Synthesis and Biological Activity of some Fused β-Lactam Peptidoglycan Analogues

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The derivatives (12), (14), (15), (19), and (22), in which a mono- or di-peptide unit is linked to 6-aminopenicillanic acid, have been prepared according to normal synthetic peptide procedures. The structures of the side chains were chosen by analogy with the D-isoglutamyl-L-lysyl sequence present in the cell-wall peptidoglycan of *Staphylococcus* species. The antibacterial activities of the derivatives were measured and the results are discussed.

It is generally accepted [1-3] that penicillin-based antibiotics owe their activity to their ability to act as irreversible inhibitors of the transpeptidase enzyme [4] which is involved in the biosynthesis of the cell-wall peptidoglycan. The structure of the latter varies with different organisms,[5,6] but its biosynthesis is regarded as occurring similarly in three essential stages.[7,†]

Stage 1. Synthesis of nucleotide precursors UDP-Ⓖ and UDP-Ⓜ (see Scheme 1) Ala-γ-D-Glu-R3-D-Ala-D-Ala where R3 is commonly L-Lys or L,L-diaminopimelic acid.

Stage 2. Assembly of disaccharide–pentapeptide repeating unit on an undecaprenyl phosphate carrier.

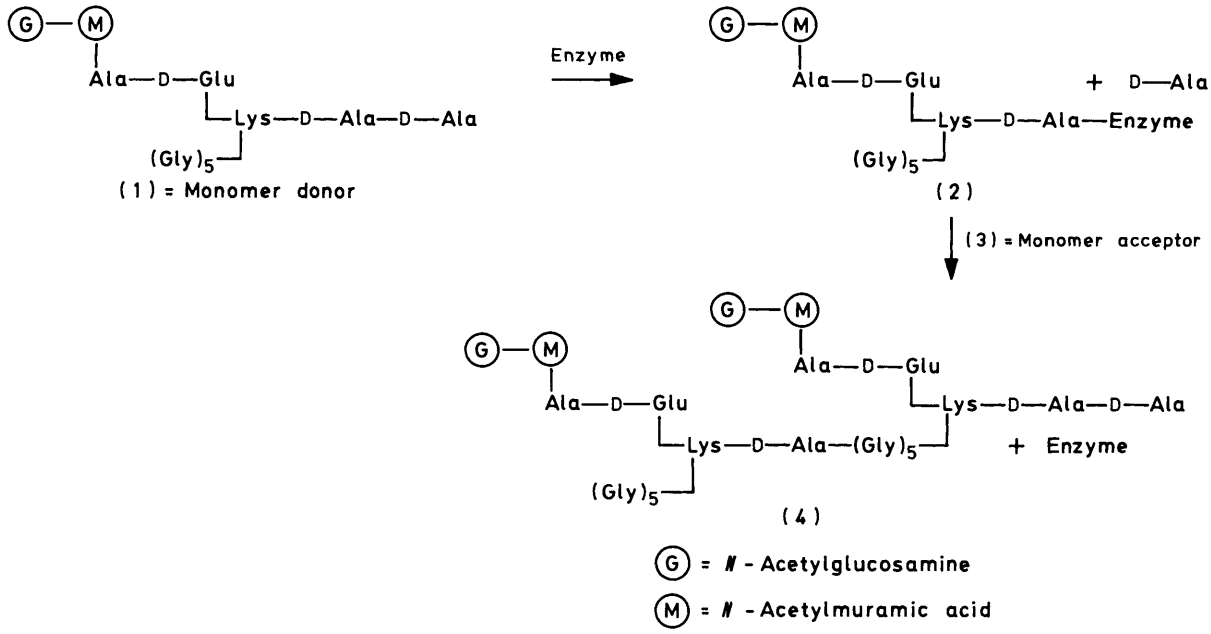
Stage 3. Polymerisation of the lipid-bound sub-units, formation and modification of cross-links between the linear chains.

The cross-linking step [8] in stage 3, which is shown for *Staphylococcus aureus* (Scheme 1), involves the aminolysis of the D-Ala-D-Ala link in one monomer by a $(Gly)_5$ moiety in a second. Certain structural similarities between the D-Ala-D-Ala unit and the penicillin nucleus have been pointed out,[9] and this is undoubtedly an important feature for the action of penicillins.

