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SYNTHESIS AND CHARACTERIZATION OF A DERIVATIVE OF 2-L-N-(L-GLUTAMYL-L-GLUTAMINYL) AMINO-3-PHENYLPROPAN-1-OL A PROPOSED C-TERMINAL PEPTIDE SEQUENCE OF METABOLITES OF TRICHODERMA SPP.

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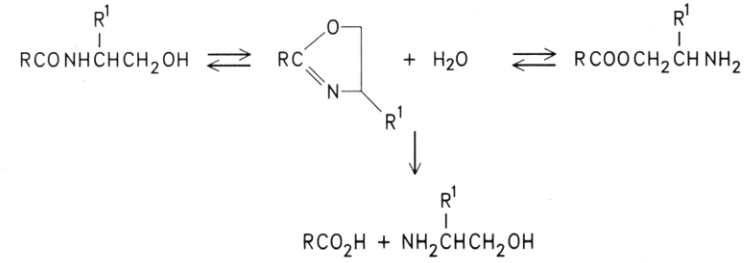
The γ-benzyl ester of the 2,4-dinitrophenyl ether of 2-L-N-(L-glutamyl-L-glutaminyl) amino-3-phenylpropan-1-ol, the C-terminal sequence of a number of antibiotic fungal peptides, has been synthesized. Evidence is presented that rearrangements did not occur at either of the two peptide bond forming steps. However, 2-L-pyroglutamylamino-3-phenyl-1-(2′, 4′-dinitrophenoxy)propane was always a byproduct in the reaction of the glutamylphenylalaninol ether with γ-benzyl N-t-butyloxycarbonylglutamic acid. The pyranyl ether protecting group of L-2-(N-L-t-butyloxycarbonyl-glutamyl)amino-3-phenyl-1-(tetrahydropyranyl-2′-)oxypropane could be selectively hydrolysed with lithium tetrafluoroborate.

On a synthésizé l'ester γ-benzyl de l'éther 2,4-binitrophenyl de 2-L-N-(L-glutamyl-L-glutaminyl) amino-3-phenyl-l-ol, la sequence terminale-C d'un nombre de peptides de mycètes antibiotiques. Les faits presentés ici démontrent que les réarrangements ont lieu à nulle étape où deux peptides forment des liaisons. Néanmoins, le propane 2-L-pyroglutamylamino-3-phenyl-1-(2′,4′-binitro-phenoxyl) a constitué un dérivé constant de la réaction de l'éther glutamylphenylalaninol avec l'acide γ-benzyl N-t-butyloxycarbonylglutamique. On a réussi à hydrolyser, au moyen du tetrafluoroborate de lithium, le groupe protegeant l'éther pyranyl du propane-L-2-(N-L-t-butyloxycarbonylglutamyl) amino-3-phenyl-1-oxy(tetrahydropyranyl-2′).

The peptide (**1**, R=R′=R″=H) is thought to be the C-terminal sequence of a large number of antibiotics produced by several species of fungi (Shaw & Taylor, 1986). A considerable effort has been directed to its synthesis which has been reported by four independent groups of workers (Nagaraj & Balaran, 1981; Gisin et al., 1981; Balasubramanian et al., 1981; Schmitt & Jung, 1985). However the physical properties of the four products reported by these workers differ by amounts greater than is normally attributable to experimental error (Shaw & Taylor, 1986). In two of these syntheses (Gisin et al., 1981; Balasubramanian et al., 1981) the compound (**2**, R=H, R′=t-C_4H_9OCO) was used as an intermediate and here again the melting points (m. p. 134°, 146°) and optical rotations ([α]$_D$ -39°, -31°) of this intermediate differed.

Scheme 1



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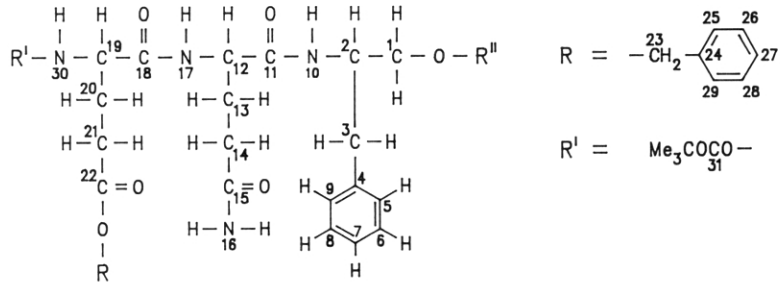
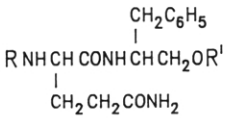
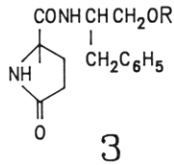
Acylaminoalcohols are known (Frump, 1971) to cyclise to oxazolines as shown in scheme 1 under a variety of reaction conditions and a mechanism involving an intermediate oxazoline has been proposed for the ready release of the phenylalaninol residue from a mixture of alamethicins (Martin and Williams, 1976). It was therefore appropriate to attempt to synthesise the compound **2** under conditions where the hydroxyl group of the phenylalaninol residue was protected, and thus unable to participate in oxazoline formation.



