SYNTHESIS OF THE RELEASING FACTOR OF GONADOTROPIC HORMONES LRF/FRF

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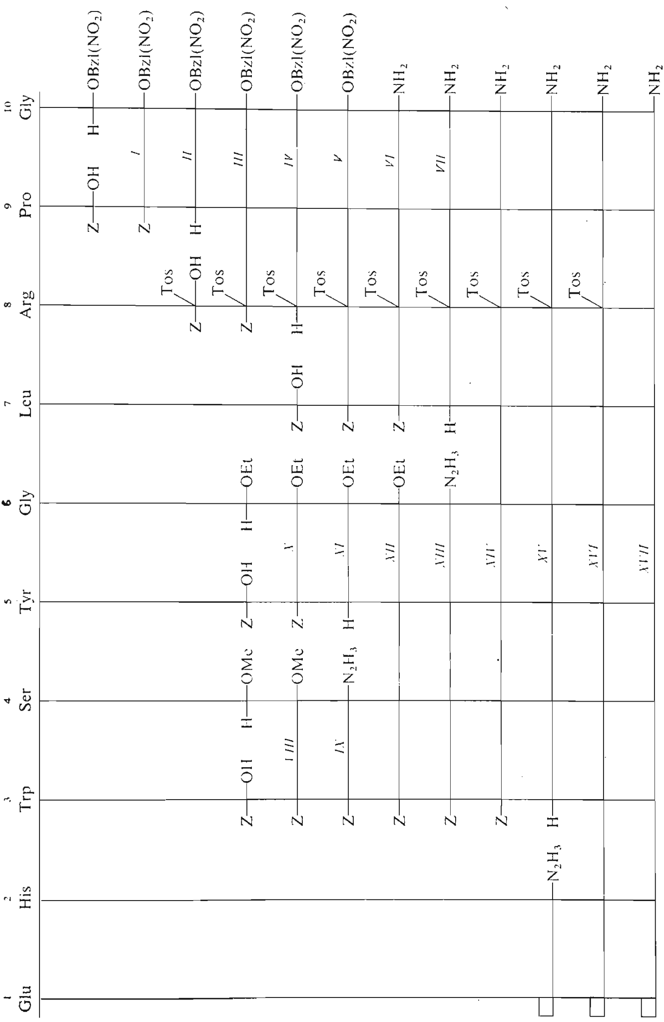
Synthesis of the gonadotropic hormone releasing factor LRF/FRF by fragment condensation is described. Of the functional groups of amino acids, only N^{g}-arginine was protected by a tosyl groups. Detosylation was done in the last step by electrolytic reduction. The final product was purified on CM-cellulose at pH 6·5.

Synthesis of the releasing factor of gonadotropic hormones, the luteotropic and the follicle-stimulating hormone, LRF/FRF, has been described by several groups, along with numerous analogues. Besides solid-state syntheses others were done by fragment condensation in solution[1-10] or by a combination of both methods[2,10]. In the present work, we used fragment condensation in solution with minimum protection of the functional side groups of amino acids, only the guanidine group of arginine being masked with a tosyl group[11]. The side groups of serine, tyrosine, as well as of pyroglutamic acid, were not protected.* For a temporary masking of the N^{α}-amino group the benzyloxycarbonyl group was solely used[15].

The tactical aim of the construction of the protected decapeptide was the preservation of the single protecting group in the side chain or arginine that could be removed during the last step of synthesis by an electrolytic reduction. The nitro group was originally used for this purpose but difficulties connected with the ammonolysis of the N^{g}-nitroarginyl-prolyl-glycine ethyl ester[16] resulted in the use of the tosyl group for protecting N^{g}-arginine. During electrolytic detosylation we used the original method[17,18], later modified for N^{g}-tosylarginine[19], employing previous experience[20]. The synthetic procedure is shown in Scheme 1.

Synthesis of the *p*-nitrobenzyl ester of benzyloxycarbonylprolyl-glycine (*I*) was accomplished either with the method of mixed anhydrides or with the DCCD method and it was comparable with the use of *p*-nitrophenyl esters[21] in respect of both the chemical and the optical purity of the product, as well as of the yield (85–90%).

* The symbols and names used follow the suggestion of the IUPAC-IUB Commission on Biochemical Nomenclature[12-14]. All amino acids except glycine are of the L-configuration. ⊏Glu stands for the pyroglutamic acid residue, DCCD for N,N'-dicyclohexylcarbodiimide.



