ^1H n.m.r. (500 MHz) spectrum of the crude incubation mixture after protein precipitation indicated the presence of one beta-lactam product and no residual starting material (18). The crude product was purified by reverse phase h.p.l.c. (ODS column, mobile phase = 10 mmolar ammonium bicarbonate, flow rate = 1 ml/min) to give:

(2S,3R,5R,6R)-1-Aza-3-methyl-3-vinyl-6-[5S-5-amino-5-carboxypentanamido]-7-oxo-4-thia-bicyclo[3.2.0]heptane-2-carboxylic acid (19)
(retention time 7.8 min)

δ_H (D_2O, 500 MHz) 1.53-1.77 (4H, m, $CH_2CH_2CH_2CO$), 1.59 (3H, s, 2β-Me), 2.25-2.38 (2H, m, CH_2CO), 3.56-3.59 (1H, m, $CHCH_2CH_2$), 4.18 (1H, s, 2-H), 5.03 (1H, d, J 10.5 Hz, $CH=CH_2$), 5.22 (1H, d, J 17 Hz, $CH=CH_2$), 5.35, 5.45 (2H, ABq, J 4 Hz, 5-H, 6-H), 5.91 (1H, dd, J 17, 10.5 Hz, $CH=CH_2$).

The stereochemistry of (19) was confirmed by n.O.e experiments: thus irradiation at 1.59 (Me) gave NOE's at: 2-H, 12%, $CH=CH_2$, 3%; irradiation at 5.91 ($CH=CH_2$) gave NOE's at: 5-H, 3%; 5.03 ($CH=CH_2$), 2%, 5.22 ($CH=CH_2$), 5%; m/e (positive argon FAB) 372 (MH^+). (19) showed antibacterial activity (ca 85% mol/mol of isopenicillan N) against gram-positive organisms (Staphylococcus aureus, Staphylococcus lutea) which was suppressed by the addition of beta-lactamase I from *Bacillus cereus*).

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