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THE SYNTHESIS OF L-α-AMINOADIPYL-L-CYSTEINYL-D-3,4-DIDEHYDRO-VALINE, A POTENT INHIBITOR OF ISOPENICILLIN SYNTHETASE

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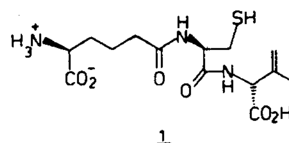
Abstract - The title peptide (1) has been synthesized and incubated with an active cell-free extract of Cephalosporium acremonium, no conversion to active antibiotics was observed; however on co-incubation with the Arnstein tripeptide (ACV) (2), strong inhibition of the conversion of ACV to isopenicillin N was observed.

It is well established that the peptide (2) is efficiently converted into isopenicillin N by cell-free extracts of C. acremonium. The nature of the conversion has aroused much interest[1]. Little is known of the active site on the enzyme complex which effects this conversion. In order to probe the chemical properties of the active site, we have synthesised the unnatural tripeptide (1) and have tested it for conversion to antibiotics. We have also co-incubated this peptide with the natural substrate (2) to observe if conversion of (2) to isopenicillin N is inhibited. Recently, Neuss et al[2] have isolated a tripeptide containing the 3,4-didehydrovaline moiety viz. α-aminoadipylserinyl-3,4-didehydro-valine (3) from the broth of Penicillium chrysogenum. This heightened our interest in the synthesis of the tripeptide (1) and in the testing of its effect on isopeniclllin N synthetase.



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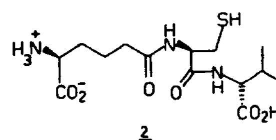

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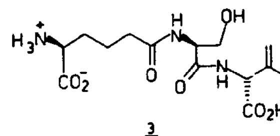

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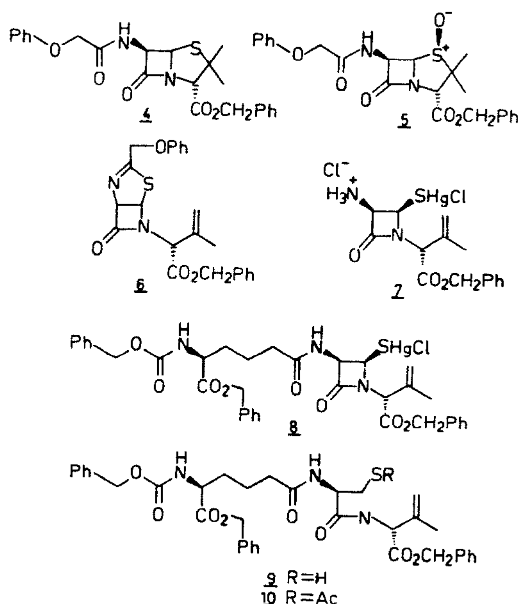
Thus penicillin N benzyl ester (4) was oxidized to the sulphoxide (5) 85%. This was converted to the thiazoline (6), 72%, by heating under reflux in benzene in the presence of trimethyl phosphite. The thiazoline was cleaved to the mercury salt (7), 66% which was coupled with N-benzyloxy-carbonyl-L-α-aminoadipic acid α-benzyl ester[3] to yield the salt (8). (8)

was converted directly to the thiol (9) (by treatment with H_2S and $NaBH_3CN$) isolated as its S-acetyl derivative (10) (Ac_2O, pyridine), 24% from (7). The crystalline thiol ester was cleanly converted to the peptide (1) and an equivalent of acetamide by treatment with sodium in liquid ammonia at -78°C. Attempts to separate the peptide from the inorganic salts formed during work up of the deprotection reaction and from acetamide by the standard techniques (treatment with Hopkin's reagent or chromatography on Biogel P2, or preparative electrophoresis or reverse phase HPLC) led to decomposition of the peptide as witnessed by nmr and analytical electrophoresis.



When the tripeptide (1) was incubated as detailed in the experimental section with a cell-free extract of C.acremonium no disappearance of (1) could be detected by ^1H nmr spectroscopy[4]. In the incubation mixture, no antibiotic activity could be detected by hole-plate assay[5] against Staphalococcus aureus.

When the tripeptide 1 (0.25 equiv) and ACV 2 (1 equiv) were co-incubated with a cell-free extract of C.acremonium the initial rate of production of isopenicillin N was reduced by a factor of 3.2 compared to a control incubation containing no inhibitor.

EXPERIMENTAL

General Procedures

All solvents were distilled prior to use. Dichloromethane was dried by distillation from calcium hydride. Benzene was dried by distillation from sodium. 2,2-dimethylpropan-1,3-diol was purified by recrystallisation from benzene. Trimethyl phosphite and benzyl bromide were purified by distillation just prior to use.