1



2



3

L-phenylalaninol readily condensed with dihydropyran to give a diasterioisomeric mixture of basic tetrahydropyranyl ethers, characterized as their 4-chlorobenzoyl derivatives. This mixture of bases on treatment with L-t-butyloxycarbonylglutamine gave a mixture of pyranyl ethers (**2**, R'=t-C_4H_9OCO, R=C_5H_9O). Under a wide range of acidic conditions these ethers gave the pyroglutamic acid derivative (**3**, R=H), but on treatment with lithium tetrafluoroborate (Lipshutz & Harvey, 1982; Taylor & Reiter, 1989), a 50% yield of the alcohol (**2**, R'=t-C_4H_9OCO, R=H) was obtained. This alcohol was characterized by conversion to its acetate, its pyranyl ethers, and as its dinitrophenyl ether. This dinitrophenyl ether was eluted as a Gausian shaped peak from a reversed phase chromatography column with a retention volume of 33.3 mL.

The structure of the dinitrophenyl ether was established from the combined ^{13}C and ^1H n.m.r. data as follows. A ^1H 2-dimensional correlation spectrum (COSY) gave the ^1H spin-spin coupling connectivities within each residue, viz H'_1, H_2, H_3, H_{10}, and H_{12}, H'_{13}, H'_{14}, H_{17} (the numbering system for the various nuclei is given on formula **1**, for compound **2** it is analogous) which subsequently permitted δ_H and J_{HH} for these protons to be determined by simulation. Resonances of the t-C_4H_9OCO group were assigned by their integrated intensities, chemical shifts and lack of long-range coupling. The resonances of the aromatic carbons C_4 to C_9 were assigned with the aid of a ^1H-coupled ^{13}C spectrum in which C_5, C_7, and C_9 each showed 3-bond coupling to 2 protons, while C_6 and C_8 were 3-bond coupled to 1 proton. Relative integrated intensities of the ^{13}C resonances distinguished C_7 from C_5 and C_9, these carbons being likely to have comparable relaxation times. The quaternary carbons of the dinitro-

phenyl group were assigned from long-range couplings and calculations of substituent effects (Breitmezer and Voelter, 1987): predicted δ_C for C_1 to C_6 of dinitrophenyl 161.8, 136.0, 120.9, 142.6, 131.4, 116.7; observed 157.2, 138.9, 122.2, 141.4, 129.7, 116.2. A ^{13}C-^1H heterocorrelation experiment indicated the ^{13}C assignments of all carbons bearing protons with distinguishable chemical shifts, the glutamine peptide CO being typically at lower field than its amide CO (Wuthrich, 1987). Finally, ^{13}C isotopic shifts, measured after exchange of N-H protons with ^2H and careful drying of the product before dissolving it in tetrahydrofuran (THF $^2H_8$) gave the following results (p.p.m., negative sign indicates upfield shift): C_{15} (-0.062), C_{11} (-0.083), C_{18} (-0.017), C_2 (-0.086), C_3 (-0.027), C_{14} (-0.049), and C_{13} (-0.052). All other shifts were either downfield or less than 0.003 p.p.m., 10 peaks changing by less than 0.001 p.p.m. Such a pattern of shifts supports the structure (**2**, R=t-C_4H_9OCO, R'=$C_6H_3(NO_2)_2$); and does not support products obtained by rearrangement via an oxazoline intermediate.

The pyroglutamyl derivative (**3**, R=$C_6H_3(NO_2)_2$) was always a component of the products obtained from solvolytic reactions of this dinitrophenyl ether (**2**, R=$C_6H_3(NO_2)_2$, R'=C_4H_9OCO). The conditions given in the Experimental section, the results of a large number of orientation experiments, are those where the minimum formation of pyroglutamate was observed. Condensation of the crude solvolytic reaction mixture with γ-benzyl t-butyloxycarbonyl-L-glutamate (Sanderin and Boissonas, 1963) under the conditions described by Neubert and Jakubke (1978) gave the tripeptide (**1**, R=C_7H_7, R'=t-C_4H_9OCO, R''=$C_6H_3(NO_2)_2$) in about 60% yield where racemisation of the glutamine residue was minimal.