SCHEME 1

Action of hydrogen bromide in glacial acetic acid yielded the hydrobromide *II* which was characterized only by chromatography. Condensation of N^{α}-benzyloxycarbonyl-N^{g}-tosylarginine with *II* was done by the mixed-anhydrides method. The amorphous tripeptide *III* was treated with hydrogen bromide in acetic acid to yield the hydrobromide *IV*. Condensation of benzyloxycarbonylleucine with *IV* was done again by the mixed-anhydrides method, which produced the *p*-nitrobenzyl ester *V* in a 70% yield: when using N-hydroxysuccinimide ester of benzyloxycarbonylleucine[22] with *IV* a quantitative yield of *V* was obtained. Ammonolysis of *V* in methanol yielded amide *VI*. Hydrogenolysis of *VI* on Pd black in 50% acetic acid yielded leucyl-N^{g}-tosylarginyl-prolyl-glycinamide *VII*; hydrogenolysis in methanol as well as in dimethylformamide proceeded slowly and incompletely. The methyl ester of benzyloxycarbonyltryptophyl-serine (*VIII*) was prepared by the DCCD method with N-hydroxybenztriazol[23]; hydrazinolysis with 80% hydrazine hydrate yielded the dipeptide hydrazide *IX*. The ethyl ester of benzyloxycarbonyltyrosyl-glycine (*X*) was prepared by the phosphoazo method or by the mixed-anhydrides method. Hydrogen bromide in acetic acid applied to *X* yielded the hydrobromide *XI* which was coupled without isolation with *IX* using the azide method of Honzl and Rudinger[24], yielding the ethyl ester of benzyloxycarbonyltryptophyl-seryl-tyrosyl-glycine (*XII*); hydrazinolysis with 80% hydrazine hydrate yielded the corresponding tetrapeptide hydrazide *XIII*. The method was used to couple[25] *XIII* with *VII*, yielding octapeptide *XIV* which was hydrogenolyzed on Pd black in 50% acetic acid and deionized on Zerolit FF (in the OH^{-} form) in methanol to yield amide *XV*. The octapeptide *XV* was coupled by the azide method with the hydrazide of pyroglutamyl-histidine[26,27] in a way similar to that with *XIV*, yielding the decapeptide *XVI* which was reduced electrolytically on a mercury cathode to the final product. The stability of the lactam ring of pyroglutamic acid under reducing conditions was followed in a model mixture of pyroglutamic acid with N^{g}-tosylarginine. After reduction, the reaction mixture was found by thin-layer chromatography to contain only arginine and the starting pyroglutamic acid. The product was desalted on Amberlite IRC 50. Because of the high salt content it was necessary to use a relatively greater amount of ion-exchange resin and to deionize in combination of input and column desalting. The crude product obtained was purified by chromatography on CM-cellulose[2]. The biological activity of the decapeptide was demonstrated semiquantitatively according to Horton and coworkers[28] using infant male rats and adult rabbits and observing the increased level of testosterone in peripheral blood.

EXPERIMENTAL

The melting points were determined in Kofler's block and are not corrected. Samples for analysis were dried *in vacuo* at 0·5 Torr over P_2O_5 at room temperature (for substances melting below 115°C) or at 105°C. Optical rotation was estimated in a photoelectric Perkin Elmer polarimeter

in methanol (c 0·2). Chromatography was done in a thin layer of silica gel (Kieselgel G, Merck) in butanol–acetic acid–water (4 : 1 : 1) (S_1) and butanol–acetic acid–pyridine–water (15 : 3 : : 10 : 6) (S_2). Electrophoresis was done on Schleicher–Schüll paper 2043B at a potential gradient of 100 V/cm in pyridine–acetic acid (pH 3·5 and 6·5) for 20 min. Detection was done with ninhydrin, Pauly and Sakaguchi reagents, treatment with chlorine and tolidine. Amino acids were analyzed on an automatic two-column analyzer (Development Workshop, Czechoslovak Academy of Sciences, Prague; type 6020). Samples for amino acid analysis were hydrolyzed for 20 h at 105°C in 6M-HCl. The standard treatment was to dissolve the reaction mixture in ethyl acetate (unless stated otherwise), wash with 1M-HCl, water, 5% sodium hydrogen carbonate, water, and dry with anhydrous sodium sulfate. The solutions were evaporated on a rotary evaporator at reduced pressure.