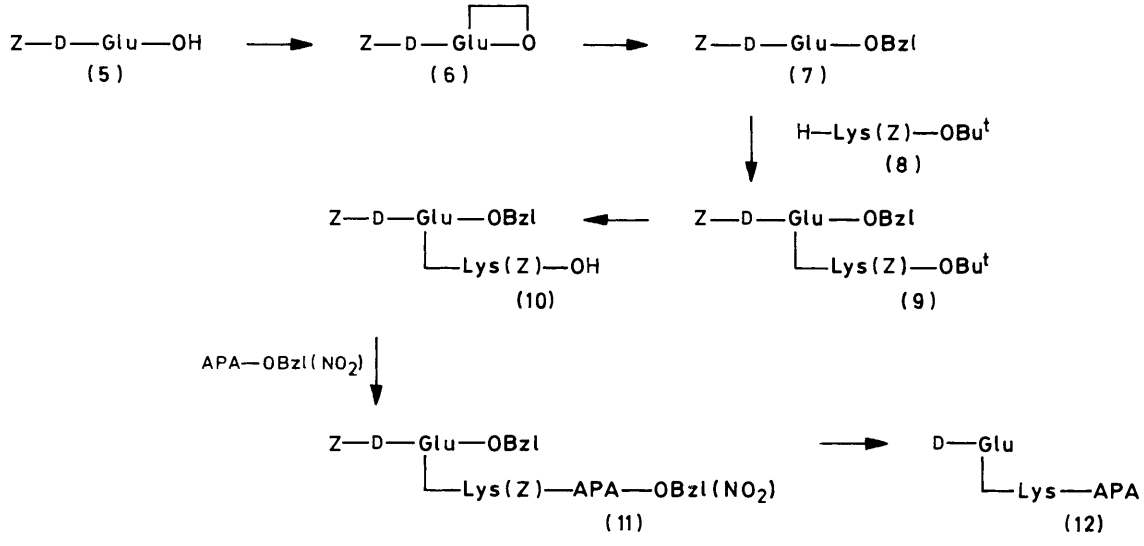
Since the isolation of 6-aminopenicillanic acid (APA) in 1959,[10] a conservative estimate would suggest that *ca.* 20 000 different semi-synthetic penicillins have been evaluated in the

(4) was converted into its α-benzyl ester (7) *via* the anhydride (6). Coupling of the ester (7) to the lysine derivative (8) afforded the dipeptide ester (9) which was obtained in good yield and smoothly deprotected to the side-chain acid (10). Standard protection, coupling and deprotection steps of peptide synthesis were applied (see Experimental section). The immediate precursor of (12), the fully protected analogue (11), was obtained in good yield from the side-chain acid (10) and the penicillin *p*-nitrobenzyl ester using a dicyclohexylcarbodiimide coupling mediated by 1-hydroxybenzotriazole. The last-named reagent is now a standard additive in peptide couplings [15] and appears quite compatible with the pencicillin nucleus, no significant degradation being observed. The reagent also worked well in the preparation of compound (21).

Disappointingly, the product (12) showed little antibacterial activity against a range of Gram-positive and Gram-negative organisms (see Table). The related analogue (14), prepared by acylation of ampicillin ‡ with the acid (7) and removal of protecting groups from the intermediate (13), showed improved activity, probably owing to the improved binding which is a general characteristic of the phenyl substituent in phenylglycine. *N*-Acetylation of the free amino group of the D-isoGlu residue of (14), which is always acylated in the monomer donor (1), gave compound (15), but this led to



Scheme 1. Cross-linking step in *Staph. aureus*



Scheme 2.

represents improved binding on replacement of L-lysine with D-phenylglycine, but further acetylation of (14) to give (15) gave very little more improvement. The activities of compounds (19) and (22) are to be compared with ampicillin and penicillin G respectively and are clearly much lower. None of the analogues (12), (14), (15), (19), and (22) show any stability to β-lactamase; this was true for β-lactamase-producing *Staph.* species as well as for Gram-negative organisms. However, the low MIC values of (14) and (15) against *E. coli* ESS imply an acceptable level of intrinsic activity, though as noted above one must be cautious in attributing this to a good affinity for the target enzyme on account of the variability of the peptidoglycan sequence with different bacteria. A very interesting comparison is provided by the monocyclic β-lactam antibiotic sulfazecin (23) [16,17] recently described by Japanese workers.* This is an example of a wider class of monocyclic β-lactam antibiotics (' monobactams ') which have recently attracted much interest and have broad-spectrum antibacterial activity.[17,18] The 1-sulphonic acid grouping is a common feature.

The γ-D-Glu-D-Ala side chain is to be compared with our peptidyl side chains, whereas the presence of a 3-methoxy β-lactam substituent doubtless improves the β-lactamase stability as evinced by the acceptable activity of (23) against Gram-negative organisms.

Experimental

M.p.s were measured in a Büchi oil immersion apparatus and are uncorrected. Satisfactory i.r. spectra were obtained for all compounds described, in particular a strong band at 1 770—1 780 cm^{-1} for the penicillins. N.m.r. spectra were recorded

* Published while this manuscript was in preparation.

Z—D—Glu—OBzl
Phg—APA
(13)

D—Glu
Phg—APA
(14)

Ac—D—Glu
Phg—APA
(15)

Z—DL—Lys—OH
(16)

Phg = D—Phenylglycine

Z—DL—Lys(Ac)—OH
(17)

Z—DL—Lys(Ac)—APA—OBzl
(18)

DL—Lys(Ac)—APA
(19)

Ac—DL—Lys(Ac)—OH
(20)

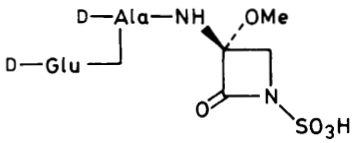
Ac—DL—Lys(Ac)—APA—OBzl(NO_2)
(21)

Ac—DL—Lys(Ac)—APA
(22)

Table. Antibacterial activities of analogues (12), (14), (15), (19), and (22) by serial dilution in nutrient agar. Compound (15) was assayed as its sodium salt, (22) as the free acid, and the other species as their zwitterions

	MIC's [a]						
Organism	(12)	(14)	(15)	(19)	(22)	Ampicillin	Penicillin G
E. coli ESS [b]	50	0.2	0.1	2.5	5.0	0.05	0.1
E. coli NCTC10418	125	25	50	50	100	2.5	25
P. mirabilis C977	125	2.5	2.5	50	50	1.25	25
B. subtilis ATCC6633	12.5	2.5	10.0	1.0	5.0	0.1	0.1
S. aureus Oxford NCTC6511	50	2.5	5.0	1.0	2.5	0.05	0.05
N. catarrhalis NCTC3622		50	<0.02	0.2	0.2	≤0.01	0.02
S. pyogenes CN10	25	0.5		0.2	1.0	≤0.01	<0.01

[a] We are most grateful to Mr. Brian Slocombe and other members of our biological team for these results. [b] Outer membrane-deficient mutant (see text). We are indebted to Professor A. Demain, M.I.T., for suggesting the introduction of this organism to our *in vitro* screening.