Though this product could be obtained as a crystalline solid, it proved unsuitable for X-ray diffraction analysis. Solutions (1%) in warm methyl, ethyl and butyl alcohols, acetone and ethyl acetate formed rigid gels on cooling to room temperature. The structure was confirmed by comparison of the n.m.r. spectra with those of (**2**, R=t-C_4H_9OCO, R'=$C_6H_3(NO_2)_2$). All resonances of corresponding nuclei were readily identifiable and the remainder were assigned by ^1H COSY, ^1H nuclear Overhauser effects (nOe), ^1H-^{13}C heterocorrelations and ^1H-coupled ^{13}C spectra. Coupling constants and chemical shifts for H'_1, H_2, H_3 and H_{10}, and for the overlapping resonances of the glutamic acid and glutamine residues, were obtained by spectral simulation, thus overlapping resonances of protons and directly bonded carbons were assigned unequivocally. The resonances of the aromatic carbons C_{24} to C_{29} (see Fig 1) were assigned from multiplicities in the ^1H-coupled ^{13}C spectrum as described for the phenyl rings in compound **2**. The lowest field signal, δ_C 175.22 and those at δ_C 172.25 and 172.44 were broad singlets in the coupled spectrum, and have counterparts in the spectrum of (**2**, R=t-C_4H_9OCO, R'=$C_6H_3(NO_2)_2$). They were therefore assigned respectively to C_{15}, and C_{11} or C_{18}. The remaining signal at 173.08 p.p.m., which showed long-range coupling to hydrogen substituents of at least 6 other carbon nuclei, is therefore C_{22}. Independent confirmation of the peptide sequence was obtained by ^1H nOe-difference measurements. Irradiation of the N-H resonances at $\delta_{H_{30}}$ 6.52, $\delta_{H_{17}}$ 8.023 and $\delta_{H_{10}}$ 7.89 produced nuclear Overhauser effects as follows: δ_H 6.52 enhanced H_{19}, $\delta_{H_{17}}$ 8.02 enhanced H_{12} and H_{19}, and δ_H 7.89 enhanced H_2 and H_{12}, in accord with structure **1**.

Thus the structure of the tripeptide **1** is firmly established and this compound will therefore serve as a point of reference in future synthetic studies in this field.

Experimental

Melting points are not corrected. Thin layer chromatography (tlc) was done on silica gel plates (Merck) and high pressure liquid chromatography (hplc) on reversed

phase columns used ammonium acetate pH 4.2 buffer-methyl alcohol mixtures as the developing solvent (proportions given for each compound). Optical rotations were measured using a Perkin-Elmer 141 polarimeter, infrared spectra on a Perkin Elmer 237 instrument and ultraviolet spectra on a Unicam SP8000 spectrometer. Nuclear magnetic resonance (n.m.r.) spectra were obtained at 20° in tetrahydrofuran (THF-$^2H_8$) or methyl alcohol [$^2H_4$] as indicated in the text. Spectra were recorded on a Bruker MSL-300 or a Nicolet NB-360 (Atlantic Magnetic Resonance Center) instruments at 300 or 360 MHz for proton spectra and 75.5 or 90.8 MHz for ^{13}C spectra. Data tables up to 64K were used where necessary. All chemical shifts are given in p.p.m. downfield from the signal of tetramethylsilane. Coupling connectivity was determined by ^1H COSY and $^{13}C/^1H$ heterocorrelation spectra. ^1H Spectral simulations using the Bruker PANIC programme gave ^1H chemical shifts and coupling constants and confirmed coupling patterns where necessary. Assignments are based on this coupling information, known chemical shifts of ^1H and ^{13}C nuclei in amino acid residues in peptides (Breitmezer & Voelter, 1987; Wuthrich, 1987), substituent effects for aromatic rings (Wuthrich, 1987), longrange coupling to quaternary carbons and isotope effects on ^{13}C chemical shifts produced by ^2H exchange. Chemical shifts and coupling constants are presented with reference to an arbitrarily numbered formula (**1**). Unless stated otherwise chemical shifts refer to single protons, and the numbering of the latter refers to the nitrogen or carbon atom whose substituent they are. All other n.m.r. data are given in the format used by Shaw & Taylor (1986).

