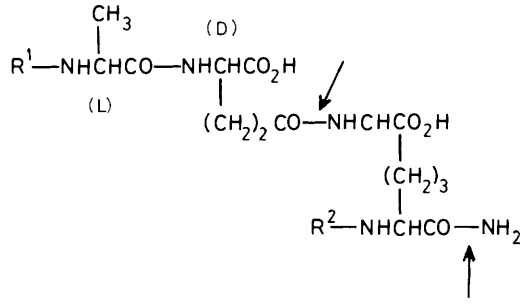
Synthesis of RP 56142: a New Immunoactive Peptide

Jean Bouchaudon,* Gilles Dutruc-Rosset, Daniel Farge, and Claude James
Rhône-Poulenc Santé, Centre de Recherches de Vitry, 94403 Vitry-sur-Seine Cedex, France

RP 56142, a new immunoactive peptide was synthesized on large scale (*ca.* 500 g) *via* L-2,6-diaminopimelic acid which was prepared by chemical or biochemical synthesis. The key derivative, N^6-benzyloxycarbonyl-L-2,6-diaminopimelamic acid was synthesized by two methods. In the first, we used a copper chelate procedure. In the second, we selectively deblocked the amine at the α-position to the free carboxylic group by the *N*-carboxyanhydride method. Condensation of N^6-benzyloxycarbonyl-L-2,6-diaminopimelamic acid and the appropriately protected lauroyl dipeptide and removal of the protecting groups afforded RP 56142.

In a programme devoted to microbial and synthetic immunoadjuvants, we became interested in the immunomodulating and adjuvant activities of crude water-soluble extracts of a strain (NRLL 5776) of *Streptomyces* and attempted, in close collaboration with P. Jollès and D. Migliore-Samour (Laboratoire des Protéines, Université de Paris V), to isolate and identify the active component of such extracts. In the course of this work, P. Jollès and D. Migliore-Samour isolated a tetrapeptide (**1**), which turned out to be inactive when tested for immunostimulating activity. Our team showed in the past[1] that chemical conjugation with lauric or palmitic acid of water-soluble peptidoglycan fragments from two strains of *M. tuberculosis var. hominis* markedly modified the adjuvant activities of these substances on both cell-mediated and humoral immune responses and, in particular, rendered them adjuvant-active in the absence of mineral oil. According to this lead, the tetrapeptide (**1**) was conjugated with lauric anhydride. Preliminary



	(**1**) R^1 = H	R^2 = H_2NCH_2CO	A_2pm:L
RP 40639	(**2**) R^1 = $CH_3(CH_2)_{10}CO$	R^2 = H_2NCH_2CO	A_2pm:rac.
RP 56142	(**3**) R^1 = $CH_3(CH_2)_{10}CO$	R^2 = H	A_2pm:L

results prompted us to prepare by total synthesis the corresponding lauroyl tetrapeptide RP 40639 (**2**) from racemic 2,6-diaminopimelic acid. This synthetic substance thus obtained was shown to stimulate *in vitro* thymidine incorporation by mouse spleen cells, enhance *in vitro* phagocytosis of sheep erythrocytes (SRBC) by mouse peritoneal macrophages and increase *in vivo* the number of anti-SRBC plaque forming cells in mouse spleen and the resistance of mice against infection with *Listeria monocytogenes*.[2–5] In our programme of studies on structure-activity of RP 40639, we synthesized 80 related compounds and it was found that the lauroyl tripeptide RP 56142 (**3**) was as active as the tetrapeptide (**2**).[6]

Synthesis

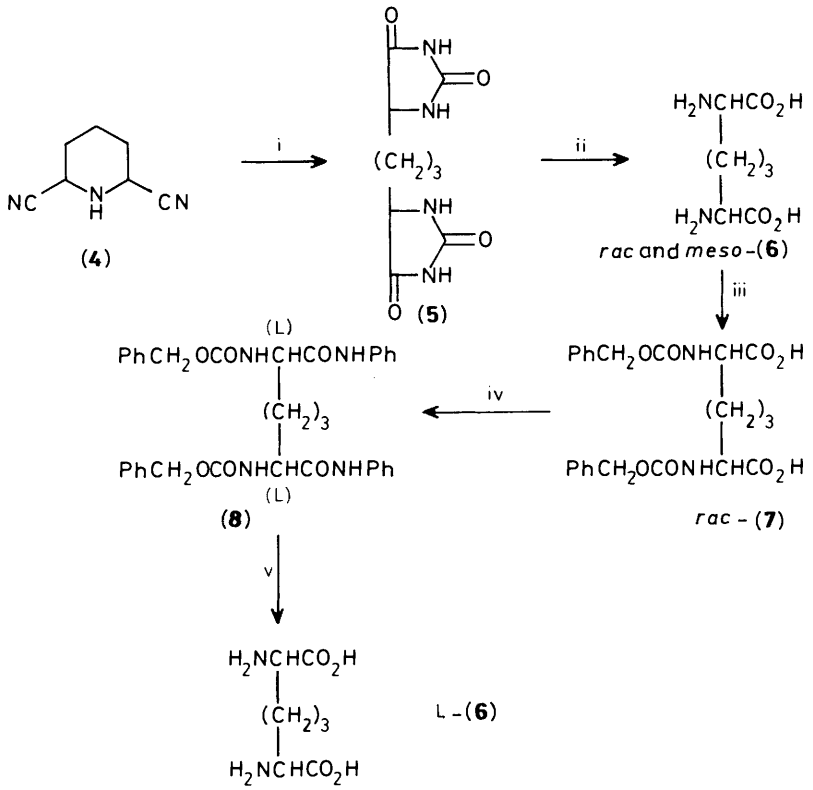
The crucial problem in the synthesis of RP 56142 was to create the peptide bonds at the positions marked by arrows with complete differentiation between the two pairs of amino acid functions in L-2,6-diaminopimelic acid. To resolve this problem, we synthesized N^6-benzyloxycarbonyl-L-2,6-diaminopimelamic acid (**14**).

Preparation of L-2,6-*Diaminopimelic Acid* L-(**6**).—The L-2,6-diaminopimelic acid was prepared by chemical and biochemical synthesis.