(23)

with a Perkin-Elmer R32 90 MHz instrument using tetramethylsilane as an internal standard. Optical rotations were measured on a Perkin-Elmer 141 polarimeter in a 1-dm cell. Qualitative thin-layer chromatograms were run on Merck silica-gel plates and visualised by fluorescence under u.v. at 254 nm and by iodine development, employing the following solvent systems: (a) n-butyl alcohol–acetic acid–water, 4 : 1 : 1; (b) n-butyl alcohol–acetic acid–water–pyridine, 10 : 3 : 12 : 15; (c) chloroform–methanol–acetic acid, 17 : 2 : 1; (d) chloroform–methanol, 9 : 1; (e) chloroform–methanol, 19 : 1. Biochromatography of penicillins was performed by descending paper chromatography in n-butyl alcohol–ethanol–water, 7 : 1 : 2, using *Bacillus subtilis* as the assay organism.

Some of the final penicillins, as zwitterions, free acids, or sodium salts, retained solvents very tenaciously, precluding meaningful elemental analysis; nor was it possible to obtain a molecular ion on these species. They were regarded as adequately characterised by their n.m.r. spectra, t.l.c. behaviour and exhibition of a single zone of inhibition on biochromatography.

Ether refers to diethyl ether throughout.

N-*Benzyloxycarbonyl*-D-*glutamic Acid* (5).—This *compound* was prepared according to a standard procedure [19] on a

$[\alpha]_D^{20}$ +7.2° (*c* 10 in AcOH) [lit.,[20] for L-enantiomer: m.p. 120—121 °C, $[\alpha]_D^{20}$ −7.9 (*c* 10 in AcOH)] (Found: C, 55.4; H, 5.3; N, 4.9. $C_{13}H_{15}NO_6$ requires C, 55.5; H, 5.3; N, 5.0%).

N-*Benzyloxycarbonyl*-D-*glutamic Anhydride* (6).—This *compound* was prepared according to the method of Le Quesne and Young [21] on a 60 mM scale and obtained in 90% yield, m.p. 92—93.5 °C; $[\alpha]_D^{20}$ +32.6° (*c* 10 in AcOH) {lit.,[21] for L-enantiomer, m.p. 93—94 °C, $[\alpha]_D^{20}$ −45.1 (*c* 10 in AcOH)} (Found: C, 59.0; H, 4.8; N, 5.4. $C_{13}H_{13}NO_5$ requires C, 59.3; H, 4.9; N, 5.3%).

N-*Benzyloxycarbonyl*-D-*glutamic Acid* α-*Benzyl Ester* (7).—The preceding anhydride (14.1 g, 53.6 mmol) was stirred with redistilled benzyl alcohol (16.9 ml) in dry ether (92 ml) and cooled to 15 °C. A solution of dicyclohexylamine (9.7 g, 53.6 mmol) in dry ether (22 ml) was run in dropwise during 0.5 h. The crude product was filtered off, washed well with ether, dried and twice recrystallised from ethanol to give the title compound as its *dicyclohexylammonium salt* (13.4 g, 45%), m.p. 162—163.5 °C; $[\alpha]_D^{20}$ +11.5° (*c* 2 in MeOH) {lit.,[22] for L-enantiomer, m.p. 163—164 °C, $[\alpha]_D^{20}$ −12.8° (*c* 2 in MeOH)} (Found: C, 69.3; H, 7.9; N, 5.1. $C_{33}H_{44}N_2O_6$ requires C, 69.6; H, 8.0; N, 5.1%).

N(ε)-*Benzyloxycarbonyl*-L-*lysine t-Butyl Ester* (8).—To liquid isobutene (*ca.* 50 ml) was added a solution of *N*(ε)-benzyloxycarbonyl-L-lysine (5.6 g, 20 mmol) in dioxan (35 ml) containing concentrated sulphuric acid (3.5 ml). The mixture was vigorously stirred under a dry ice–acetone cold trap for 6 h with occasional addition of more dioxan to maintain a clear solution; then the trap was removed and excess of isobutene