2-(L-2'-Amino-3'-phenylpropan-1'-oxy)tetrahydropyranyl ethers - L-2-Amino-3-phenylpropan-1-ol (10 g, 66 mmol) was dissolved in hydrochloric acid (*N*, 85 mL) and the solution was evaporated. The crude hydrochloride (12.4 g) in chloroform (300 mL) was treated with dihydropyran (18.8 mL, 206 mmol) and toluene-4-sulphonic acid (90 mg, .5 mmol). The solution was kept for 9 h at room temperature when it was washed with sodium carbonate solution (5%, 2 x 120 mL) and saturated brine (100 mL). The dry (Na_2SO_4) chloroform solution was evaporated to give an oil (17.7 g). This oil (1.8 g) was applied to a silica gel column (100-200 mesh, packed with diethyl ether, 4.5 x 27 cm) which was developed with diethyl ether (500 mL), diethyl ether-methyl alcohol (49:1, 500 mL), diethyl ether-methyl alcohol (24:1, 500 mL) and diethyl ether-methyl alcohol (19:1, 500 mL). The pyranyl ethers (0.7 g) were collected in the fractions eluted with the last 2 solvents and were purified for analysis by distillation; b. p. 120-130°/0.1 mm Hg, (Found, C, 71.1; H, 8.8; N, 5.85; O, 13.5. $C_{14}H_{21}NO_2$ requires C, 71.4; H, 9.0; N, 5.95; O, 13.6%), pK_a 8.1 (E. Wt. 243), $[\alpha]^{25}_D$ +7° (c, 3, MeOH), $\lambda_{max.}$ (KBr) 1603, 1495, 1200, 1030 cm^{-1}, δc (C^2HCl_3) 139.0 (q. arom. C), 129.2 (2C, arom.), 128.6, 128.3, 126.2, 99.4 (ketal CH), 72.5 (propanol CH_2), 62.3 (pyranyl-2 CH_2), 52.4 (propanol CH), 40.9 (benzyl CH_2), 30,6, 25.5, 19.6. This amine (121 mg) in THF (5 mL) was treated with 4-chlorobenzoyl azide (Shaw & Taylor 1986, 100 mg) and the resulting solution kept at 20° for 4 days. The solution was then evaporated, the residue digested with several portions of petroleum ether (b. p. 30-60°). The petroleum ether was decanted after each digestion. *(2-(L-2'-(N-4''-chlorobenzoylamino)-3'*-phenyl-propan-1'-oxy) tetrahydropyranyl ethers separated from isopropyl ether as needles, m. p. 118-129°, R_v (3:17) 10.4 and 11.2 mL, (Found, C, 67.65; H, 6.5; Cl, 9.3; N, 3.7; O, 12.9. $C_{21}H_{24}ClNO_3$ requires C, 67.5; H, 6.5; Cl, 9.5; N, 3.75; O, 12.8%), $[\alpha]^{22}_D$ -41° (c, 1.3, MeOH), υ_{max} (KBr) 1640, 1600, 1500 cm^{-1}, δ_H (C^2HCl_3) 7.72 (2H, $^3J_{HH}$ 8.5 Hz, $^4J_{HH}$ 3 Hz), 7.32 (2H, $^3J_{HH}$ 8.5 Hz), 7.25 (5H), *ca.* 4.6 (2H, m), ca. 3.7 (4H, m), 3.6 (2H, $^2J_{HH}$ 7 Hz), *ca.* 1.6 (6H, m).

L-2-(N-2'-(N'-t-butyloxycarbonyl)-L-glutaminyl-amino-3'-phenylpropan-1'-oxy) tetrahydropyranyl ethers - (a) The tetrahydropyranyl ethers described in the previous paragraph (8.62 g 36.6 mmol), N-t-butyloxycarbonyl-L-glutamine (Hofmann et al., 1965, 9.02 g, 36.6 mmol), 1-ethoxy-carbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ, 9.93 g, 36.5 mmol), and toluene (300 mL) were stirred together at 35° for 4 days. The

reaction mixture was cooled to 4° and kept at this temperature for 1 h when the precipitate (13.5 g, m. p. 130-150°, 80%) was collected. The *L-glutaminyltetrahydropyranyl ethers* (**2**, R=C_5H_9O, R'=C_4H_9OCO) separated from ethyl acetate as rods, m. p. 132-153°, R_v 6.9 and 7.7 mL (3:17), (Found, C, 62.4; H, 8.0; N, 9.05; O, 21.0. $C_{24}H_{37}N_3O_6$ requires C, 62.2; H, 8.0; N, 9.1; O, 20.7%), $[\alpha]^{21}_D$ - 25.0° (c, 2.3, $CHCl_3$), $\upsilon_{max.}$ (KBr) 1660, 1550, 1520, 1250 cm^{-1}, δ_C (C^2HCl_3) 175.2 (175.15), 171.1 (170.0), 155.9 (urethane CO), 138.1, 129.4 (2C, $^1J_{CH}$ 160 Hz), 128.4 (2C, $^1J_{CH}$ 160 Hz), 126.4 ($^1J_{CH}$ 159 Hz), 100.2 (99.3) ($^1J_{CH}$ 160 Hz), 79.99 (t-Bu C), 68.3 (68.0) J_{CH_2} 142 Hz, benzyl), 63.3 (62.7) ($^1J_{CH_2}$ 139 Hz), 52.4 (b, CH_2), 50.8 (50.35) (J_{CH_2} 136 Hz), 37.8 (37.5), 31.8, 30.8 (30.6), 29.3 (29.1), 28.35 (t-butyl), 25.3, 20.1 (19.7) (J_{CH_2} 131 Hz).