Chemical synthesis (Scheme 1). A modification of the method developed by R. Roy *et al.*[7] was employed. Thus piperidine-2,6-dicarbonitrile (**4**) was treated in an autoclave at 100 °C for 4 h with a mixture of ammonium hydroxide and ammonium hydrogen carbonate. The resulting 5,5′-trimethylenedihydantoin (**5**) was hydrolysed without purification with hydrobromic acid and the mixture of three isomeric 2,6-diaminopimelic acids *rac*- and *meso*-(**6**) obtained was neutralized with Amberlite IR 120. The overall yield was 65—69%. The *rac* and *meso* forms were separated by fractional crystallization of the dibenzyloxycarbonyl derivatives according to the method described by J. Van Heijenoort *et al.*[8] The determination of the relative proportion of the different isomers of 2,6-diaminopimelic acid was obtained after hydrolysis, methanolysis, and trifluoroacetylation of the derivatives. The separation of the volatile derivatives was performed by gas chromatography on an optically active capillary column. Following the method of J. Van Heijenoort,[8] we obtained, after two recrystallizations, racemic 2,6-dibenzyloxycarbonylaminopimelic acid *rac*-(**7**), containing 5% of the *meso* isomer. For the resolution of the racemic *rac*-(**7**), we followed a method used by Y. Izumi.[9] For the separation of the isomers of 2,6-diaminopimelic acid, Y. Izumi used a papain-catalyzed reaction of aniline with a bis-benzoyl derivative of 2,6-diaminopimelic acid. In the same way, we obtained the dianilide derivative (**8**). After removing the benzyloxycarbonyl groups and the amide functions with hydrochloric acid in acetic acid, we obtained L-2,6-diaminopimelic acid L-(**6**) containing $<0.3\%$ of the *meso* form and $<0.2\%$ of the D form.

Biochemical synthesis. L-2,6-Diaminopimelic acid was also prepared from cultures of a mutant of *Pseudomonas aeruginosa* called PAC 7, following the method of F. Saleh *et al.*[10] Using this method L-2,6-diaminopimelic acid (4 kg) was obtained with high optical purity ($>99.8\%$).

Preparation of N^6-*Benzyloxycarbonyl*-L-2,6-*diaminopimelamic acid* (**14**).—*First method* (Scheme 2). The esterification of L-2,6-dibenzyloxycarbonylaminopimelic acid L-(**7**) with benzyl alcohol in the presence of toluene-*p*-sulphonic acid gave the dibenzyl ester (**9**) which was saponified with 0.86 equiv. of NaOH to give the monoester (**10**) following the method of A. Arendt *et al.*[11] Amidification of (**10**) with ammonia in methanol gave the monoamide (**11**) which was hydrogenolyzed to yield L-2,6-diaminopimelamic acid (**12**). For selective protection of



Scheme 1. *Reagents and conditions:* i, NH_4OH, NH_4HCO_3; ii, HBr; iii, $PhCH_2OCOCl$, recrystallization; iv, $PhNH_2$, papaine; v, H^+

the amino groups on (**12**), we investigated benzyloxycarbonylation of (**12**) under copper chelate conditions at different pH values. Using the method of C. Nicot *et al.*,[12] we obtained a yield <10% at pH 11.5 of the insoluble copper complex. However, at pH 8 with 0.5 equiv. of cupric bromide and 1.28 equiv. of benzyl chloroformate, a better yield was obtained (67%). Treatment of this complex (**13**) with hydrogen sulphide gave N^6-benzyloxycarbonyl-L-2,6-diaminopimelamic acid (**14**) (yield: 29%). Contrary to the results of C. Nicot *et al.*,[12] in our case, the benzyl chloroformate reacted with the amino group adjacent to the carboxamide function and not the free carboxylic acid, giving (**14**) instead of (**15**). The structure of (**14**) was assigned by the titrimetric method for continuous determination of carbon dioxide of A. Patchornik.[12,13] Ninhydrin in acid medium on (**14**) provoked a rapid evolution of CO_2 which is characteristic of a free α-amino acid (Figure 1). These results were confirmed by the identical spectral characteristics and α_D of (**14**) prepared according to the unequivocal second method.

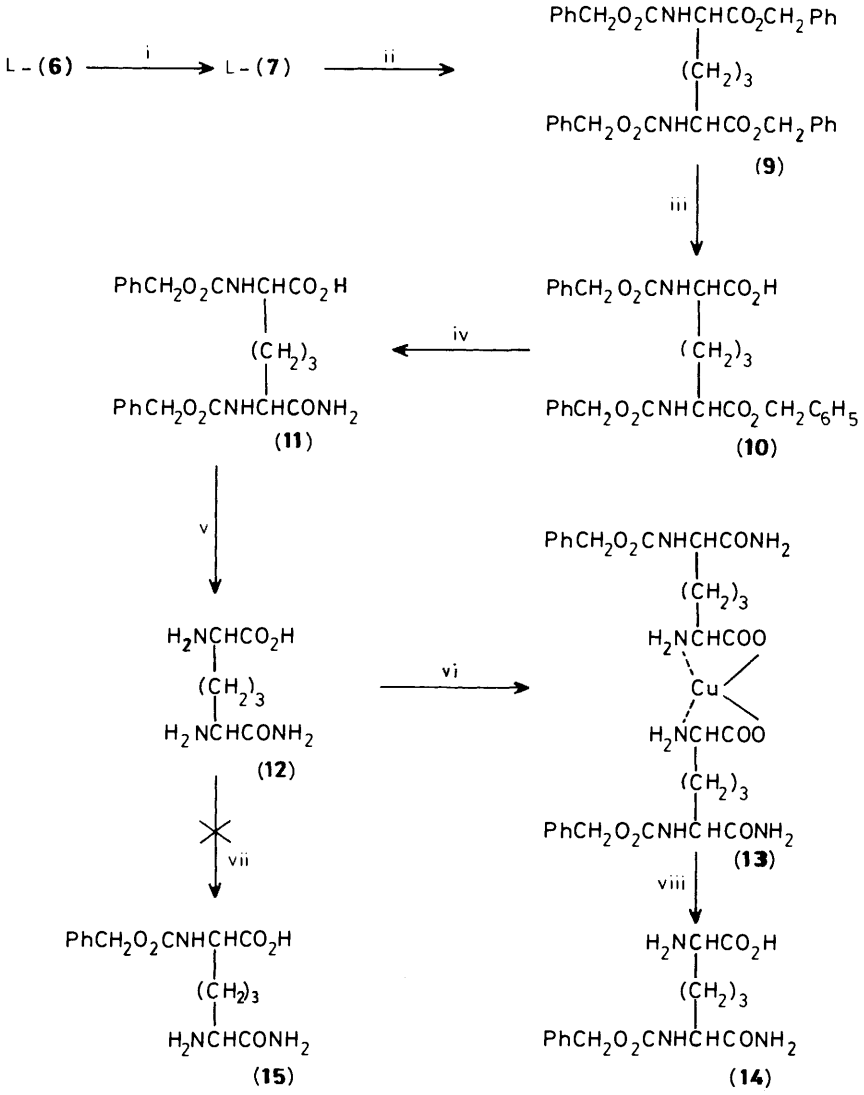
Second method (Scheme 3). The monoesterification of the triethylammonium salt of L-2,6-dibenzyloxycarbonylaminopimelic acid L-(**6**) with an equimolecular quantity of *p*-nitrobenzyl bromide gave the monoester (**16**) according to the method of A. Arendt *et al.*[14] The monoester was treated with phosphorus pentachloride in dichloromethane to afford the *N*-carboxy-anhydride (**17**), which upon hydrolysis, yielded O^1-*p*-nitrobenzyl-N^2-benzyloxycarbonyl-L-2,6-diaminopimelic acid (**18**) (81%). Amidification of (**18**) with ammonia gave N^6-benzyloxycarbonyl-L-2,6-diaminopimelamic acid (**14**) (66%).