Nmr spectra were measured on a Bruker WH 300 spectrometer. Spectra were taken in deuterochloroform unless otherwise stated. Mass spectra were obtained using VG Micromass ZAB 1F and 16F instruments. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. Infra-red spectra were determined on a Perkin-Elmer 257 spectrometer. TLC was carried out using glass-supported silica gel 60 plates. Preparative chromatography was carried out using Merck Silica Gel 60.

Penicillin V benzyl ester (4)

A mixture of freshly dried DMF (100 ml), potassium salt of penicillin V (19.40 g, 0.05 mole) and benzyl bromide (8.545 g, 0.05 mole) was stirred under nitrogen for 24 h at room temperature. The reaction mixture was filtered, poured into water (250 ml) and extracted with dichloromethane (4 x 80 ml). The combined extracts were washed with water (10 x 60 ml), dried over sodium sulphate, and the solvent removed *in vacuo* to yield a yellow oil which was crystallised by dissolving it in chloroform and adding petrol ether (40°-60°C). The ester 4, was obtained as a white solid (20.94 g, 95%) m.pt. 70-72°C δ^1H 1.43 (3H, s, CH_3-C-S-), 1.58 (3H, s, CH_3-C-S-), 4.56 (2H, q, J = 15.2Hz, -O-CH_2-), 4.58 (1H, s, -CH-CMe_2S-), 5.20 (2H, s, -CH_2O-), 5.59(1H, d, J = 4.2Hz, H-7), 5.75 (1H, dd, J = 9.2, 4.2 Hz, H-6), 6.92-7.30 (11H, m, ArH + NH); ν_{max} ($CHCl_3$) 3415, 3035, 3015, 1785, 1745, 1690, 1600, 1580, 1540, 1495, cm^{-1}; λ_{max} (MeOH), 261, 267, 274; m/e 440 ($M^{+\cdot}$); $[\alpha]_D^{20}$= 130° (c = 0.32, $CHCl_3$).

Penicillin V sulphoxide benzyl ester (5)

Penicillin V benzyl ester (4, 20.42 g, 0.046 mole) was dissolved in dry dichloromethane (200 ml) and the solution was cooled to 5°C. A solution of m-chloroperbenzoic acid (8.396 g, 0.0487 mole) in dry dichloromethane was added with stirring over 30 min and the reaction was stirred for a further 30 min at 10°C. The solution was washed with a saturated solution of sodium hydrogen carbonate then with brine. The solution was dried over sodium sulphate, and the solvent removed *in vacuo* to give a white foam, which was crystallised from dichloromethane/diethyl ether. Penicillin V sulphoxide benzyl ester (5) was obtained as a white solid (18.106 g, 85%) m.pt. 125°C (lit[6] 124-125°C), δ^{1}H 1.077 (3H, s, CH_3-C-S-), 1.68 (3H, s, CH_3-C-S-), 4.53 (2H, s, -CH_2-O-), 4.70 (1H, s, -CH-CMe_2-S-), 5.02 (1H, d, J = 4.8 Hz, H-7), 5.23 (2H, q, J = 11.9 Hz, -CH_2-O-), 6.10 (1H, dd, J = 10.5, 4.6 Hz, H-6), 6.91 to 7.38 (10H, m, ArH), 8.25 (1H, d, J = 10.5 Hz, NH); ν_{max} ($CHCl_3$) 3380, 3010, 1800, 1740, 1690, 1600, 1515, 1490, cm^{-1}; λ_{max} (MeOH) 267, 273 nm, m/e 456 (M^{+}·), 438, $[\alpha]^{20}_{D}$ + 169° (c = 0.58, $CHCl_3$).

4,7-diaza-7-(1-benzyloxycarbonyl-2-methylprop-2-enyl)-3-phenoxymethyl-2-thiabicyclo-[3.2.0]-hept-3-en-6-one (6)