(b) L-2-(N-(N'-t-butyloxycarbonyl-L-glutaminyl)amino-3-phenylpropan-1-ol (see below, 100 mg, 0.26 mmol), chloroform (5 mL) and dihydropyran (0.08 mL, 0.94 mmol) were mixed together and the mixture treated with a solution (3 mL) of toluene-4-sulphonic acid (1 mg) in chloroform. The mixture was stirred at 20° for 4 h, was then diluted with chloroform (50 mL) and the solution washed with sodium bicarbonate solution (5%, x2) and brine. The dry (Na_2SO_4) chloroform solution was evaporated and the residue (120 mg) recrystallized from ethyl acetate gave the tetrahydropyrenyl ethers (110 mg, m.p. 140-150°, 90%) identical with the material prepared as described in the preceeding paragraph.

L-2-(N-L-pyroglutamyl)amino-3-phenylpropan-1-ol - (a) The tetrahydropyranyl ethers, prepared in the preceeding paragraph (**2**, R=C_5H_9O, R'=t-C_4H_9OCO, 1 g, 2.15 mmol) in THF (50 mL) were treated at 0° with a slow stream of hydrogen chloride for 15 min. Excess hydrogen chloride was removed with a stream of nitrogen, the precipitate collected, washed with ice-cold THF and dried. *2-L-N-Pyroglutamylamino-3-phenylpropan-1-ol* separated from ethyl alcohol as colorless needles m.p. 173-175°, R_v 3.85 (3:17), (Found, C, 64.05; H, 5.9; N, 10.7). $C_{14}H_{18}N_2O_3$ requires C, 64.1; H, 6.9; N, 10.7%), $[\alpha]^{23}_D$ - 39° (c, 2, MeOH), $\upsilon_{max.}$ (KBr) 1695, 1655, 1560, 1045 cm^{-1}, δ_H ($C^2H_3O_2H$) 7.23 (5H), 4.25-4.0 (2H, m), 3.65 (2H, CH_2OH), 2.95-2.70 (2H, benzyl CH_2), 2.4-2.0 (4H, m).

(b) L-pyroglutamic acid (1.29 g, 10 mmol), L-2-amino-3-phenylpropan-1-ol (1.51 g, 10 mmol), EEDQ (2.72 g, 11 mmol), and toluene (100 mL) were stirred together at 45° for 4 days. The reaction mixture was cooled to 4°, kept at this temperature for 18 h and the colorless crystalline precipitate collected. The pyroglutamylphenylalaninol was recrystallized from ethyl alcohol as needles m. p. 174°, $[\alpha]^{22}_D$ - 38° (c, 1.5, MeOH), 2.48 g, 94%.

L-2-(-N-(N'-t-Butyloxycarbonyl-L-glutaminyl)amino-3-phenylpropan-1-ol - The tetrahydropyranyl ethers (**2**, R=C_5H_9O, R'=t-C_4H_9OCO, 2.32 g, 5 mmol) were dissolved in a solution (20 mL) of water (0.4 mL) in acetonitrile by heating the mixture to 45°. The solution was cooled to 15° and was then treated with a solution (5 mL) of lithium tetrafluoroborate (98%, 0.467 g, 5 mmol) in aqueous acetonitrile (2%, v/v). The resulting solution was kept at room temperature for 72 h, was then evaporated at <10° to ca. 5 mL and the concentrate diluted with ice-cold saturated brine (50 mL). The mixture was extracted with ethyl acetate (5 x 50 mL) and the combined extracts washed with sodium bicarbonate soultion (5%, 2 x 20 mL). The dry (Na_2SO_4) extract was filtered and the filtrate evaporated. The residue (2 g) crystallized on tituration with ethyl acetate. The *phenylalaninol* (**2**, R=H, R'=t-C_4H_9OCO) separated from ethyl acetate as long thin rods m. p. (138°) 149-150°, R_v 5.8 mL (3:17), (Found C, 60.2; H, 7.7; N, 11.1; O, 21.1. $C_{19}H_{29}N_3O_5$ requires C, 60.1; H, 7.7; N, 11.1; O, 21.1%), $[\alpha]^{24}_D$ - 41° (c, 1.8, MeOH), $\upsilon_{max.}$ (KBr) 1680, 1660, 1525 cm^{-1}, δ_H (THF, $^2H_8$) 1.40 (9H), 1.85 ($^2J_{H_{13}H_{13}}$ -14.0 Hz, $^3J_{H_{13}H_{14}}$ 7.2 Hz, $^3J_{H_{13}H_{14}}$ 7.6 Hz, $^3J_{H_{13}H_{12}}$ 7.9 Hz), 1.95 ($^3J_{H_{13}H_{12}}$ 5.9 Hz, $^3J_{H_{13}H_{14}}$ 7.2 Hz, $^3J_{H_{13}H_{14}}$ 6.8 Hz), 2.15 ($^2J_{H_{14}H_{14}}$ -15.2 Hz), 2.19, 2.72 (2H, e), 2.77 ($^2J_{H_3H_3}$ -13.5 Hz, $^3J_{H_3H_2}$ 6.7