Preparation of O^1-*Benzyl*-N-(N-*lauroyl*-L-*alanyl*)-D-*glutamic Acid* (**21**).—The remaining fragment necessary for constructing the framework of RP 56142 was the appropriately protected lauroyl dipeptide (**21**). O^1-Benzyl-*N*-(t-butoxycarbonyl-L-alanyl)-D-glutamic acid (**19**) was prepared in the standard manner.[15] Treatment with hydrogen chloride in acetic acid removed the t-butoxycarbonyl protecting group to afford (**20**). Lauroyl chloride was allowed to react with silylated (**20**) prepared *in situ* by treatment with bis(trimethylsilyl)acetamide (BSA). A 79% yield of the condensation product (**21**) was thus obtained which was sufficiently pure without column chromatography.

Preparation of RP 56142 (**3**).—Compound (**21**) was converted into the mixed anhydride *in situ* with isobutyl chloroformate and then allowed to react with an aqueous solution of the sodium salt of (**14**) to afford the blocked lauroyl tripeptide (**22**). When the condensation was performed using equimolecular quantities of the reagents, we obtained (**22**) as a mixture of diastereoisomers containing *ca.* 5% of the diastereoisomer with *meso*-2,6-diaminopimelic acid. This racemisation was proved by hydrolysis of (**22**) with 6M HCl at 80 °C over 16 h in a sealed tube and measurement of the percentage of the different isomers of 2,6-diaminopimelic acid in the hydrolysate by the method described above (Figure 2). However, by coupling the mixed anhydride with 1.1 equiv. of the sodium salt of (**14**), we eliminated racemisation and obtained (**22**) in a 76% yield. Removal of the benzyloxycarbonyl and the benzyl protecting groups by hydrogenolysis over 10% palladium–charcoal yielded after silica gel column chromatography the final product (**3**) (74%).

Experimental

All solvents and reagents were of analytical grade and used without further purification. Petroleum refers to the fraction boiling 35—60 °C. M.p.s were determined on a Kofler melting point apparatus. The values are uncorrected. Optical activity was measured by determining the optical rotation of sodium D



Scheme 2. *Reagents and conditions:* i, $PhCH_2OCOCl$; ii, $PhCH_2OH$, *p*-$MeC_6H_4SO_3H$; iii, 1 equiv. NaOH; iv, NH_3; v, H_2–Pd/C; vi, $CuBr_2$, $PhCH_2OCOCl$, pH 8; vii, $CuBr_2$/$PhCH_2OCOCl$, pH 11.5/H_2S; viii, H_2S

light with a Perkin-Elmer polarimeter model 241. Determination of the percentage of the different isomers of A_2pm: a mixture of a sample of the derivative of A_2pm (2 mg) and anhydrous HCl in MeOH (6M, 1 ml) was refluxed over 16 h in the absence of moisture and concentrated to dryness under reduced pressure. The residue was treated with trifluoroacetic anhydride (200 µl) and the mixture was allowed to stand 0.5 h at 20 °C and then concentrated to dryness. A solution (1 µl) of the residue in EtOAc (1 ml) was injected as outlined in Figure 2. The optical isomers of A_2pm were eluted in the order: D-A_2pm, L-A_2pm and *meso*-A_2pm (retention time: *ca.* 11 min). The calculation of the percentage of the different isomers was made by internal normalization, supposing that the response factors of the three isomers were the same. The Chirasil-Val column separated D-A_2pm from the mixture of L- and *meso*-A_2pm and the Wax-57 column separated *meso*-A_2pm from the mixture of L- and D-A_2pm. The percentage of the L isomer was obtained by difference.

Thin-layer chromatography was performed on silica gel precoated plates 60 F_{254} (Merck). The solvent systems (by vol.) were: A, EtOAc–toluene–AcOH (70:30:10); B, AcOH–EtOAc (80:20); C, BuOH–pyridine–AcOH-H_2O (50:20:6:24); D, EtOAc–AcOH–H_2O (40:12:10); E, $ClCH_2CH_2Cl$–MeOH (65:35); F, EtOAc–MeOH (65:35); G, EtOAc–AcOH (60:40); and H, EtOAc–AcOH (99:1). Detection was mainly carried out with ninhydrin, the chlorine-*o*-toluidine reagent and iodine sulphuric acid.

^1H N.m.r. spectra were obtained at 250.13 MHz on a Bruker WM 250 and at 400.13 MHz on a Bruker AM 400. The frequencies (δ in p.p.m.) were given compared with the central line of [$^2H_6$]DMSO (2.5 p.p.m.).

2,6-*Diaminopimelic Acid* rac- *and* meso-(**6**).—A mixture of 2,6-dicyanopiperidine (**4**) (337.5 g, 2.5 mol), ammonium bicarbonate (1 687 g, 20.8 mol) and NH_4OH (5M, 1.5 l) was heated at 100—105 °C in a 5 l autoclave for 4 h. The insoluble material was filtered off, washed with water (400 ml), and the aqueous phases were concentrated to dryness. The resultant oil (589 g, 98%) was combined with another batch of 5,5′-trimethylenedihydantoin (**5**) (589 g, 2.45 mol) obtained *via* an identical operation and refluxed with HBr (48%, d = 1.49, 5.6 l, 49 mol) for 36 h. The reaction mixture was cooled to 0 °C, filtered and concentrated to dryness. The residue was dissolved in water (4 l), then concentrated again to dryness and