p-Nitrobenzyl Ester of Benzyloxycarbonylprolyl-glycine (*I*)

The anhydride method: Solution of benzyloxycarbonylproline (50 g; 0·2 mol) in dichloromethane (200 ml) was combined at 0°C with N-ethylpiperidine (28 ml) and secondary butyl chloroformate (28 ml). After 6 min of stirring, the solution was combined with a suspension of glycine *p*-nitrobenzyl ester, prepared by liberating the corresponding hydrochloride[29] (49·6 g; 0·2 mol) in dimethylformamide (40 ml) and dichloromethane (200 ml) by adding N-ethylpiperidine (28·2 ml). After stirring for 30 min at 0°C and for 2 h at room temperature the solution was processed in a standard way. Crystallization from a mixture of 200 ml 2-propanol and 300 ml light petroleum yielded 78·1 g (88%) product melting at 97—99°C. Sample for analysis was crystallized from methanol and water, the m.p. remaining unchanged. For $C_{22}H_{23}N_3O_7$ (441·4) calculated: 59·86% C, 5·25% H, 9·52% N; found: 59·82% C, 5·12% H, 9·20% N. $[\alpha]_D^{20}$ −56·0°.

The DCCD *method*: A solution of *p*-nitrobenzyl ester of glycine in 130 ml dimethylformamide prepared from the hydrochloride (12·3 g; 50 mmol) by adding 7·2 ml N-ethylpiperidine was added to a solution of benzyloxycarbonylproline (12·5 g; 50 mmol) in dimethylformamide (50 ml); the solution was combined at 0°C with 11 g DCCD, the solution was stirred for 1 h at 0°C and, after standing for 12 h at room temperature, N,N′-dicyclohexylurea was filtered. The filtrate was processed in a standard way and crystallized from a mixture of 2-propanol and light petroleum to yield 17·7 g (80%) product melting at 95—97°C.

p-Nitrobenzyl Ester of Benzyloxycarbonyl-N^g-tosylarginyl-prolyl-glycine (*III*)

Solution of *I* (44·1 g; 0·1 mol) in 30 ml glacial acetic acid was combined with 35% hydrogen bromide in 100 ml acetic acid and after 1 h at room temperature, hydrobromide *II* was precipitated with ether, dried for 3 h over P_2O_5 and KOH, dissolved in dimethylformamide (150 ml) and neutralized with N-ethylpiperidine (14·2 ml); R_F 0·52 in S_1 and 0·62 in S_2. N-Ethylpiperidine (14 ml) and secondary butyl chloroformate (14 ml) were added at −10°C to a solution of benzyloxycarbonyl-N^g-tosylarginine (47·5 g; 0·1 mol) in 150 ml dimethylformamide. After 10 min, a solution of the dipeptide ester released from *II* was added. Standard processing yielded 52·1 g (69%) amorphous product.

Hydrobromide of the *p*-Nitrobenzyl Ester of N^g-Tosylarginyl-prolyl-glycine (*IV*)

This was prepared from *III* (15 g; 20 mmol) by treatment with 2M-HBr in acetic acid (40 ml) for 1 h at room temperature; crystalline hydrobromide was dried for 3 h over P_2O_5 and KOH and processed immediately. Sample for analysis was crystallized from a mixture of acetic acid,

2-propanol and ether, m.p. 133—135°C. For $C_{27}H_{35}N_7O_8S.2\,HBr.H_2O$ (797·5) calculated: 40·67% C, 4·93% H, 12·29% N; found: 40·20% C, 5·01% H, 11·97% N. $[\alpha]_D^{20}$ −19·9° R_F: 0·48 (S_1), 0·63 (S_2).

p-Nitrobenzyl Ester of Benzyloxycarbonylleucyl-N^g-tosylarginyl-prolyl-glycine (*V*)

The anhydride method: Solution of benzyloxycarbonylleucine (5·3 g; 20 mmol) in dichloromethane (50 ml) and N-ethylpiperidine (2·8 ml) was combined at 0°C with secondary butyl chloroformate (2·8 ml). After 5 min, the mixture was combined with the tripeptide ester released from hydrobromide *IV* in dichloromethane (50 ml); the release was accomplished with a mixture of chloroform-5% sodium hydrogen carbonate. Standard processing yielded 12·1 g (70%) amorphous product.