and ether (300 ml). The ether was separated and the aqueous phase further extracted with ether (3 × 100 ml). The combined ether extracts were dried (Na_2SO_4), concentrated to *ca.* 50 ml, and saturated with dry hydrogen chloride gas; then further ether (50 ml) was added. After being cooled, the solid was filtered off, washed well with ether, dried and recrystallised from ethyl acetate containing a little ethanol to give the title ester as its hydrochloride (3.02 g, 40%), m.p. 139—140 °C; $[\alpha]_D^{20}$ +12.3° (*c* 2 in EtOH) {lit.,[23] m.p. 147—149 °C, $[\alpha]_D^{20}$ +13.6° (*c* 2 in EtOH)}; R_F(c) 0.4; δ ($CDCl_3$) 1.0—2.2 [15 H, br m, $(CH_3)_3C$ and $(CH_2)_3$], 3.0—3.3 (2 H, br m, CH_2NH), 3.95 (1 H, t, *J* 6 Hz on D_2O exchange, $CHNH$), 5.06 (2 H, s, OCH_2Ph), 5.5 (1 H, br m, D_2O exchanged, NH), 7.30 (5 H, s, C_6H_5), 8.70 (3 H, br s, D_2O exchanged, NH_3) (Found: C, 56.7; H, 7.6; N, 7.6. Calc. for $C_{18}H_{29}ClN_2O_4 \cdot 0.5H_2O$: C, 56.6; H, 7.9; N, 7.3%).

N-*Benzyloxycarbonyl*-D-*glutamyl* α-*Benzyl Ester* γ-[N(ε)-*Benzyloxycarbonyl*]-L-*lysine t-Butyl Ester* (9).—This dipeptide could be obtained from the γ-2,4,5-trichlorophenyl or γ-*N*-hydroxysuccinimido ester of (7) but the following procedure was simpler. The preceding hydrochloride salt (8) (1.86 g, 6 mmol) and *N*-benzyloxycarbonyl-D-glutamic acid α-benzyl ester (7) as its dicyclohexylammonium salt (2.76 g, 5 mmol) were dissolved together in dry methylene dichloride (70 ml). The solution was cooled in ice–water and a solution of *N,N'*-dicyclohexylcarbodi-imide (1.13 g, 5.25 mmol) in methylene dichloride (5 ml) was added in one portion. The mixture was allowed to return to room temperature and stirred overnight. After 21 h in all the precipitated *N,N*-dicyclohexylurea was filtered off and the filtrate evaporated to dryness. The residue was partitioned between ethyl acetate (50 ml) and water (50 ml). The organic phase was separated and washed with 10% citric acid, water, saturated aqueous sodium hydrogen carbonate, water, and brine, then dried (Na_2SO_4). Evaporation afforded the title *dipeptide diester* (92%); recrystallisation was effected from ethyl acetate–light petroleum (2.45 g, 71%); m.p. 120.5—121.5 °C; $[\alpha]_D^{20}$ −1.6° (*c* 1 in MeOH); R_F (e) 0.6; δ ($CDCl_3$) 1.1—2.4 [19 H, m, $(CH_3)_3C$, $(CH_2)_3$ and $(CH_2)_2$], 3.12 (2 H, m, t, *J* 6 Hz on D_2O exchange, CH_2N), 4.40 (2 H, m, 2 × CHN), 4.95 (1 H, m, D_2O exchanged, $NHCH_2$), 5.06 (4 H, s) and 5.13 (2 H, s, 3 × $OCH_2C_6H_5$), 5.75 (1 H, br d, D_2O exchanged, NHCH), 6.35 (1 H, br d, D_2O exchanged, NHCH), and 7.30 (15 H, br s, 3 × C_6H_5) (Found: C, 65.9; H, 6.8; N, 6.0. $C_{38}H_{47}N_3O_9$ requires C, 66.2; H, 6.8; N, 6.1%).

N-*Benzyloxycarbonyl*-D-*glutamyl* α-*Benzyl Ester* γ-[N(ε)-*Benzyloxycarbonyl*]-L-*lysine* (10).—The preceding dipeptide diester (9) (1.65 g, 2.4 mmol) was dissolved in a 19 : 1 v/v mixture of redistilled trifluoroacetic acid and water (10 ml) at 0 °C. The solution was allowed to return to room temperature and kept for 0.5 h, then evaporated to dryness. Trituration of the residue with ether–light petroleum (3 : 1) afforded a white solid which was recrystallised from ethyl acetate–light petroleum to give the *protected acid* (1.29 g, 85%); m.p. ill-defined, range 95—120 °C; $[\alpha]_D^{20}$ +10.2° (*c* 1 in MeOH); R_F (d) 0.15, δ [$CDCl_3$ + 3 drops $(CD_3)_2SO$] 1.1—2.4 [10 H, m, $(CH_2)_3$ + $(CH_2)_2$], 3.09 (2 H, m, t, *J* 6 Hz on D_2O exchange, CH_2NH), 4.35 (2 H, m, 2 × *CH*NH), 5.05 (4 H, s), and 5.11 (2 H, s, 3 × $OCH_2C_6H_5$), 6.15 (1 H, m, D_2O exchanged, $NHCH_2$), 6.90 (2 H, m, 2 × CHCH), and 3.30 (15 H, br s, C_6H_5) (Found: C, 63.9; H, 5.9; N, 6.5. $C_{34}H_{39}N_3O_9 \cdot 0.5H_2O$ requires C, 63.6; H, 6.2; N, 6.5%).