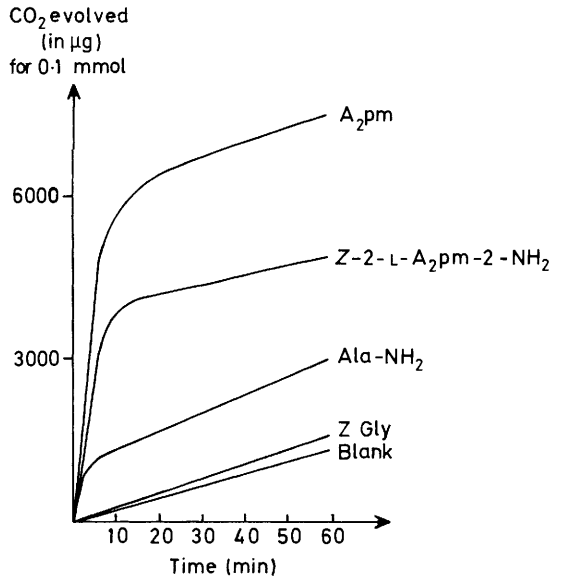


Figure 1. Evolution of CO_2 under the action of ninhydrin as a function of the heating time at 100 °C. The evolved CO_2 obtained with 0.1mM of product dissolved in 0.3M citrate buffer (pH 2.5) (3 ml) under the action of ninhydrin (100 mg) was absorbed by benzylamine and titrated with 0.16M sodium ethoxide in presence of Thymol Blue

redissolved in water (4 l). This solution was adsorbed on an Amberlite IR 120 column (H^+ form, 10 l, i.d.: 16 cm). The column was washed with water (20 l) until no bromide ion was eluted, then the 2,6-diaminopimelic acid was eluted with NH_4OH (4M, 50 l). The eluate was concentrated to dryness and the residue was treated with EtOH (4 l), filtered, washed with EtOH (1 l), isopropyl ether (1 l) and dried to give a white solid *rac*- and *meso*-(**6**) (658 g, 70%).

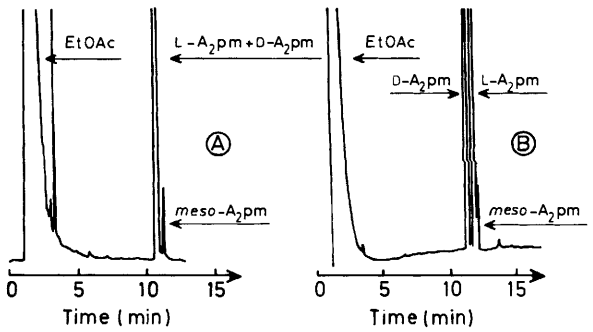
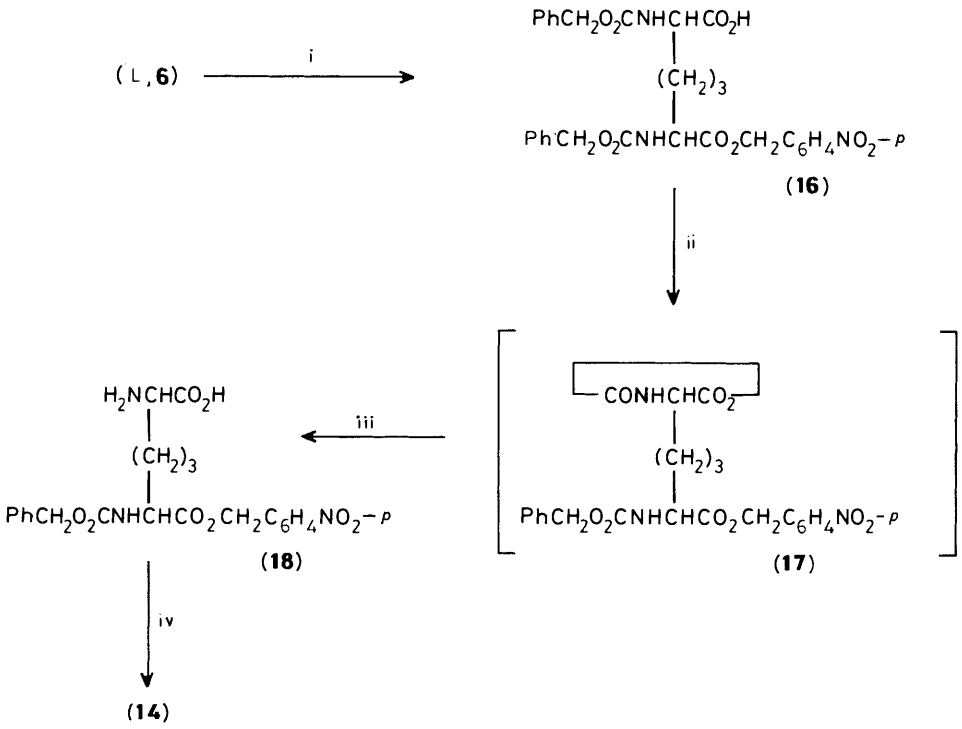


Figure 2. Determination of the relative proportions of the isomers of A_2pm by g.l.c. Chromatograph, Carlo Erba HRGC 4160; capillary column, 25 m; vector gas, He (0.5 bar); detection by flame ionization

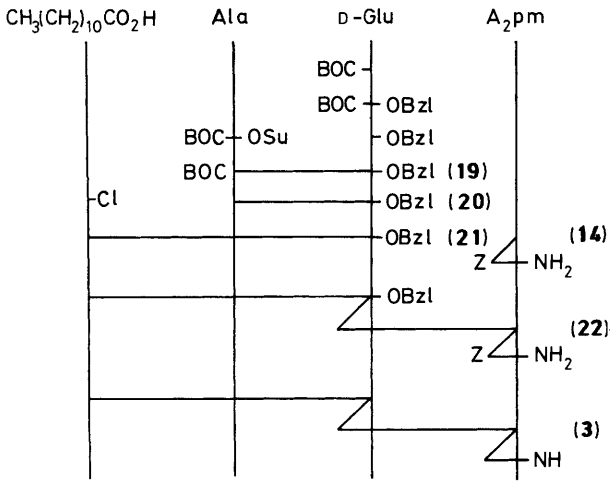
		Ⓐ	Ⓑ
	Phase	WAX-57	CHIRASIL-VAL
Temperature (°C)	Injector	270	270
	Detector	300	300
	Oven	180	160

2,6-*Dibenzyloxycarbonylaminopimelic Acid* rac-(**7**).—Following the method of J. Van Heijenoort *et al.*,[8] the reaction of 2,6-diaminopimelic acid (510 g, 2.68 mol) with benzyl chloroformate (1 236 g, 7.24 mol) gave *rac*-(**7**) (380.3 g) after recrystallization from EtOAc; m.p. 160 °C, *meso* 9.4%. A second recrystallization of combined batches of the acid (1 036 g, *meso* 10%) gave a white crystalline solid *rac*-(**7**) (762 g, total yield; 45%), m.p. 167 °C softening 150 °C (lit.,[8] m.p. 165.5 °C softening 164—165 °C); *meso* 5%.