Hz), 2.85 ($^3J_{H_3H_2}$ 7.5 Hz), 3.38 ($^2J_{H_1H_1}$ -10.8 Hz, $^3J_{H_1H_2}$ 5.3 Hz), 3.45 ($^3J_{H_1H_2}$ 4.4 Hz), 4.02 ($^3J_{H_9H_{10}}$ 7.9 Hz), 4.03 ($^3J_{H_{12}H_{17}}$ 8.0 Hz), 6.35 (H_{17}, e), 7.11 (m H_7), 7.20-7.22 (4H), 7.45 (H_{10}, e), δ_C 28.66 (3C), 29.68 (C_{13}), 32.20 (C_{14}), 37.74 (C_3), 53.73 (C_2), 54.80 (C_{12}), 63.06 (C_1), 79.01 (Me_3C), 126.68 (C_7), 128.89 (C_6 & C_8), 130.21 (C_5 & C_9), 140.05 (C_4), 156.4 (BuO C O), 172.0 (C_{11}), 175.31 (C_{15}). This alcohol (0.2 g, 0.53 mmol) was suspended in acetic acid (0.35 mL) and the stirred suspension treated with acetic anhydride (1.13 mL, 12 mmol). The mixture was stirred for 4 days at room temperature, the resulting solution evaporated and the residue, recrystallized from ethyl *acetate* gave the acetate (**2**, R=Ac, R'=t-C_4H_9OCO) as needles m. p. 152-153°, (Found C, 59.9; H, 7.4; N, 10.0; O, 22.7. $C_{21}H_{31}N_3O_6$ requires C, 59.8; H, 7.4; N 10.0; O, 22.8%), $\upsilon_{max.}$ (KBr) 1720, 1680, 1665, 1560, 1525, 1275 cm^{-1}.

L-2(-N-(N'-t-Butyloxycarbonyl-L-glutaminylamino)-3-phenyl-1-(2',4'-dinitro-phenoxy)propane - The glutaminylphenylalaninol (**2**, R=H, R'=t-C_4H_9OCO, 5.69 g, 15 mmol) was suspended in a solution (100 mL) of triethylamine (2.1 mL, 15 mmol) in acetone in the dark and under an atmosphere of nitrogen. A solution (30 mL) of 2,4-dinitrofluorobenzene (2.8 g, 15 mmol) was added and the stirred mixture heated under reflux for 4 days. The mixture was evaporated, the residue dissolved in ethyl acetate (650 mL) and the solution washed with dilute hydrochloric acid (5%, 250 mL), then with sodium bicarbonate solution (5%) until the aqueous phase was colorless and finally with brine. The dry (Na_2SO_4), filtered ethyl acetate solution was evaporated and the residue (8.1 g) taken up in hot ethyl alcohol (125 mL). The filtered solution was allowed to cool very slowly (ca. 5 deg. C h^{-1}) and after 18 h the crystalline solid (=A, 3.56 g, m. p. 159-167°) was collected. The residue (3 g) from evaporation of the mother liquors from this crystallization was dissolved in ethyl acetate (50 mL) and applied to a silica gel (180 g, 100-200 mesh) column. The column was developed with ethyl acetate and the first 50mL of eluate were discarded. The following 1.1 L of eluate were collected and evaporated to give yellow needles (1.14 g). The *dinitrophenyl ether* separated from ethyl alcohol as very fine yellow needles m. p. 164-168°, R_v 12.4 mL (1:5), (Found C, 55.4; H, 6.1: N, 12.8 $C_{25}H_{31}N_5O_9$ requires C, 55.0; H, 5.7: N, 12.8%), $[\alpha]^{24}_D$ -41° (c, 0.9, MeOH), $\lambda_{max.}$ (MeOH) 251,289 nm (ε 10800 15000), $\upsilon_{max.}$ (KBr) 1680, 1660, 1610, 1525, δ_H (THF $^2H_8$) 1.37 (9H), 1.77 (H_{13}), 1.88 (H_{13}), 2.12 (H_{14}), 2.59 ($2H_{16}$, e) 2.98 (H_3), 3.19 (H_3), 4.02 (H_{12}), 4.20 (H_1), 4.31 (H_1), 4.42 H_2, 6.20, 6.38 (H_{17}, e), 6.65, 7.15 (H_7), 7.12-7.30 (4H), 7.47 (H_6 of 2,4-dinitrophenyl=DNP), 7.73 (H_{10}, e), 8.43 (H_5 of DNP), 8.77 (H_3 of DNP), δ_C (THF, $^2H_8$) 28.6 (3C), 29.52 (C_{13}), 32.23 (C_{14}), 37.39 (C_3), 50.93 (C_2), 54.93 (C_{12}), 71.29 (C_1), 79.09 ($Me_3C.OCO$), 116.17 (C_6 of DNP), 122.22 (C_3 of DNP), 127.21 (C_7). 129.22 (C_6 & C_8), 129.74 (C_5 of DNP), 130.12 (C_5 & C_9), 138.91 (C_2 of DNP), 140.08 (C_4), 141.40 (C_4 of DNP), 156.44 (C_{18}), 157.23 $9C_1$ of DNP), 172.60 (C_{11}), 174.83 (C_{15}). Further elution of the column with ethyl aceate-methyl alcohol (49:1, 200 mL) gave the starting alcohol (m. p. (138°) 150°, 0.83 g).