The ester method: A solution of the tripeptide ester (1·98 g; 3·28 mmol) in 50 ml dichloromethane was combined at room temperature with three portions of N-hydroxysuccinimide ester of benzyloxycarbonylleucine (1·27 g; 3·5 mmol) added in 5 min intervals, stirred for 2 h at room temperature and left to stand for 12 h. Then it was processed in the standard way. Precipitation from 2-propanol (25 ml) and light petroleum (75 ml) yielded 2·5 g (90%) product which underwent ammonolysis to *VI* (82%; m.p. 127—129°C).

Amide of Benzyloxycarbonylleucyl-N^g-tosylarginyl-prolyl-glycine (*VI*)

Solution of *V* (17·3 g; 20 mmol) in methanol (50 ml) was flushed at 0°C with a stream of ammonia for 4 h, evaporated *in vacuo*, the residue combined with ethyl acetate (50 ml) and the solution was left for 12 h at 0°C. The precipitated amide was crystallized from methanol (50 ml) and ether (100 ml). A total of 9·9 g (68%) product melting at 137—139°C was obtained. Sample for analysis was crystallized in a similar way, the m.p. was not altered. For $C_{34}H_{48}N_8O_8S$ (728·9) calculated: 56·03% C, 6·64% H, 15·37% N; found: 55·74% C, 6·65% H, 15·41% N. $[\alpha]_D^{20}$ −39·2°. R_F: 0·57 (S_1), 0·75 (S_2).

Amide of Leucyl-N^g-tosylarginyl-prolyl-glycine (*VII*)

Solution of *VI* (14·6 g; 20 mmol) in 50% acetic acid (100 ml) was hydrogenated on Pd black for 7 h, evaporated, twice evaporated with methanol and deionized on Zerolit FF (in the OH^- form) using a 2·8 cm × 26 cm column, in methanol. Evaporation and drying over P_2O_5 yielded 12·6 g (100%) amorphous, homogeneous product. Sample for analysis was crystallized from a mixture of methanol, 2-propanol and ether, m.p. 128—131°C. For $C_{26}H_{42}N_8O_6S.H_2O$ (607·8) calculated: 51·38% C, 7·28% H, 18·43% N; found: 50·90% C, 7·15% H, 18·27% N. $[\alpha]_D^{20}$ −26·0°. R_F: 0·18 (S_1), 0·65 (S_2). Amino acid analysis: Arg 1·00, Pro 1·02, Gly 1·01, Leu 0·99.

Methyl Ester of Benzyloxycarbonyltryptophyl-serine (*VIII*)

Solution of benzyloxycarbonyltryptophan (84·95 g; 250 mmol) and hydroxybenztriazol (35 g) in dimethylformamide (150 ml) was combined with a solution of serine methyl ester in dimethylformamide (150 ml) released from the corresponding hydrochloride (39 g; 250 mmol) with N-ethylpiperidine (35 ml). The mixture was combined with DCCD (55 g) at 0°C, stirred for 1 h at 0°C and, after 12 h of standing at room temperature, standard treatment (with 10% citric acid) and crystallization from ethyl acetate (120 ml) and light petroleum (170 ml) yielded 68·5 g (63%) product melting at 132—135°C (it softens at 122°C). Sample for analysis was crystallized

in a similar way, m.p. 138—140°C. For $C_{23}H_{25}N_3O_6$ (439·5) calculated: 62·86% C, 5·73% H, 9·56% N; found: 62·84% C, 5·97% H, 9·61% N. $[\alpha]_D^{20}$ —12·4°.

Hydrazide of Benzyloxycarbonyltryptophyl-serine (*IX*)

A solution of *VIII* (39·6 g; 90 mmol) in methanol (270 ml) was combined at room temperature with 80% hydrazine hydrate (22·5 ml). After 12 h of standing at room temperature the precipitated hydrazide was filtered and washed with methanol. Crystallization from methanol (900 ml) and water (300 ml) yielded 30·1 g (76%) product melting at 169—173°C. Sample for analysis was crystallized in a similar way, m.p. 179—182°C. For $C_{22}H_{25}N_5O_5$ (439·5) calculated: 60·13% C, 5·73% H, 15·94% N; found: 59·82% C, 5·88% H, 15·41% N.