L-N,N-*Diphenyl*-2,6-*dibenzyloxycarbonylaminopimelamide* (**8**).—To 0.8M NaOH (726 ml) were added *rac*-(**7**) (*meso* 5%) (133 g, 0.29 mol) and aniline (26.5 ml, 0.29 mol). The solution



Scheme 3. *Reagents and conditions:* i, *p*-$NO_2C_6H_4CH_2Br$, Et_3N; ii, PCl_5; iii, H^+; iv, NH_4OH



Scheme 4.

was adjusted to pH 5.2 with AcOH. A solution of papain (Merck; biochemical utilisation) (7.25 g) and L-cysteine (2 g) in a mixture of water (145 ml) and phosphate buffer pH 5.2 (0.1M KH_2PO_4–0.1M Na_2HPO_4 100:2) (218 ml) was then added followed by phosphate buffer (870 ml). The reaction mixture was allowed to stand for 24 h at 37 °C and then filtered. The solid was washed with water (200 ml) and dried. The product (74.7 g) was recrystallized from AcOH (1 040 ml) (50.3 g, 57%, m.p. 244 °C; $[\alpha]_D^{20}$ +18.9° (c 1 in MeOH); L-A_2pm 99.7%, *meso*-A_2pm 0.3%; δ_H (400 and 250 MHz; [$^2H_6$]DMSO) 1.49 [2 H, m, CH_2 (A_2pm-4)], 1.69 [4 H, m, CH_2 (A_2pm-3,5)], 4.17 (2 H, m, CH) 5.03 (4 H, AB, CH_2OCO), 7—7.65 and 7.55 (22 H, m and d, aromatic and OCONH), 10.03 (2 H, br m, CONH).

L-2,6-*Diaminopimelic Acid Hydrate* L-(**6**).—Compound (**8**) (53.8 g, 88.4 mmol) was added to a mixture (1:1, 1 080 ml) of HCl (d = 1.19) and AcOH. The mixture was refluxed for 24 h and then concentrated to dryness. The residue was dissolved in water (110 ml), neutralized to pH 6.4 with LiOH and EtOH (600 ml) was added. The resultant white solid was filtered off, washed with EtOH (300 ml) and dissolved in water (180 ml). Addition of EtOH (1 l) gave a white solid which was washed with EtOH (200 ml), filtered off and dried (16.8 g, 91%), $[\alpha]_D^{20}$ +43° (c 0.88 in 5M HCl) [lit.,[8] +44.5° (c 1 in 5M HCl)].

L-2,6-*Dibenzyloxycarbonylaminopimelic Acid* L-(**7**).—A vigorously stirred solution of L-(**6**) (obtained by biochemical synthesis) (238 g, 1.14 mol) in NaOH (1M; 6.25 l) at 0 °C was treated during 1 h with benzyl chloroformate (490 ml, 3.43 mol), then stirred for a further 1 h at 0 °C and allowed to warm to room temperature over 16 h. NaOH (1M, 400 ml) was added during the first 5 h to maintain the pH at 8—9. The unreacted benzyl chloroformate was removed by extraction with EtOAc (3 l), the aqueous layer was acidified with HCl (4M, 800 ml) and kept overnight. The precipitated solid was isolated by decantation, dissolved in a mixture of methyl ethyl ketone and EtOAc (1:1, 2 l), washed with HCl (1M, 500 ml), dried ($MgSO_4$) and then concentrated. A solution of the residue in EtOH (1 l) was treated during 0.5 h with dicyclohexylamine (500 ml), then diluted with EtOH (1 l) and kept at 10 °C for 2 h. The precipitated solid was filtered off, washed successively with EtOH (900 ml) and ether (250 ml) and dried to afford L-(**7**) as the dicyclohexylammonium salt (723 g), $[\alpha]_D^{20}$ +9° (c 1 in EtOH) [lit.,[8] $[\alpha]_D$ +9.5° (c 1 in EtOH)]. A second crop of 123 g was obtained by concentration to dryness of the mother liquors and addition of EtOAc (500 ml). The combined crops (846 g) were suspended in a mixture of water (5 l) and EtOAc (3 l) and treated with methanesulphonic acid (4M; 620 ml). The organic layer was separated and the aqueous layer was washed with EtOAc (3 l). The combined organic layers were dried (Na_2SO_4), concentrated to 1.5 l and allowed to stand at 0 °C overnight. The precipitated solid was filtered off, washed with ether and dried to afford 222 g of L-(**7**) as a white solid. The mother liquors were concentrated to dryness and the residue was partitioned between EtOAc (2 l) and water (3 l). The organic layer was washed with methanesulphonic acid (4M, 300 ml) and treated as above to yield a second crop of 260 g. Total yield 482 g (92%), $[\alpha]_D^{20}$ −4.7° (c 2 in EtOH) (lit.,[8] $[\alpha]_D$ −4.4°); m.p. 150 °C (lit.,[8] 153—155 °C); R_F(A) 0.65.

L-2,6-*Dibenzyloxycarbonylaminopimelic Acid Dibenzyl Ester* (**9**).—A solution of L-(**7**) (44.7 g, 97 mmol) in benzyl alcohol (30 ml) and toluene (300 ml) was refluxed during 5 h with toluene-*p*-sulphonic acid (3 g), the water being removed azeotropically using a Dean and Stark distilling receiver. The mixture was allowed to warm to room temperature and was then filtered. The solid was washed with aqueous 5% sodium carbonate (400 ml) and water (400 ml) and dried (55.4 g, 89%), m.p. 118 °C; R_F(B) 0.53.

L-2,6-*Dibenzyloxycarbonylaminopimelic Acid Monobenzyl Ester* (**10**).—A solution of (**9**) (55 g, 86 mmol) in benzyl alcohol (400 ml) at 40 °C was treated dropwise with a solution of 86% pure KOH pellets (4.8 g, 74 mmol) in benzyl alcohol (400 ml) over 6.5 h, maintaining the temperature at 40 °C for 1 h. The reaction mixture was stirred at 20 °C for 16 h and then concentrated to dryness. The oily residue was dissolved in water (1 l) and extracted with EtOAc (900 ml). The aqueous phase was acidified to pH 2 with HCl (4M, 45 ml) and extracted with EtOAc (1.5 l). The organic extract was washed with a saturated solution of NaCl (500 ml) and dried (Na_2SO_4). The dried solution was treated with dicyclohexylamine (17 ml, 86 mmol) and concentrated to dryness. The residual yellow oil was dissolved in EtOH (100 ml), treated with water (100 ml) and left to stand for 20 h at 0 °C. The white solid thus obtained, was filtered off, washed with water (100 ml) and dissolved in a mixture of EtOAc and water (1:1, 400 ml). The aqueous phase was acidified with methanesulphonic acid (1M, 40 ml), the organic phase was decanted and washed with water (100 ml), dried (Na_2SO_4) and concentrated to dryness (15 g, 32%) to give an orange oil; R_F(C) 0.76.