The penicillin sulphoxide (5, 4.41 g, 9.66 mmole), dry benzene (72.5 ml) and trimethyl phosphite (1.14 ml, 1.20 g, 9.66 mmole) were heated for 15 h under reflux with a Dean-Stark attachment. The mixture was cooled and evaporated to leave an orange oil which was purified by column chromatography (1:1, ethyl acetate: hexane) to give the desired thiazoline (6) as white needles (2.945 g, 72%) m.pt. 55-57.5°C; δ^{1}H 1.73 (3H, s, -CH_3), 4.91 (2H, dq, J = 14.2, 1.2 Hz, ΦO-CH_2-), 4.87 (2H, s, -C = CH_2), 5.05 (1H, s, -CH-$CMeCH_2$-), 5.18 (2H, q, J = 12.2 Hz, ΦCH_2O-), 5.91 (1H, d, J = 4.3 Hz, -S-CH-N-), 6.00 (1H, dt, J = 4.3, 1.2 Hz, H-5), 6.90-7.38 (10H, m); ν_{max} 3030, 3010, 1770, 1740, 1650, 1620, 1600, 1580, 1495, 1240, 1160, m/e 422 (M^{+}·) $[\alpha]^{20}_{D}$ -124° (c = 0.58, $CHCl_3$). Found: C, 65.17%, H, 5.23%, N, 6.71%; $C_{23}H_{22}N_2O_4S$ requires: C, 65.40%, H, 5.21%, N, 6.63%.

Preparation of 3-amino-1-(1-benzyloxycarbonyl-2-methylprop-2-enyl)-4-[thiomercury (II) chloride]-azetidin-2-one hydrochloride salt (7)

The thiazoline (6, 0.200 g, 0.47 mmole) was dissolved in dry dichloromethane (2 ml) in a dry nitrogen atmosphere. Mercuric chloride (0.128 g, 0.47 mmole) and 2,2-dimethylpropan-1,3-diol (0.049 g) were added in one lot. After stirring for 14 h, the fine white precipitate was filtered and washed with dichloromethane, then dried to give the desired hydrochloride salt (7, 0.182 g, 66%) m.pt. 158-163°C, δ^{1}H (d6 - DMSO) 1.82 (3H, s, CH_3-), 4.70 (1H, d, J = 4.7 Hz, H-4), 4.86 (1H, s, -CH-$CMeCH_2$-), 5.04 and 5.09 (2H, 2 x s, -C = CH_2), 5.20 (2H, q, J = 12.4 Hz, -OCH_2-), 5.70 (1H, d, J = 4.7 Hz, H-5), 7.3-7.5 (5H, m, ArH), 8.70 (3H, br. s, $RN^{+}H_3$); λ_{max} 206 nm, m/e: 561 (M^{+}·) Found: C, 30.9%, H, 3.4%, N, 4.8%; $C_{15}H_{18}Cl_2HgN_2O_3S$ requires: C, 31.2%, H, 3.1%, N, 4.8%

N-benzyloxycarbonyl-α-benzyl-δ-(L-α-aminoadipyl)-S-acetyl-L-cysteinyl-D-3,4-didehydrovaline benzyl ester (10)

The amine hydrochloride (7, 0.58 g, 1.00 mole) was added to dichloromethane (100 ml) and the suspension was washed with saturated sodium hydrogen carbonate solution under nitrogen. The organic phase was dried over sodium sulphate, filtered and concentrated to approximately 30 mls then added, under nitrogen, to a solution of N-carbethoxy-2-ethoxy-1,2-dihydroquinoline (EEDQ, 0.256 g, 1.04 mmole) and N-benzyloxycarbonyl-L-α-aminoadipic acid α-benzyl ester (0.38 g, 0. 987 mmole) in dichloromethane. After stirring for 3 days, the mixture was diluted with dichloromethane, washed in turn with 2N hydrochloric acid, saturated sodium carbonate solution and water. The solution was dried over sodium sulphate, filtered and the solvent removed *in vacuo* to yield a yellow foam. [If desired this material could be purified at this stage by chromatography on silica gel using i) 1 CH_2Cl_2: 9 EtOAc and ii) 5 CH_2Cl_2: 5 EtOAc. The resulting white foam had a ^{1}H nmr spectrum consistent with the thiomercuric chloride (8)]. Routinely, crude 8 was used directly without further purification. Crude 8 (0.15 g) was added to freshly dried methane, (20 mls, distilled from magnesium methoxide) under nitrogen. Hydrogen sulphide was vigorously bubbled through the suspension for 10 minutes at 0°C and the resulting slurry was filtered through Celite under nitrogen. Acetic acid (2 ml) was added to the filtrate and then a solution of sodium cyanoborohydride (0.200 g) in dry methanol (10 ml) was added dropwise over 10 mins. After 1 h, the solvent was removed *in vacuo* to leave a white foam. Acetic anhydride (2 ml) was added under nitrogen followed by pyridine (0.2 ml) and the mixture was stirred for 1.5 h, then washed with 2N hydrochloric acid. The organic solution was dried over sodium sulphate filtered and the solvent removed *in vacuo*. The residue was purified by column chromatography (i) 7 $CHCl_3$: 3 EtOAc: ii) 5 CH_2Cl_2:5 EtOAc) to yield pure protected tripeptide (10) as white prisms (28.3 mg, 24% from 7) m.pt. 133-134°C, δ^{1}H 1.73 (3H, s, CH_3 -C = CH_2), 1.58-1.74 (4H, m, -COCH_2 CH_2CH_2-), 2.16-2.25 (2H, m, -COCH_2CH_2 CH_2-), 2.35 (3H, s, CH_3CCS-), 3.26 (2H, m, -CH_2S-), 4.41 (1H, m, -N-CH-CO-), 4.59 (1H, m, -N-CH-CO-), 4.95-