N-*Benzyloxycarbonyl*-D-*glutamyl* α-*Benzyl Ester* γ-[N(ε)-*Benzyloxycarbonyl*]-L-*lysyl*-6-*aminopenicillanic Acid* 4-*Nitrobenzyl Ester* (11).—The preceding protected acid (10) (0.63 g, 1 mmol) was dissolved in dry methylene dichloride (7 ml) together with 6-aminopenicillanic acid 4-nitrobenzyl ester toluene-4-sulphonate (0.52 g, 1 mmol). To this solution were added triethylamine (0.14 ml, 1 mmol) and 1-hydroxybenzotriazole monohydrate [15] (0.15 g, 1 mmol). The solution was cooled to 0 °C and a solution of *N,N'*-dicyclohexylcarbodiimide (0.23 g, 1.05 mmol) in methylene dichloride (3 ml) was added in one portion. The reaction mixture was stored at 0 °C for 16 h, filtered and the filtrate evaporated to dryness. The residue was taken up in ethyl acetate (30 ml) and washed with 10% aqueous citric acid, water, saturated aqueous sodium hydrogen carbonate, water and brine, then dried (Na_2SO_4). Trituration with ether–light petroleum gave a pale yellow solid which was reprecipitated from chloroform–light petroleum to give *protected penicillin* (0.73 g); m.p. 96.5—100 °C; $[\alpha]_D^{20}$ +52.5° (*c* 1 in $CHCl_3$); R_F (d) 0.60; δ ($CDCl_3$) 1.0—2.4 [16 H, m, $(CH_3)_2C$ + $(CH_2)_3$ + $(CH_2)_2$], 3.10 (2 H, m, t, *J* 6 Hz on D_2O exchange, CH_2NH), 4.38 (2 H, m, 2 × *CH*NH), 4.45 (1 H, s, 3-H of penicillin), 5.06 (4 H, s), 5.13 (2 H, s) and 5.26 (2 H, s, 4 × $OCH_2C_6H_5$), 5.51 (2 H, m, ABq t on D_2O exchange, 5- and 6-H of penicillin), 5.85 (1 H, br d, NHCH), 6.55 (1 H, br d, NHCH), 7.30 (17 H, br s, 3 × C_6H_5 + 2 × NH; 15 H on D_2O exchange), 7.50 (2 H, d, *J* 9 Hz), and 8.21 (2 H, d, *J* 9 Hz, *p*-$O_2NC_6H_4$−). An analytical sample was obtained by a further reprecipitation from chloroform–ether, m.p. 97—100 °C (Found: C, 60.5; H, 5.5; N, 8.8; S, 3.3. $C_{49}H_{54}N_6O_{13}S \cdot 0.5H_2O$ requires C, 60.3; H, 5.6; N, 8.6; S, 3.3%).

D-*Isoglutamyl*-L-*lysyl*-6-*aminopenicillanic Acid* (12).—The fully protected penicillin (11) (200 mg, 0.21 mmol) was dissolved in redistilled tetrahydrofuran (5 ml) and water (2.5 ml) was added, followed by two further drops of tetrahydrofuran to clear the solution. 10% Palladium-on-carbon (Engelhard, 400 mg) was added and the mixture hydrogenated for 16 h. Further catalyst (100 mg) was added and the hydrogenation contained for another 4 h. The mixture was filtered through a tight Celite pad and the latter was well washed with water. The aqueous solution was concentrated to *ca.* 5 ml and further small traces of catalyst filtered off through a Whatman No. 50 paper. Lyophilisation gave the dipeptidyl penicillin (82 mg, 84%) as a pale yellow powder of indefinite m.p., $[\alpha]_D^{20}$ +129.4° (*c* 1 in H_2O); R_F (a) *ca.* 0, R_F (b) 0.30; biochromatogram, single zone, R_F *ca.* 0.02; δ (D_2O) 1.1—2.7 [16 H, m, $(CH_3)_2O$ + $(CH_2)_3$ + $(CH_2)_2$], 2.95 (2 H, m, CH_2NH_2), 3.6 (1 H, m, $CHNH_2$), 4.20 (1 H, s, 3 H of penicillin), 4.30 (1 H, br m, CHNH), and 5.50 (2 H, ABq t, 5- and 6-H of penicillin). Solvent traces were also visible.

N-*Benzyloxycarbonyl*-D-*glutamyl* α-*Benzyl Ester* γ-D-*phenylglycyl*-6-*aminopenicillanic Acid* (13).—The dicyclohexylammonium salt of (7) (5.5 g, 10 mmol) was converted into its free acid by partitioning between ethyl acetate (50 ml) and 10% aqueous citric acid (50 ml). The organic phase was separated, washed with water and brine and dried (Na_2SO_4). Evaporation of solvent gave the acid as a pale yellow oil which crystallised on storing under ether–light petroleum overnight at 0 °C. Recrystallisation from ethyl acetate–light petroleum afforded a colourless solid (2.52 g, 68%); m.p. 81—82.5 °C; $[\alpha]_D^{20}$ +9.9° (*c* 3 in AcOH) {lit.,[24,25] m.p. 94—96 °C; m.p. 96.5—98.5 °C, both for L-enantiomer; $[\alpha]_D^{20}$ −11.7° (*c* 3.15 in AcOH) for L-enantiomer}; R_F(c) 0.60 (Found: C, 64.7; H, 5.4; N, 3.7. Calc. for $C_{20}H_{21}NO_6$: C, 64.7; H, 5.7; N, 3.8%).