L-2,6-*Dibenzyloxycarbonylaminopimelamic Acid* (**11**).—A solution of (**10**) (19 g, 35 mmol) in MeOH (190 ml) was cooled to 0 °C, saturated with NH_3 and then immediately transferred into a 1 l autoclave which was kept closed for 6 days at 20 °C. The solution obtained was degassed and concentrated. The residue was dissolved in water (250 ml), acidified to pH 2 with HCl (4M, 30 ml) and extracted with EtOAc (300 ml). The organic extracts were washed with a saturated solution of NaCl (100 ml), dried (Na_2SO_4) and concentrated to dryness (15 g, 95%) to give an orange oil; R_F(C) 0.68.

L-2,6-*Diaminopimelamic Acid Dichlorhydrate* (**12**).—A solution of (**11**) (15 g, 33 mmol) in a mixture of MeOH (300 ml) and concentrated HCl (*d* = 1.19) (5.9 ml) was treated with 3% palladium–charcoal (15 g) and a slow stream of H_2 was passed through the stirred mixture for 4 h. The mixture was then filtered and concentrated to dryness (8.5 g, 99%) to give a hard foam; R_F(C) 0.1.

L-2,6-*Dibenzyloxycarbonylaminopimelic Acid Mono* p-*Nitrobenzyl Ester* (**16**).—Following the method of A. Arendt *et al.*,[14] L-(**6**) (480 g, 1.047 mol) and *p*-nitrobenzyl bromide (223 g, 1.033

mol) yielded (**16**) (459 g, 74%) as an orange oil; R_F(E) 0.59, R_F(F) 0.73; δ_H(250 MHz, [$^2H_6$]DMSO) 1.44 [2 H, m, CH_2 (A_2pm-4)], 1.55—1.85 [4 H, m, CH_2 (A_2pm-3,5)], 3.90 (1 H, m, C*H*-CO_2H), 4.12 (1 H, m, C*H*-CO_2), 5.03 and 5.06 [4 H, 2 AB, (CH_2OCONH) × 2], 5.3 (2 H, s, *p*-$NO_2C_6H_4CH_2$), 7.35 (11 H, m, aromatics and N*H*CHCO$_2$H), 7.65 (2 H, d, *J* 9 Hz, aromatics *meta* to NO_2), 7.86 [1 H, d, *J* 7.5 Hz, (N*H*CHCO)], 8.24 (d, *J* 9.24 Hz, aromatics *ortho* to NO_2).

O^1-p-*Nitrobenzyl*-N^2-*benzyloxycarbonyl*-L-2,6-*diaminopimelic Acid* (**18**).—Powdered PCl_5 (112.8 g, 542 mmol) was added portionwise over 15 min to a stirred, ice-cooled solution of (**16**) (268 g, 451 mmol) in CH_2Cl_2 (4 l). The resulting suspension was stirred for 50 min at 5 °C and then for 15 min at 20 °C. Over the next 50 min, CH_2Cl_2 (2 l) was distilled off from the reaction mixture. The concentrate was cooled to 5 °C and treated with petroleum–ether (6.66 l). The mixture was then left to stand at 5 °C for 24 h. The oily product was decanted, triturated with petroleum–ether (1.5 l), dissolved in CH_2Cl_2 (1 l) and concentrated to dryness. The hard foam obtained was dissolved in a mixture of AcOH (1.74 l) and water (0.87 l), left to stand for 24 h at 20 °C and then partitioned between water (7.2 l) and ether (2 l). The aqueous phase was neutralized to pH 5 by slow addition of Na_2CO_3 (400 g) and the precipitated solid was filtered off, washed successively with water (1 l) and ether (1.5 l) and dried (Na_2SO_4) (169.3 g, 81%), m.p. 110—115 °C, $[\alpha]_D^{20}$ +11.6° (c 1.25 in AcOH); R_F(C) 0.61, R_F(G) 0.13; δ_H(250 MHz; [$^2H_6$]DMSO) 1.48 [2 H, m, CH_2 (A_2pm-4)], 1.55—1.85 [4 H, m, CH_2 (A_2pm-3,5)], 3.15 [1 H, br t, CH (A_2pm-6)], 4.10 [1 H, m, CH (A_2pm-2)], 5.07 (2 H, AB, *J* 11 Hz, CH_2OCONH), 5.31 (2 H, s, *p*-$NO_2C_6H_4CH_2$), 7.37 (5 H, m, aromatics), 7.65 (2 H, d, *J* 9 Hz, aromatics *meta* to NO_2), 7.90 (1 H, d, *J* 7.5 Hz, CONH), 8.25 (2 H, d, *J* 9 Hz, aromatics *ortho* to NO_2).

N^6-*Benzyloxycarbonyl*-L-2,6-*diaminopimelamic acid* (**14**).—*First method.* A solution of (**12**) (5 g, 19 mmol) in water (35 ml) was treated with $CuBr_2$ (2.14 g, 9.6 mmol), basified to pH 10 with NaOH (1M, 45 ml) and stirred for 2 h at 20 °C. A small amount of insoluble material was filtered off and the filtrate was cooled to between −3 and 0 °C. $NaHCO_3$ (9.6 g) was added, followed by benzyl chloroformate (4.1 ml, 29 mmol) added dropwise over 30 min. The reaction mixture (pH 8) was then stirred for 18 h at 20 °C. The blue precipitate formed was filtered off, washed with water (90 ml), EtOH (90 ml), ether (90 ml) and dried to yield 4.18 g (67%) of the copper complex (**13**). The complex was then stirred for 1 h with HCl (1M, 28 ml) at 20 °C. The insoluble material was filtered off, MeOH (14 ml) was added to the filtrate and a stream of H_2S was then passed through the mixture for 6 h. The mixture was left to stand for 16 h and the resulting black slurry was filtered and washed with water (15 ml). The combined filtrates were concentrated to 10 ml, brought to pH 7 by additon of Et_3N (5 ml) and then brought to pH 6.8 by addition of HCl (1M, 5 ml). The white slurry thus obtained was kept at 0 °C for 2 h, filtered off, washed successively with water (30 ml), EtOH (30 ml) and ether (30 ml) and dried (1.78 g, 29%), m.p. 248 °C; $[\alpha]_D^{20}$ +18° (c 0.3 in AcOH); L-A_2pm 99.8%, *meso*-A_2pm 0.2%; R_F(C) 0.46, R_F(D) 0.44; δ_H(250 MHz; [$^2H_6$]DMSO) 1.3—1.75 [6 H, m, CH_2 (A_2pm-3,4,5)], 3.10 [1 H, m, C*H*CO$_2$H], 3.90 (1 H, m, C*H*CONH$_2$), 5.06 (2 H, s, C*H*$_2$OCONH), 7 (1 H, br s, CON*H*$_2$], 7.29 and 7.30—7.45 (7 H, d and m, CONH and aromatics, CON*H*$_2$).