Ethyl Ester of Benzyloxycarbonyltyrosyl-glycine (*X*)

The phosphoazo method: Suspension of ethyl ester glycine hydrochloride (14 g; 100 mmol) in 50 ml pyridine was stirred for 30 min at room temperature and, after cooling to —20°C, a solution of phosphorus trichloride (4·5 ml) in 30 ml pyridine was added at —20°C. After 30 min of standing at that temperature the mixture was combined with benzyloxycarbonyltyrosine (32 g; 100 mmol) and the mixture was refluxed for 3 h. After evaporation, the residue was processed in a standard way and crystallization from methanol (160 ml) and water (300 ml) yielded 23·9 g (57%) product melting at 162—164°C. Sample for analysis was crystallized from 2-propanol, m.p. 169—171°C. For $C_{21}H_{24}N_2O_6$ (400·4) calculated: 62·99% C, 6·04% H, 7·00% N; found: 63·17% C, 6·42% H, 6·90% N. $[\alpha]_D^{20}$ —13·4°.

The anhydride method: A solution of benzyloxycarbonyltyrosine (6·4 g; 20 mmol) and N-ethylpiperidine (2·8 ml) in dichloromethane (50 ml) kept at —5°C was combined with secondary butyl chloroformate (2·8 ml). After 10 min, glycine ethyl ester in 50 ml dichloromethane was added after release from the corresponding hydrochloride (2·9 g; 20 mmol) with N-ethylpiperidine (2·9 ml). Standard treatment and crystallization from ethanol (150 ml) and water (50 ml) yielded 3·8 g (49%) product melting at 168—170°C.

Ethyl Ester of Benzyloxycarbonyltryptophyl-seryl-tyrosyl-glycine (*XII*)

A solution of *IX* (8·8 g; 20 mmol) in tetrahydrofuran (100 ml) and 20% HCl (8 ml) was combined under stirring at —10°C with a solution of sodium nitrite (1·38 g) in 5·6 ml water. After 8 min of stirring at —10°C, the solution was combined with ethyl acetate (100 ml) and the organic phase was washed with precooled saturated solution of sodium hydrogen carbonate in 16·8% NaCl, dried with sodium sulfate and added to a solution of tyrosyl-glycine ethyl ester in dimethylformamide (60 ml) released by N-ethylpiperidine (2·8 ml) from the corresponding hydrobromide *XI* which was prepared from *X* (8·0 g; 20 mmol) with 2M-HBr in acetic acid; R_F 0·50 in S_1 and 0·69 in S_2. On the following day the solution was processed in a standard way with 10% citric acid. Crystallization from 2-propanol (50 ml), tetrachloromethane (50 ml) and light petroleum (300 ml) yielded 9·4 g (70%) product melting at 148—150°C. For $C_{35}H_{39}N_5O_9$ (673·7) calculated: 62·40% C, 5·83% H, 10·39% N; found: 62·52% C, 5·80% H, 10·24% N. $[\alpha]_D^{20}$ —17·4°.

Hydrazide of Benzyloxycarbonyltryptophyl-seryl-tyrosyl-glycine (*XIII*)

A solution of *XII* (33·7 g; 50 mmol) in methanol (100 ml) was combined at 40—50°C with 80% hydrazine hydrate (12·5 ml). After 12 h of standing at room temperature the precipitated hydrazide was filtered and washed with methanol. Crystallization from ethanol (600 ml) and water

(25 ml) yielded 28·8 g (86%) product melting at 190—193°C (it softens at 180°C). Sample for analysis was crystallized in a similar way, m.p. 178—180°C. For $C_{33}H_{37}N_7O_8$ (659·7) calculated: 60·10% C, 5·65% H, 14·86% N; found: 59·59% C, 5·83% H, 14·66% N.

Amide of Benzyloxycarbonyltryptophyl-seryl-tyrosyl-glycyl-leucyl-N^g-tosylarginyl-prolyl-glycine (*XIV*)

The product was prepared like with *XII*, proceeding from *XIII* and *VII* on a 5 mmol scale. The mixture was processed by evaporation, dissolved in 1-butanol (250 ml) and processed in a standard way with 10% citric acid. Crystallization from methanol (15 ml) and ether (50 ml) yielded 5·11 g (84%) product melting at 151—155°C. Sample for analysis was crystallized in a similar way, m.p. 157—159°C (it softens at 150°C). For $C_{59}H_{75}N_{13}O_{14}S.3\ H_2O$ (1276) calculated: 55·52% C, 6·40% H, 14·26% N; found: 55·59% C, 6·08% H, 14·39% N. $[\alpha]_D^{20}$ —30·6°. R_F: 0·61 (S_1), 0·76 (S_2).