This acid (17) (1.86 g, 5 mmol) was dissolved in dry methylene dichloride (15 ml) containing triethylamine (0.69 ml, 5 mmol) and cooled to −10 °C. Ethyl chloroformate (0.48 ml, 5 mmol) was added and the solution kept at −10 °C with stirring for 0.5 h. Meanwhile a solution of anhydrous D-phenylglycyl-6-aminopenicillanic acid (1.75 g, 5 mmol) in dry

methylene dichloride (15 ml) containing triethylamine (1.4 ml, 10 mmol) was prepared. When the mixed anhydride formation was complete, the two solutions were combined and stirred at ambient temperature for 1 h. After this time the solvent was evaporated and the residue partitioned between water (30 ml) and ether (30 ml). The separated aqueous phase was covered with ethyl acetate (30 ml) and acidified to pH 2 with vigorous stirring. Separation of the organic phase followed by washing with water and brine, drying (Na_2SO_4) and evaporation gave a pale yellow gum. Trituration with ether–light petroleum gave the *protected penicillin acid* (2.50 g, 71%) as an off-white powder of indefinite m.p.; $[\alpha]_D^{20}$ +114.1° (*c* 1 in MeOH); R_F (d) 0.20; R_F (c) 0.70; biochromatogram, single zone, R_F 0.82; δ ($CDCl_3$) 1.40 and 1.45 [6 H, 2s, $(CH_3)_2$], 1.80—2.50 [4 H, m, $(CH_2)_2$], 4.25 (1 H, m, C*H*NH), 4.34 (1 H, s, 3 H of penicillin), 5.03 and 5.06 (4 H, 2s, 2 × $OCH_2C_6H_5$), 5.3—6.0 (4 H, m, 5- and 6-H of penicillin, C_6H_5CHNH and one NHCH), 7.0—7.5 (16 H, br s, 3 × C_6H_5 and one NHCH), and 7.6—8.0 (1 H, br s, N*H*). On D_2O exchange the δ 5.3—5.7 region simplifies: 5.35 and 5.50 (2 H, Abq t, 5- and 6-H of penicillin), 5.58 (1 H, s, C_6H_5CHNH), and the δ 7.3 signal sharpens considerably with loss of the δ 7.6—8.0 signal (Found: C, 60.9; H, 5.45; N, 7.55. $C_{36}H_{38}N_4O_9S\cdot0.5H_2O$ requires C, 60.8; H, 5.5; N, 7.8%).

D-*Isoglutamyl*-D-*phenylglycyl*-6-*aminopenicillanic Acid* (14).—The protected penicillin acid (13) (1.25 g, 1.78 mmol) was dissolved in ethanol (30 ml) containing water (20 ml) and hydrogenated over 10% palladium-on-carbon (1.25 g). After 3.5 h, further catalyst (0.5 g) was added and hydrogenation was continued for a further 2 h. The catalyst was filtered off using Celite and the filtrate was lyophilised to give the *dipeptidyl penicillin* (0.53 g, 52%) as a white powder; $[\alpha]_D^{20}$ +160.7° (*c* 1 in H_2O); R_F (a) 0.15; biochromatogram, single zone, R_F 0.12; δ (D_2O) 1.37 and 1.42 [6 H, 2s, $(CH_3)_2C$], 2.13 and 2.44 [4 H, 2m, $(CH_2)_2$], 3.76 (1 H, m, C*H*NH), 4.21 (1 H, s, 3-H of penicillin), 5.4 (3 H, s, 5- and 6-H of penicillin and C_6H_5CHNH), and 7.28 (5 H, s, C_6H_5) (Found: C, 47.1; H, 6.3; N, 10.3. $C_{21}H_{26}N_4O_7S\cdot3H_2O$ requires C, 47.4; H, 6.0; N, 10.5%).

N-*Acetyl*-D-*isoglutamyl*-D-*phenylglycyl*-6-*aminopenicillanic Acid Disodium Salt* (15).—The preceding penicillin (14) (50 mg, 0.1 mmol) was dissolved in 1M-sodium hydrogen carbonate solution (1 ml). Redistilled acetic anhydride (2 drops) was added and the progress of the reaction monitored by t.l.c. Further acetic anhydride (to a total of 0.5 ml) was added so long as starting material could be seen. After 4 h at ambient temperature the pH of the solution was lowered to 2 with concomitant extraction into ethyl acetate (3 × 10 ml). The total organic extract was washed with brine, dried (Na_2SO_4) and evaporated. The residue was dissolved in methanol (2 ml) and 2M-sodium ethyl hexanoate in methyl isobutyl ketone (0.03 ml) was added. The resulting solution was again evaporated and triturated with dry ether to afford the product disodium salt (26 mg, 44%) as an amorphous white solid; R_F (a) 0.35; biochromatogram, single zone, R_F 0.07; δ [$(CD_3)_2SO$] 1.40 and 1.52 [6 H, 2s, $(CH_3)_2C$], 1.69 (3 H, s, CH_3CO), 2.18 [4 H, m, $(CH_2)_2$], 3.80 (1 H, s, 3-H of penicillin), 3.81 (1 H, s, C*H*NH), 5.25 and 5.35 (2 H, m, ABq t on D_2O exchange, 5- and 6-H of penicillin), 5.69 (1 H, d, *J* 7 Hz, s on D_2O exchange, C_6H_5CHNH), 7.20—7.50 (5 H, m, C_6H_5), 8.65, 8.73, and 8.92 (3 H, all ds, D_2O exchanged, N*H*s). Solvent traces were also visible.

