SYNTHESIS OF PEPTIDE AND GLYCOPEPTIDE FRAGMENTS OF BACTERIAL CELL WALLS*

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Peptides, ranging from tetrapeptide *I*, *II* to octadecapeptide *XXI* and from N-acetylmuramyl-hexapeptide *XXIII* to tris(N-acetylmuramyl)-octadecapeptide *XXVII*, were prepared using synthesis in solution as well as solid-phase synthesis. H-L-Ala-D-iGln-L-Lys-D-Ala-(L-Ala)$_2$-OMe (*VIII*) and its lysine-acetylated analogue *X* were pyrogenic and the former (*VIII*) exhibited weak immunoadjuvant activity. The immunoadjuvant activity of tris-(N-acetylmuramyl)-octadecapeptide *XXVII* was comparable with that of MDP and the compound was not pyrogenic.

Peptidoglycan from bacterial cell wall and its fragments possess a number of biological activities[1,2] whose investigation has a considerable theoretical and prospective practical importance.

For the pyrogenic and immunoadjuvant activity of fragments of bacterial wall peptidoglycan the sugar component is important but not essential. Peptides, containing a meso-diaminopimelic acid residue in position 3, have *per se* an immunoadjuvant effect[3,4]. The simplest compound of this type is a dipeptide, γ-D-glutamyl-meso-diaminopimelic acid**.

Contradictory data exist on analogous peptides with L-lysine instead of meso-diaminopimelic acid in position 3. According to some authors[6,7] muramic acid is essential for the pyrogenic and immunoadjuvant activity, according to others[8,9] its presence is not necessary.

The tetrapeptide H-L-Ala-D-Glu(X-D-Ala-OH)-OH (where X is meso-diaminopimelic acid or N$^{\varepsilon}$-substituted lysine) was isolated from a natural material[8] and its activity may thus be due to an immunoadjuvant impurity[1,2].

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** The symbols and abbreviations follow the published recommendations[5]. Further we use: BBM benzyl 2-acetamido-4,6-O-benzylidene-3-O-[(*R*)-1-carboxyethyl]-2-deoxy-α-D-glucopyranoside; MDP N-acetylmuramyl-L-alanyl-D-isoglutamine; $\mathscr{R}$ resin.

The synthetic tetrapeptide H-L-Ala-D-iGln-L-Lys-D-Ala-OH is inactive[10]. The synthetic[11] nonapeptide *XXVIII* was claimed to possess immunoadjuvant activity[9], later repeated experiments afforded inconsistent results.

To clarify the question of pyrogenic and immunoadjuvant effect of lysine-containing peptides of bacterial walls, we synthesized a series of peptides, ranging from tetrapeptides to octadecapeptide, and their glyco derivatives and determined their pyrogenic and immunoadjuvant effects. Shorter peptides were prepared by synthesis in solution[11-13], more complex compounds exclusively by solid phase synthesis[11,14-16]. Compounds with identical amino acids at the amino-terminus of both or all three chains can be synthesized in two ways: the chain is lengthened either at each step by one amino acid (compounds *XVI*, *XVIII*) or a suitably protected intermediate is deprotected and the synthesis proceeds simultaneously in all branches. The second way, more advantageous and less time-consuming, was used in the preparation of octadecapeptide *XX* and tris-(N-Ac-Mur)-octadecapeptide *XXVI* (Scheme 1, ref.[16]). The synthesized compounds were liberated from the polymer support by transesterification in methanol or by ammonolysis[11,15], the peptide-acids were cleaved from the resin with liquid hydrogen fluoride[17]. The remaining protecting groups were removed with sodium in liquid ammonia.

The prepared compounds were systematically tested for pyrogenicity and for the ability to affect the cellular immune response to the given antigen using the delayed-type hypersensitivity test.

An indistinct immunoadjuvant activity was found with the tetrapeptide amide *I*, containing free lysine ε-amino group (see Table I). Compound *I* had no pyrogenic effect. A distinct immunoadjuvant activity was observed for the linear and branched hexapeptide esters *VIII* and *XII*. The linear derivative *VIII* had a considerable pyrogenic effect whereas the branched compound *XII* was apyrogenic. The linear hexapeptide ester *X* differing from the peptide ester *VIII* by the acetylated lysine ε-amino group, exhibited the same pyrogenic effect as *VIII* but its immunoadjuvant activity was lower. Immunoadjuvant activity of the heptapeptide amide *XIII* was indistinct and the compound was not pyrogenic. In our hands, the nonapeptide amide *XXIX* (refs[11,16]) was biologically inactive[33] both in the pyrogenic and immunoadjuvant activity test. The same results with this compound were obtained at the Sandoz Forschungsinstitut in Vienna. A weak immunoadjuvant activity, together with a significant pyrogenicity, was observed with the branched nonapeptide amide *XIV*, whereas the corresponding branched nonapeptide-acid *XV* was inactive. The protected nonapeptide *XXX* (refs[11,16]) exhibited a significant immunoadjuvant activity but was not pyrogenic. The enhanced activity of partially protected peptides is known. Thus, *e.g.* the partially protected tetrapeptide ester *XXXII* has a significant pyrogenic effect[11,16] whereas tetrapeptides with free lysine ε-amino group are inactive. Peptides higher than nonapeptides exhibited only negligible pyrogenic effect. All the glycopeptides studied by us were biologically active. The glyconona-

peptide *XXXI* (refs[11,16]) and glycooctadecapeptide ester *XXVII* are worth notice: both possess strong immunoadjuvant activity and are not pyrogenic[16].