Second method. (**18**) (57.9 g, 126 mmol) was added to a mixture of aqueous 20% ammonia (1.24 l) and EtOAc (175 ml). The resultant mixture was stirred at 20 °C for 24 h. The aqueous phase was separated, washed with EtOAc (600 ml), cooled to 10 °C and neutralized to pH 6 by addition of AcOH (250 ml). The resulting slurry was stirred over 0.5 h at 5 °C and then filtered. The solid was washed successively with AcOH (400 ml) and EtOAc (400 ml) and dried (23.5 g, 58%), m.p. 248 °C, $[\alpha]_D^{20}$ +18.2° (c 0.3 in AcOH); L-A_2pm 99.8%, *meso*-A_2pm 0.2%; R_F(C) 0.46, R_F(D) 0.44; δ_H(250 MHz; [$^2H_6$]DMSO): identical spectra with the sample above.

L-*Alanyl*-O^1-*benzyl*-D-*glutamic Acid Hydrochloride* (**20**).—Compound (**19**)[15] (50 g, 122 mmol) was dissolved in an anhydrous solution of HCl in AcOH (1.7M, 425 ml). The resulting solution was stirred for 2 h, then added slowly to ether (2.5 l). The mixture was left to stand for 16 h at 0 °C, and the oily precipitate formed was decanted off the supernatant liquor and dissolved in acetone (300 ml). The resultant solution was concentrated and dried under reduced pressure to give an oil (33.5 g, 79%), R_F(C) 0.56; δ_H(250 MHz; [$^2H_6$]DMSO) 1.38 [3 H, d, *J* 7.5 Hz, CH_3 (Ala)], 1.85 and 2.01 [1 H each, 2m, CH_2 (β-Glu)], 2.31 [2 H, t, *J* 7.5 Hz, CH_2 (γ-Glu)], 3.89 (1 H, q, *J* 7.5 Hz, CH (Ala)], 4.38 [1 H, m, CH (Glu)], 5.14 (2 H, AB, *J* 13 Hz, CO_2CH_2Ar), 7.3—7.45 (5 H, m, aromatics), 9.04 (1 H, d, *J* 7.5 Hz, CONH).

O^1-*Benzyl*-N-(N-*lauroyl*-L-*alanyl*)-D-*glutamic Acid* (**21**).—Lauroyl chloride (58 ml, 251 mmol) was added over 5 min to a solution of (**20**) (61.77 g, 179 mmol) and bis(trimethylsilyl)-acetamide (151 ml, 616 mmol) in CH_2Cl_2 (770 ml) maintained at −40 °C. The mixture was stirred for 1 h at −10 °C, 3 h at 20 °C and then concentrated to dryness. The residual oil was treated with water (1.5 l) and the resultant solid formed filtered off, washed successively with water (600 ml), HCl (0.1M, 200 ml), H_2O (600 ml), and ether (600 ml) and dried. Recrystallization from CH_3CN (250 ml) afforded a white solid (66.56 g, 75.8%), m.p. 131 °C; $[\alpha]_D^{20}$ −13.9° (c 1 in MeOH); R_F(H) 0.46; δ_H(250 MHz; [$^2H_6$]DMSO) 0.85 [3 H, t, *J* 7.5 Hz, CH_3 (lauric acid)], 1.17 [3 H, d, *J* 7.5 Hz, CH_3 (Ala)], 1.25 [16 H, br m, CH_2 (C_4-C_{11} lauric acid)], 1.47 [2 H, m, CH_2 (C_3 lauric acid)], 1.82 and 2 [1 H each, 2m, CH_2 (β-Glu)], 2.08 [2 H, t, *J* 7.5 Hz, CH_2 (C_2 lauric acid)], 2.25 [2 H, t, *J* 7.5 Hz, CH_2 (γ-Glu)], 4.32 [2 H, m, CH (Glu and Ala)], 5.09 (2 H, s, CO_2CH_2Ar), 7.35 (5 H, m, aromatics), 7.88 and 8.27 (1 H, each, 2d, *J* 7.5 Hz, CONH (Ala, Glu)], 12.15 (1 H, br m, CO_2H).

N^2-[O^1-*Benzyl*-N-(N-*lauroyl*-L-*alanyl*)-γ-D-*glutamyl*]-N^6-*benzyloxycarbonyl*-L-2,6-*diaminopimelamic Acid* (**22**).—Isobutyl chloroformate (54 ml, 410 mmol) was added over 2 min to a stirred solution of (**21**) (200.2 g, 410 mmol) in a mixture of tetrahydrofuran (THF) (8.3 l) and Et_3N (57 ml) maintained at −6 °C. The resultant mixture was stirred for 20 min at −6 °C and then treated with a solution of (**14**) (120 g, 371 mmol) in a mixture of water (3.7 l) and NaOH (1M, 371 ml) cooled to 5 °C. The reaction mixture was stirred for 1 h at 0 °C, 16 h at 20 °C and then acidified to pH 1.5 with HCl (1M, 800 ml). The THF was removed by distillation and the resultant solid was filtered off, washed with water (3 l) and dried. The solid was dissolved in refluxing propan-2-ol (6 l), treated with EtOAc (6 l) and kept at 0 °C for 64 h. The precipitate formed was filtered off, washed with cold EtOAc (2 l) and dried 221 g, 75%), m.p. 180 °C; $[\alpha]_D^{20}$ −13.5° (c 1 in AcOH); R_F(C) 0.73, R_F(F) 0.42; L-A_2pm 99.8%, *meso*-A_2pm 0.2%; δ_H(400 MHz; [$^2H_6$]DMSO) 0.83 [3 H, t, *J* 7 Hz, CH_3 (lauric acid)], 1.17 [3 H, d, *J* 7.5 Hz, CH_3 (Ala)], 1.10—1.4 [18 H, br m, CH_2 (C_4-C_{11} lauric acid and A_2pm-4)], 1.4—1.7 [6 H, m, CH_2 (C_3 lauric acid and A_2pm-3,5)], 1.83 and 1.97 [1 H each, 2 m, CH_2 (β-Glu)], 2.09 [2 H, t, *J* 7.5 Hz, CH_2 (C_2 lauric acid)], 2.16 [2 H, t, *J* 7.5 Hz, CH_2 (γ-Glu)], 3.85 [1 H, m, CH (A_2pm-6)], 4.02 [1 H, m, CH (A_2pm-2)], 4.21 [1 H, m, CH (α-Glu)], 4.34 [1 H, m, CH (Ala)], 5 and 5.1 (2 H each, AB and s, $COOCH_2Ar$), 6.94 (1 H, br m, CON*H*$_2$), 7.23 (1 H, d, *J* 7.5 Hz, CON*H*$_2$), 7.25—7.4 (11 H, m, aromatics and CON*H*$_2$), 7.88

[1 H, d, *J* 7.5 Hz, CONH (A_2pm-2)], 7.94 [1 H, d, *J* 7.5 Hz, CONH (lauroyl-Ala)], 8.45 [1 H, d, *J* 7.5 Hz, CONH (Ala-Glu)].