5.18 (9H, m, 3 x $-OCH_2Ph$, $-CH-CMeCH_2$), 5.63 (1H, d, J = 9.0 Hz, NH), 6.44 (1H, d, J = 7.1 Hz, NH), 7.29-7.40 (m, 15H, Ar-H); ν (CH_2Cl_2) 3420, 1740, 1730, 1690, m/e 717 (M^{+}·); Found:717. 2737: $C_{38}H_{43}N_3O_9S$ requires 717.2720. Found: C,63.37%, H,5.97%, N, 6.01%, $C_{38}H_{43}N_3O_9S$ Requires: C, 63.58%, H, 6.07%, N, 5.85%, $[\alpha]_D^{20}$- 40.7° (c = 1.3, $CHCl_3$).

Preparation of δ-(L-α-aminoadipyl)-L-cysteinyl-D-3,4-didehydrovaline (1)

Ammonia (10 mls) was distilled onto the protected peptide (10 8.4 mg), at -78°C under an argon atmosphere. Sodium metal was added in small amounts until a permanent blue colour developed in the solution. Ammonium sulphate was added until the blue colour faded and the ammonia was evaporated. The residue was brought to pH 2.0 with 0.05M sulphuric acid and then back to pH 6.85 with 3N sodium hydrogen carbonate solution. The product was then freeze dried. The total solid weight after freeze drying was 0.367 g containing 4.23 mg of the tripeptide (1). The ^1H nmr spectrum of the product was cleanly consistent with the desired peptide plus one equivalent of acetamide. δ ^1H (D_2O) 1.51 (3H, s, $CH_3C = CH_2$), 1.41-1.70 (4H, m, $-CH_2CH_2CH_2CO-$), 1.80 (3H, s, acetamide), 2.24 (2H, t, J = 7.4 Hz, $-CH_2CO-$), 2.72 (2H, m, $-CH_2S-$), 3.55 (1H, t, J = 5.9 Hz, -COCHNH-), 4.35 (1H, t, J = 5.5 Hz -COCHNH-), 4.45 (1H, s, CH_2=CMeCH-), 4.83 (2H, br.s., MeC = CH_2).

Electrophoresis was performed on the product at pH 4.5 (4kv, 1 hour). This afforded a single mobile zone sensitive to cadmium/ninhydrin. The zone gave a positive reaction with a thiol-active[7] spray.

Incubation of peptide 1 with cell-free extract of C. acremonium
(a) Production of β-lactam antibiotics

The tripeptide L-α-aminoadipyl-L-cysteinyl-D-3,4-didehydrovaline (initial concentration 3mM) was incubated for 12 h at 20°C with a cell-free extract of *C. acremonium* in the presence of dithiothreitol (10 mM), L-ascorbate (750 μM), Fe^{2+} (75 μM) and bovine liver catalase (10000 units/ml) in an ammonium hydrogen carbonate buffer at pH 8.1 (50 mM). No disappearance of ACD could be detected by ^1H nmr spectroscopy, and no antibiotic activity could be detected by hole-plate assay against *S. aureus*.

(b) Inhibition of the conversion of ACV to isopenicillin N

The tripeptide ACD (initial concentration 0.75 mM) and ACV (initial concentration 3 mM) were co-incubated with a cell-free extract of *C.acremonium* at 20°C in the presence of the cofactors and buffer (as described above). The rate of conversion of ACV to isopenicillin N was measured directly[4] by ^1H nmr spectroscopy by monitoring the disappearance of the valinyl-methyl resonances of ACV (δ0.97, δ1.02 ppm) and the appearance of the α- and β-methyl resonances of isopenicillin N (δ1.62, δ1.74) in aliquots taken as the incubation progressed. The rate of production of isopenicillin N was reduced by a factor of 3.2 relative to a control incubation containing no ACD. The inorganic salt and acetamide which arise during the deprotection of the tripeptide were shown to have no effect on the rate of production of isopenicillin N in a separate control experiment.

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