Boc-L-Lys(Z)-D-Ala-O-$\mathscr{R}$
↓
Tos-L-Ala-D-iGln-L-Lys(Z)-D-Ala-O-$\mathscr{R}$
↓
Boc-$(Gly)_5$¬
Tos-L-Ala-D-iGln-L-Lys-D-Ala-O-$\mathscr{R}$
↓
Tos-L-Ala-D-iGln-L-Lys(Z)-D-Ala-$(Gly)_5$¬
↓ Tos-L-Ala-D-iGln-L-Lys-D-Ala-O-$\mathscr{R}$
tridecapeptide
XIX

Boc-L-Lys(Ac)-D-Ala-$(L\text{-Ala})_2$¬
Z-L-Lys-D-Ala-$(L\text{-Ala})_2$¬
↓ Z-L-Lys-D-Ala-$(L\text{-Ala})_2$-O-$\mathscr{R}$
BBM-L-Ala-D-iGln-L-Lys(Ac)-D-Ala-$(L\text{-Ala})_2$¬
BBM-L-Ala-D-iGln-L-Lys-D-Ala-$(L\text{-Ala})_2$¬
↓ BBM-L-Ala-D-iGln-L-Lys-D-Ala-$(L\text{-Ala})_2$-O-$\mathscr{R}$
tris-(N-Ac-Mur)-octadecapeptide
XXVII

SCHEME 1

Scheme of solid phase synthesis[16] of tridecapeptide *XIX* and tris-(N-Ac-Mur)-octadecapeptide *XXVII*

I, H-L-Ala-D-iGln-L-Lys-D-Ala-NH_2
II, H-L-Ala-D-iGln-L-Lys-D-Ala-OH
III, Z-L-Ala-D-iGln-L-Lys(Tos)-D-Ala-D-Ala-OMe
IV, H-L-Ala-D-iGln-L-Lys-D-Ala-D-Ala-OMe
V, H-L-Ala-D-iGln-L-Lys-D-Ala-$(L\text{-Ala})_2$-OH
VI, Z-L-Lys(Tos)-D-Ala-L-Ala-L-Ala-OMe
VII, Z-L-Ala-D-iGln-L-Lys(Tos)-D-Ala-$(L\text{-Ala})_2$-OMe
VIII, H-L-Ala-D-iGln-L-Lys-D-Ala-$(L\text{-Ala})_2$-OMe
IX, Boc-L-Ala-D-iGln-L-Lys(Ac)-D-Ala-$(L\text{-Ala})_2$-OMe
X, H-L-Ala-D-iGln-L-Lys(Ac)-D-Ala-$(L\text{-Ala})_2$-OMe
XI, Ac-L-Ala-L-Ala¬
Boc-L-Ala-D-iGln-L-Lys-D-Ala-OMe
XII, Ac-L-Ala-L-Ala¬
H-L-Ala-D-iGln-L-Lys-D-Ala-OMe
XIII, H-L-Ala-D-iGln-$(Gly)_5$-NH_2
XIV, H-$(Gly)_5$¬
H-L-Ala-D-iGln-L-Lys-D-Ala-NH_2
XV, H-$(Gly)_5$¬
H-L-Ala-D-iGln-L-Lys-D-Ala-OH

XVI, Tos-L-Ala-D-iGln-L-Lys(Ac)-D-Ala-(L-Ala)$_2$┐
Tos-L-Ala-D-iGln-L-Lys-D-Ala-OMe

XVII, H-L-Ala-D-iGln-L-Lys(Ac)-D-Ala-(L-Ala)$_2$┐
H-L-Ala-D-iGln-L-Lys-D-Ala-OMe

XVIII, Tos-L-Ala-D-iGln-L-Lys(Z)-D-Ala-(Gly)$_5$┐
Tos-L-Ala-D-iGln-L-Lys-D-Ala-OMe

XIX, H-L-Ala-D-iGln-L-Lys-D-Ala-(Gly)$_5$┐
H-L-Ala-D-iGln-L-Lys-D-Ala-OMe

XX, Boc-L-Ala-D-iGln-L-Lys(Ac)-D-Ala-(L-Ala)$_2$┐
Boc-L-Ala-D-iGln-L-Lys-D-Ala-(L-Ala)$_2$┐
Boc-L-Ala-D-iGln-L-Lys-D-Ala-(L-Ala)$_2$-OMe