N^2-(N-*Lauroyl*-L-*alanyl*-γ-D-*glutamyl*)-L-2,6-*diamino-pimelamic Acid. RP* 56142 (**3**).—A solution of (**22**) (680 g, 854 mmol) in AcOH (16 l) was treated with 10% palladium–charcoal (210 g) and a slow stream of H_2 was passed through the stirred mixture for 12 h. The mixture was then filtered and concentrated to dryness. The residual solid was stirred with EtOAc (5 l), filtered, washed with EtOAc (1.5 l) and dried to afford a white solid, (498 g) purity (h.p.l.c.) 93%; L-A_2pm ≥99.8%. The peptide was purified in 70 g portions by chromatography (Jobin-Yvon Modulprep with column id. 80 mm). Thus a portion (70 g) of crude (**3**) was dissolved in AcOH (400 ml) at 45 °C and treated with neutral silica gel (0.04—0.063 mm, Merck) (200 g). The mixture was concentrated to dryness and introduced onto the column containing neutral silica gel (0.04—0.063 mm Merck) (1 200 g). Elution was carried out successively with a mixture of EtOAc–AcOH–H_2O (90:12:10, 19.5 l) and a mixture of EtOAc–AcOH–H_2O (60:12:10, 24 l), 500 ml fractions being collected every 5 min. Fractions 61 to 82 were combined and concentrated to dryness (54.76 g, 78%); purity (HPLC on a Gilson apparatus) 99.6% [column Sup-Rs S 5 ODS-2 (Prolabo), solvent: CH_3CN–Na_2SO_4 (25mM) 30:70, flow rate 1 ml/min, optical absorption monitored at 210 nm, retention time of the peak, 18min]; $[\alpha]_D^{20}$ −17° (c 0.1 in AcOH); R_F(C) 0.36, R_F(D) 0.45 (Found: C, 54.3; H, 9.0; N, 11.8. Calc. for $C_{27}H_{49}N_5O_8$. 1.35 H_2O: C, 54.41; H, 8.74; N, 11.75); δ_H(250 and 400 MHz and COSY experiment; [$^2H_6$]DMSO) 0.85 [3 H, t, *J* 6.5 Hz, CH_3 (lauric acid)], 1.18 [3 H, d, *J* 7 Hz, CH_3 (Ala)], 1.20—1.32 [16 H, br m, CH_2 (C_4-C_{11} lauric acid)], 1.35—1.50 [4 H, m, CH_2 (C_3 lauric acid and A_2pm-4)], 1.66 [4 H, m, CH_2 (A_2pm-3,5)], 1.83 and 1.90 [1 H each, 2m, CH_2 (β-Glu)], 2.10 [4 H, t, *J* 7 Hz, CH_2 (C_2 lauric acid and γ-Glu)], 3.6 [1 H, t, $J_{\varepsilon-\delta}$ 5.5 Hz, CH (A_2pm-6)], 4.01 [1 H, q, $J_{\alpha\text{-NH}}$ 6.5 Hz, $J_{\alpha-\beta}$ 6.5 Hz, CH (Glu)], 4.07 [1 H, q, $J_{\alpha\text{-NH}}$ 6.5 Hz, $J_{\alpha-\beta}$ 65 Hz, CH (A_2pm-2)], 4.29 [1 H, quin., $J_{\alpha\text{-NH}}$ 7 Hz, $J_{\alpha\text{-CH}}$ 7 Hz, CH (Ala)], 7.33 and 7.88 (1 H each, 2 br s, $CONH_2$), 7.8 [1 H, d, *J* 6.5 Hz, CONH (Ala-Glu)], 7.91 [1 H, d, *J* 6.5 Hz, CONH (Glu-A_2pm)], 7.99 [1 H, d, *J* 7 Hz, CONH (lauroyl-Ala)].

Acknowledgements

The authors are indebted to the analytical service of the Centre de Recherches de Vitry, especially to Mrs. E. Pacaud and Miss C. Rombi for the determination of the isomers of A_2pm and Mr. M. Vuilhorgne who performed the n.m.r. measurements. We thank also Mr. H. Halys for his technical assistance and Mrs. H. Mason for her assistance in translating the manuscript into English.

References

1 D. Migliore-Samour, F. Floc'h, R. Maral, G. Werner, and P. Jollès, *Immunology*, 1977, **33**, 477.
2 D. Migliore-Samour, J. Bouchaudon, F. Floc'h, A. Zerial, L. Ninet, G. Werner, and P. Jollès, *Life Sci.*, 1980, **26**, 883.
3 D. Migliore-Samour, J. Bouchaudon, F. Floc'h, A. Zerial, L. Ninet, G. Werner, and P. Jollès, *C. R. Hebd. Seances Acad. Sci., Ser. D*, 1979, **289**, 473.
4 G. Werner, F. Floc'h, A. Zerial, J. Bouchaudon, L. Ninet, D. Migliore-Samour, and P. Jollès, in 'Current Chemotherapy and Infections Disease,' eds. J. Nelson and C. Grassi, American Society for Microbiology, Washington, D.C., 1980, pp. 1728—1730.
5 F. Floc'h, J. Bouchaudon, G. Werner, D. Migliore-Samour, and P. Jollès, *Ann. Immunol. (Paris)*, 1981, **132D**, 265.
6 F. Floc'h and J. Poirier, *Int. J. Immunopharmacol*, in press.
7 R. Roy and M. Karel, *Can J. Biochem.*, 1973, **51**, 942.
8 J. Van Heijenoort and E. Bricas, *Bull. Soc. Chim. Fr.*, 1968, 2828.
9 Y. Izumi, *J. Chem. Soc. Jap.*, 1954, **75**, 1152.
10 F. Saleh and P. White, *J. Gen. Microbiol.*, 1976, **96**, 253.
11 A. Arendt, A. Kolodziejczyk, and T. Sokolowska, *Rocz. Chem.*, 1974, **48**, 1305.
12 C. Nicot and E. Bricas, *Acta Chim. Acad. Sci. Hung.*, 1965, **44**, 229.
13 A. Patchornik and Y. Shalitin, *Anal. Chem.*, 1961, **33**, 1887.
14 A. Arendt, A. Kolodziejczyk, T. Sokolowska, and E. Szulfer, *Rocz. Chem.*, 1974, **48**, 1501.
15 P. Dezélée and E. Bricas, *Biochemistry*, 1970, **9**, 823.

Received 11*th July* 1988; *Paper* 8/02791I