L-3-Phenyl-2-L-pyroglutamylamino-1-(2', 4'-dinitrophenoxy)propane - 2-L-N-Pyroglutamylamino-3-phenylpropan-1-ol (83 mg), 0.32 mmol) was suspended in acetone (3 mL) and triethylamine (32 mg, 0.33 mmol) was added A solution of dinitrofluorobenzene (59 mg, 0.33 mmol) in acetone was added and the mixture heated in the dark in an atmosphere of nitrogen under reflux for 5 days. The reaction mixture was evaporated, the residue dissolved in ethyl acetate (20 mL) and the solution washed with sodium carbonate (5%) solution until the washings were colorless. The dry (Na_2SO_4) ethyl acetate solution was filtered, evaporated and the residue obtained (69 mg) was applied to a silica gel column (100-200 mesh, 25 g). The column was developed with ethyl acetate; the first yellow band eluted in 60 mL provided 2,4-dinitrofluorobenzene and the second 60 mL of eluate gave the *dinitrophenyl ether* which separated from methyl alcohol as yellow needles m. p. 200-201°, R_v 7.5 mL

(3:17), (Found C, 55.9; H, 4.7; N, 13.0; O, 25.9. $C_{20}H_{20}N_4O_7$ requires C, 56.1; H, 4.7; N, 13.1; O, 26.1%), $[\alpha]^{22}_D$ -39.5° (c, 0.54, dimethylsuphoxide).