Amide of Tryptophyl-seryl-tyrosyl-glycyl-leucyl-N^g-tosyl-arginyl-prolyl-glycine (*XV*)

Hydrogenolysis of *XIV* (8·7 g; 7 mmol) on Pd black (like with *VII*) yielded 8·6 g (100%) amorphous product; R_F: 0·35 in S_1, 0·52 in S_2.

Amide of Pyroglutamyl-histidyl-tryptophyl-seryl-tyrosyl-glycyl-leucyl-N^g-tosylarginyl-prolyl--glycine (*XVI*)

A solution of hydrazide of pyroglutamyl-histidine (2·5 g; 8·9 mmol) in dimethylformamide (100 ml) and concentrated hydrochloric acid (7 ml) was combined at —20°C with a solution of sodium nitrite (625 mg) in water (2·5 ml). After 15 min, the solution was neutralized with N-ethylpiperidine (about 8·5 ml) to pH 6·9 and precooled solution of *XV* (7·6 g; 7 mmol) in 30 ml dimethylformamide was added. After 12 h at 0°C, the solution was evaporated and the residue stirred with saturated NaCl (200 ml), the aqueous phase was decanted and the remaining oil was then triturated four times with a solution of NaCl. Finally, the residue was mixed with cold water (20 ml), decanted and the residue dried *in vacuo*. Dissolving in methanol (15 ml) and pouring into 150 ml 2-propanol yielded 7·6 g (76%) product melting at 188—192°C. A sample for analysis was precipitated in a similar way, m.p. 184—186°C (it sinters at 180°C). For $C_{62}H_{80}N_{17}O_{15}S.5H_2O$ (1425) calculated: 52·24% C, 6·36% H, 16·70% N; found: 52·04% C, 6·09% H, 16·70% N. $[\alpha]_D^{20}$ —29·9°. Amino acid analysis: His 1·04, Arg 1·00, Ser 0·93, Glu 1·15, Pro 1·09, Gly 2·04, Leu 1·03, Tyr 1·02.

Amide of Pyroglutamyl-histidyl-tryptophyl-seryl-tyrosyl-glycyl-leucyl-arginyl-prolyl-glycine (*XVII*)

A solution of *XVI* (710 mg; 0·5 mmol) and tetramethylammonium chloride (1·5 g) in a mixture of methanol (10 ml), 2-propanol (10 ml) and dimethylformamide (10 ml) was reduced electrolytically on a mercury cathode for 80 min (I = 200—250 mA). The anolyte used was tetramethylammonium chloride (1·5 g) in the same mixture as the catholyte. Cellophane served as the diaphragm. The temperature of the cathode and anode spaces was 12—15°C, the anode was made of graphite. After the reduction, the catholyte was acidified with acetic acid (about 0·5 ml) to pH 4, evaporated, the residue was diluted with water to 50 ml and desalted on Amberlite IRC 50 (H^+ form), first in a mixture, then on a column. The total consumption of the resin was about 30 ml. After washing the resin with 0·25% acetic acid, the peptide was eluted with 50% acetic acid. The combined eluates were evaporated, gradually dried with tetrahydrofuran and a mixture of tetra-

hydrofuran with benzene and finally the crude product was precipitated from a methanol solution with ether. A total of 275 mg product was obtained; it was chromatographed on a 2·5 cm × × 50 cm column of CM-cellulose, using ammonium acetate buffer (pH 6·5) in a 0—0·05M gradient for elution. A homogeneous product was thus obtained (195 mg). The final product produces a single cathodic spot after high-voltage electrophoresis (49 mm distand at pH 3·5, 25 mm distant at pH 6·5) which gives positive Pauly and Sakaguchi reactions and is negative to ninhydrin. A sample for analysis was precipitated from methanol and ether. For $C_{55}H_{74}N_{17}O_{13}.2\,C_2H_4O_2.5\,H_2O$ (1331) calculated: 53·22% C, 6·96% H, 17·88% N; found: 53·08% C, 6·34% H, 17·40% N. $[\alpha]_D^{20}$ −40·4°. Amino acid analysis: His 1·05, Arg 1·00, Ser 1·00, Glu 1·06, Pro 1·06, Gly 2·01, Leu 1·06, Tyr 1·00.

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