N(α)-*Benzyloxycarbonyl*-DL-*lysine* (16).—This was prepared *via* the *N*-(ε)-benzylidene derivative following the procedure of Handford *et al.*[26] on a 0.1-M scale to give the mono-protected acid, which was recrystallised from water (6.2 g, 30%); m.p. 214.5—216 °C (decomp.) (lit.,[24] 230—233 °C for L-compound) (Found: C, 59.9; H, 6.9: N, 10.1. Calc. for $C_{14}H_{20}N_2O_4$: C, 60.0; H, 7.1; N, 10.0%).

N(α)-*Benzyloxycarbonyl*-N(ε)-*acetyl*-DL-*lysine* (17).—The preceding *N*-(α)-protected derivative (16) (2.8 g, 10 mmol) was suspended in 1.0M-sodium hydrogen carbonate (20 ml) and cooled to 0 °C. Acetic anhydride (2.75 ml) was added dropwise during 0.1 h, and after a further 0.3 h another batch (5 ml) was added. Further acetic anhydride (2 × 2.5 ml) was added during 0.5 h, after which time no starting material was visible by t.l.c. After 2.5 h the solution was acidified to pH 1 with 2M-hydrochloric acid and the product extracted into ethyl acetate (3 × 25 ml). The combined organic extract was washed with water and brine, dried and evaporated to a colourless, viscous oil. Trituration with ether afforded the *protected acid* (2.39 g) as a white solid which was recrystallised from ethyl acetate–light petroleum containing a little ethanol (2.16 g, 67%); m.p. 132—134 °C; R_F (c) 0.35; δ [$(CD_3)_2SO$] 1.10—1.70 [6 H, m, $(CH_2)_3$], 1.75 (3 H, s, CH_3CO), 2.97 (2 H, m, t, *J* 6 Hz on D_2O exchange, CH_2NH), 3.92 (1 H, m, C*H*NH), 5.01 (2 H, s, $OCH_2C_6H_5$), 7.33 (5 H, s, C_6H_5), 7.8 (1 H, d, *J* 7 Hz, D_2O exchanged, CHN*H*), 7.75 (1 H, t, *J* 6 Hz, D_2O exchanged, CH_2NH), 12.45 (1 H, br s, D_2O exchanged, CO_2H) (Found: C, 60.0; H, 7.1; N, 8.7. $C_{16}H_{22}N_2O_5$ requires C, 59.6; H, 6.8; N, 8.7%).

N(α)-*Benzyloxycarbonyl*-N(ε)-*acetyl*-DL-*lysyl*-6-*amino-penicillanic Acid Benzyl Ester* (18).—The preceding acid derivative (17) (1.29 g, 4 mmol) was dissolved with 6-amino-penicillanic acid benzyl ester toluene-4-sulphonate (1.91 g, 4 mmol) in dry acetonitrile (10 ml) and triethylamine (0.56 ml, 4 mmol) was added. The solution was cooled to 0 °C and 1-hydroxybenzotriazole monohydrate (0.61 g, 4 mmol) was added, followed by a solution of *N*,*N*′-dicyclohexylcarbodi-imide (0.82 g, 4 mmol) in acetonitrile (2 ml). The mixture was left at 0 °C overnight, then worked up exactly as described for (16) to give a pale yellow gum, which on trituration with ether–light petroleum gave a solid (1.86 g). Reprecipitation from ethyl acetate–light petroleum gave the *product di-astereoisomers* (1.47 g, 60%) as a powder of ill-defined m.p. which retained solvent traces tenaciously; R_F (d) 0.45, 0.50; δ ($CDCl_3$) 1.10—1.80 [6 H, m, $(CH_2)_3$], 1.40 and 1.57 [6 H, 2s, $(CH_3)_2$], 1.91 (3 H, s, CH_3CO), 3.18 (2 H, m, *ca.* t, *J* 6 Hz on D_2O exchange, CH_2NH), 4.20 (1 H, m, *ca.* t, *J* 5 Hz on D_2O exchange, CH_2CHNH), 4.47 (1 H, s, 3-H of penicillin), 5.10 (2 H, s, $OCH_2C_6H_5$), 5.17 (2 H, s, $OCH_2C_6H_5$), 5.55 (2 H, m, ABq t on D_2O exchange, 5- and 6-H of penicillin), 5.65 and 5.85 (2 H, 2 br m, D_2O exchanged, NHs), 7.35 (10 H, br s, 2 × C_6H_5), and 7.2—7.4 (1 H, m, D_2O exchanged, NH).

N(ε)-*Acetyl*-DL-*lysyl*-6-*aminopenicillanic Acid* (19).—The preceding fully protected penicillin (18) (1.35 g, 2.2 mmol) was dissolved in a mixture of ethanol (15 ml) and water (5 ml). 10% Palladium-on-carbon (1 g) was added and the mixture hydrogenated for 16 h. In the morning the catalyst was filtered off (Celite) and a fresh batch (1 g) added, then hydrogenation was resumed for 2.5 h. This cycle was repeated once more, then the catalyst was finally filtered off and washed well with water. The filtrate was washed with ethyl acetate (3 × 10 ml) to remove traces of protected material remaining, then the aqueous layer was freeze-dried to give the *product diastereo-isomers* (350 mg, 41%); R_F (a) 0.1; biochromatogram, R_F 0.12, single zone; δ [$(CD_3)_2SO$] 1.10—1.70 [6 H, m, $(CH_2)_3$], 1.47 and 1.57 [6 H, 2s, $(CH_3)_2C$], 1.77 (3 H, s, CH_3CO), 3.00