L-2-(γ-Benzyl N-t-butyloxycarbonyl-L-glutamyl-L-glutaminylamino)-1-(2',4'-dinitrophenoxy)-3-phenylpropane - The t-Butyloxycarbonyldipeptide (**2** R=$C_6H_3(NO_2)_2$, R'=t-C_4H_9OCO, 2.73 g, 5.9 mmol) was dissolved in a solution (38 mL) of concentrated hydrochloric acid in acetic acid (1:19). After 1 h at 20° the solution was evaporated at <20° (1 mm) and the residue was kept at 4° for 18 h at 1 mm pressure over sodium hydroxide pellets. The residue and γ-benzyl N-t-butyloxycarbonyl-L-glutamate (Sanderin & Boissonnas, 1963, 1.68 g, 5 mmol) were dissolved in THF (50 mL). The resulting solution was treated with triethylamine (0.7 mL, 5 mmol) and was stirred for 10 min at 0° when dicyclohexylcarbodiimide-pentafluorophenol complex (Kovacs et al., 1967, 1:3, 4.25 g, 5.2 mmol) was added. The reaction mixture was stirred at 0° for 2 h and was then kept at 4° for 70 h, when it was filtered and the filtrate evaporated. The residue was taken up in ethyl acetate (1 L), the solution washed with dilute sodium bicarbonate (2%, 2 x 250 mL) and then with saturated brine (250 mL). The combined aqueous phases were washed with ethyl acetate, the ethyl acetate solutions dried (Na_2SO_4), filtered and evaporated. The crude residues from 2 runs were dissolved in methyl alcohol (50 mL) and the warm solution absorbed onto silica gel (100-200 mesh, 13 g). The methyl alcohol solution was evaporated and the residue packed on the top of a silica gel column (135 g, 3 x 30 cm). The column was developed with ethyl acetate (650 mL) and then with methyl alcohol-ethyl acetate (1:49). The product (**1**, R=benzyl, R'=C_4H_9OCO, R''=2,4-dinitrophenyl, 2.62 g) was eluted in the first 1 L of the latter solvent and was recrystallized from methyl alcohol to give 2.05 g of a yellow powder m. p. 152-155°. Recrystallization of this material (30 mg) from n-butyl alcohol (3 mL) gave very fine yellow needles m. p. 153°, (Found C, 57.8; H, 5.8; N, 10.9; O, 25.3. $C_{37}H_{44}N_6O_{12}$ requires C, 58.1; H, 5.8; N, 11.0; O, 25.1%), $[\alpha]^{24}_D$ -26.3° (c, 1.8, THF), $\lambda_{max.}$ (MeOH) 254, 291 nm (ε 7700, 10800), δ_H 1.41 (9H), 1.83 ($^2J_{H_{13}H_{13}}$ -15.5 Hz, $^3J_{H_{13}H_{14}}$ 6.6 Hz, $^3J_{H_{13}H_{14}}$ 7.5 Hz, $^3J_{H_{13}H_{12}}$ 5.9 Hz), 1.94 ($^3J_{H_{13}H_{14}}$ 6.6 Hz, $^3J_{H_{13}H_{14}}$ 6.6 Hz, $^3J_{H_{13}H_{12}}$ 7.9 Hz), 2.13 ($^2J_{H_{14}H_{14}}$ -15.6 Hz), 2.20 (H_{14}), 1.85 ($^2J_{H_{20}H_{20}}$ -13.8 Hz, $^3J_{H_{20}H_{21}}$ 7.7 Hz, $^3J_{H_{20}H_{21}}$ 7.2 Hz, $^3J_{H_{20}H_{19}}$ 8.0 Hz), 2.02 ($^3J_{H_{20}H_{21}}$ 7.7 Hz, $^3J_{H_{20}H_{21}}$ 7.2 Hz, $^3J_{H_{20}H_{19}}$ 5.7 Hz), 2.41 ($2H_{21}$), 2.70 ($2H_{16}$, e), 2.95 ($^2J_{H_3H_3}$ -13.7 Hz, $^3J_{H_3H_2}$ 7.3 Hz), 3.13 ($^3J_{H_3H_2}$ 7.3 Hz), 4.00 (H_{19}), 4.19 ($^2J_{H_1H_1}$ -9.8 Hz, $^3J_{H_1H_2}$ 4.2 Hz), 4.30 ($^3J_{H_1H_2}$ 5.1 Hz), 4.43 ($^3J_{H_2H_{10}}$ 7.9 Hz), 5.07 ($2H_{23}$), 6.36, 6.52 (e, $^3J_{H_{30}H_{19}}$ 7.2 Hz), 6.81, 7.14 H_7), 7.21-7.27 (m, $H_5H_6H_8H_9$), 7.26-7.35 (m, $H_{25}H_{26}H_{28}H_{29}$), 7.47 ($^3J_{HH}$ 9.3 H_6 of DNP), 7.89 (e, $^3J_{H_{10}H_2}$ 7.85 Hz), 8.02 (e, $^3J_{H_{17}H_{12}}$ 7.4 Hz), 8.41 (H_5 of DNP), 8.74 (H_3 of DNP, $^4J_{HH}$ 2.8 Hz), δ_C 28.42 (C_{20}), 28.68 (3C), 28.84 (C_{13}), 31.08 (C_{21}), 32.23 (C_{14}), 37.31 (C_3), 51.00 (C_2), 53.84 (C_{12}), 55.08 (C_{19}), 66.54 (C_{23}), 71.20 (C_1), 79.39 (t-butyl), 116.16 (C_6 of DNP), 122.23 (C_3 of DNP), 127.15 (C_7), 128.62 (C_{27}), 128.86 (C_{25} & C_{29}), 129.11 (C_{26} & C_{28}), 129.18 (C_6 & C_8), 129.70 (C_5 of DNP), 130.12 (C_5 & C_9), 137.64 (C_{24}, by elimination), 138.98 (C_2 of DNP), 140.07 (C_4), 141.28 (C_4 of DNP), 156.80 (C_{31}), 157.25 (C_1 of DNP), 172.25 (C_{11} or C_{18}), 172.44 (C_{18} or C_{11}), 173.08 (C_{22}), 175.22 (C_{15}). The column was then eluted with methyl alcohol-ethyl acetate (1:19) and the next 800 mL discarded and the following 450 mL collected. This eluate was evaporated and the residue (0.166 g) recrystallized from methyl alcohol (5 mL) gave the pyroglutamic acid derivative (**3**, R=$C_6H_3(NO_2)_2$, 72 mg, m. p. 198-200°.

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