(2 H, m, CH_2NH), 3.4—3.8 (1 H, m, simplified on D_2O exchange, CH_2CHNH), 4.03 (1 H, s, 3-H of penicillin), 4.8—5.6 (3 H, m, D_2O exchanged, NH_2 and one NH), 5.40 (2 H, m, ABq t on D_2O exchange, 5- and 6-H of penicillin), 7.83 (1 H, m, D_2O exchanged, NH) (Found: C, 45.4; H, 7.3; N, 13.0. $C_{16}H_{26}N_4O_5S\cdot 2H_2O$ requires C, 45.5; H, 7.1; N, 13.3%).

Owing to the low biological activity of (19), no subsequent effort was made to separate the diastereoisomers of (18) and hence of (19).

N(α)-N(ε)-*Diacetyl*-DL-*lysine* (20).—This was prepared according to the procedure of Greenstein and Winitz [27] on a 50-mmol scale. The product was recrystallised from acetone (4.35 g, 38%), m.p. 139—140.5 °C (lit.,[27] m.p. 141 °C).

N(α),N(ε)-*Diacetyl*-DL-*lysyl*-6-*aminopenicillanic Acid* 4-*Nitrobenzyl Ester* (21).—The diacetyl acid (20) (1.15 g, 5 mmol) was dissolved in dry dimethylformamide (20 ml) together with 6-aminopenicillanic acid 4-nitrobenzyl ester (1.75 g, 5 mmol) which had been obtained from its toluene-4-sulphonate by extraction into ethyl acetate from 1.0M-sodium hydrogen carbonate solution. 1-Hydroxybenzotriazole monohydrate (0.77 g, 5 mmol) was added and the solution cooled to 0 °C with stirring. Finally *N,N'*-dicyclohexylcarbodi-imide (1.03 g, 5 mmol) in dimethylformamide (2 ml) was added, and the solution was allowed to reach room temperature. After 2 h the urea was filtered off and the filtrate evaporated to dryness at room temperature and <1 mmHg. Work-up as described for (11) afforded 2.31 g of crude product, which was reprecipitated from ethyl acetate–ether to give the *product diastereoisomers* (1.72 g, 61%) as an off-white powder of indefinite m.p.; R_F (d) 0.30, 0.35; δ ($CDCl_3$) 1.30—1.90 [6 H, m, $(CH_2)_3$], 1.45 and 1.65 [6 H, 2s, $(CH_3)_2C$], 1.98 and 2.03 (6 H, 2s, 2 × CH_3CO), 3.30 (2 H, m, CH_2NH), 4.50 (1 H, m, $CHNH$), 4.53 (1 H, s, 3-H of penicillin), 5.35 (2 H, s, $OCH_2C_6H_4NO_2$), 5.65 (2 H, m, ABq t on D_2O exchange, 5- and 6-Hs of penicillin), 6.45 (1 H, t, *J* 6 Hz, D_2O exchanged, CH_2NH), 7.05 (1 H, d, *J* 7 Hz, D_2O exchanged, $CHNH$), 7.60 and 8.30 (4 H, 2d, *J* 9 Hz, $CH_2C_6H_4NO_2$-*p*), and 7.90 (1 H, br d, D_2O exchanged, NH). Again the n.m.r. spectrum showed no clear resolution of the diastereoisomers (Found: C, 52.5; H, 5.8; N, 12.3. $C_{25}H_{35}N_5O_8S\cdot 0.5H_2O$ requires C, 52.4; H, 6.0; N, 12.2%).

N(α),N(ε)-*Diacetyl*-DL-*lysyl*-6-*aminopenicillanic Acid* (22).—The preceding protected derivative (0.62 g, 1.1 mmol) was dissolved in ethanol (20 ml) and hydrogenated with 10% palladium-on-carbon (0.7 g). After 2 h, t.l.c. analysis showed a little starting material remaining; further catalyst (0.3 g) was added and hydrogenation resumed for a further 1 h. The catalyst was filtered off (Celite) and the filtrate concentrated to low volume (5 ml), then the product (0.24 g) was precipitated by addition of ether (50 ml). This crude product was dissolved as far as possible in water (10 ml) and a little insoluble material filtered off. Lyophilisation gave the *diastereoisomeric penicillin acids* (0.21 g, 45%) as a freeze-dried powder; R_F (a) 0.35; biochromatogram, R_F 0.22, single zone; δ (D_2O) 1.20—1.90 [6 H, m $(CH_2)_3$], 1.58 and 1.68 [6 H, 2s, $(CH_3)_2C$], 8.00 and 8.07 (6 H, 2s, 2 × CH_3CO), 3.20 (2 H, t, *J* 6 Hz, CH_2NH), 4.35 (1 H, br t, *J* 7 Hz, CH_2CHNH), 4.53 (1 H, br s, 3-H of penicillin), and 5.55 (2 H, ABq t, 5- and 6-H of penicillin) (Found: C, 48.0; H, 6.6; N, 12.5. $C_{18}H_{28}N_4O_6S\cdot H_2O$ requires C, 48.4; H, 6.7; N, 12.6%).

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