XXI, H-L-Ala-D-iGln-L-Lys(Ac)-D-Ala-(L-Ala)$_2$┐
H-L-Ala-D-iGln-L-Lys-D-Ala-(L-Ala)$_2$┐
H-L-Ala-D-iGln-L-Lys-D-Ala-(L-Ala)$_2$-OMe

XXII, BBM-L-Ala-D-iGln-L-Lys(Tos)-D-Ala-(L-Ala)$_2$-OMe

XXIII, N-Ac-Mur-L-Ala-D-iGln-L-Lys-D-Ala-(L-Ala)$_2$-OMe

XXIV, Ac-L-Ala-L-Ala┐
BBM-L-Ala-D-iGln-L-Lys-D-Ala-OMe

XXV, Ac-L-Ala-L-Ala┐
N-Ac-Mur-L-Ala-D-iGln-L-Lys-D-Ala-OMe

XXVI, BBM-L-Ala-D-iGln-L-Lys(Ac)-D-Ala-(L-Ala)$_2$┐
BBM-L-Ala-D-iGln-L-Lys-D-Ala-(L-Ala)$_2$┐
BBM-L-Ala-D-iGln-L-Lys-D-Ala-(L-Ala)$_2$-OMe

XXVII, N-Ac-Mur-L-Ala-D-iGln-L-Lys(Ac)-D-Ala-(L-Ala)$_2$┐
N-Ac-Mur-L-Ala-D-iGln-L-Lys-D-Ala-(L-Ala)$_2$┐
N-Ac-Mur-L-Ala-D-iGln-L-Lys-D-Ala-
-(L-Ala)$_2$-OMe

XXVIII, H-L-Ala-D-iGln-L-Lys(Ac)-D-Ala-(Gly)$_5$-OMe

XXIX, H-L-Ala-D-iGln-L-Lys(Ac)-D-Ala-(Gly)$_5$-NH_2

XXX, Z-L-Ala-D-iGln-L-Lys(Z)-D-Ala-(Gly)$_5$-OMe

XXXI, N-Ac-Mur-L-Ala-D-iGln-L-Lys(Ac)-D-Ala-(Gly)$_5$-NH_2

XXXII, H-L-Ala-D-iGln-L-Lys(Tos)-D-Ala-OMe

Our results lead to the following conclusions: Peptide fragments of peptidoglycan containing lysine in position 3 and their analogues exhibit low, mostly just observable immunoadjuvant and pyrogenic effects. Nevertheless, it is obvious that these peptides *per se* also show both the pyrogenic and immunoadjuvant activity and the sugar component is thus important only for the magnitude but not for the existence of these activities. The magnitude and character of the biological effects depend on the length and structure of the peptide chain, on the functional groups and their substitution and on the character of the substituents. Weak biological effects can be observed already with tetrapeptides, significant effects are found with hexapeptides and weak effects again with nonapeptides and higher peptides. The biological activities of linear peptides differ clearly from those of branched peptides. The linear hexapeptide ester *VIII* is strongly pyrogenic and has a distinct immunoadjuvant

effect whereas the branched hexapeptide ester *XII* has a distinct immunoadjuvant but not pyrogenic effect. It seems that compounds with modified C-terminal carboxy group (ester or amide) are biologically more active than compounds with free carboxyl and that linear peptides with free lysine ε-amino group have stronger immunoadjuvant effect than linear peptides with acylated ε-amino group. The activity of the partially and fully protected compounds – tetrapeptide *XXXII* (refs[11,16]) and nonapeptide *XXX* (refs[11,16]) – is interesting. The obtained results indicate many ways how to separate the immunoadjuvant and the pyrogenic activities.

TABLE I

Synthetic peptide and glycopeptide fragments of bacterial cell walls and their biological activities

Compound	Method of preparation[a]	Pyrogenicity[b]	Immunoadjuvant activity[c]		References
			M 24	OVA	
I	SP	–	–	±	16, 34
II	SP	–	–	–	16
IV	CL	–			16
V	SP	–	–	±	16
VIII	SP, CL	++	+	–	16, 33–35
X	SP	++	±	–	16, 34
XII	SP	–	–	+	16
XIII	SP	–	±		16
XIV	SP	+	–	±	16
XV	SP	–	–	–	16
XVII	SP	±			16, 34
XIX	SP	±	–		16, 18, 33, 34, 36
XXI	SP	–	–		16, 18, 34
XXIII	SP, CL	+	+	+	16, 33–36
XXV	SP		–	+	16
XXVII	SP	–	++	++	16, 34
XXVIII	SP	–			9, 11, 16
XXIX	SP	–	–	–	11, 16, 33–36
XXX	SP	–	–	+	11, 16
XXXI	combined	–		++	11, 16, 33–36
XXXII	CL	+			11, 16, 37, 38

[a] CL = classical synthesis in solution, SP = solid phase synthesis. [b] Pyrogenicity was tested on rabbits in comparison with MDP as positive standard (+++). [c] Immunoadjuvant activity was measured as delayed type hypersensitivity by skin test on guinea pigs in comparison with MDP as positive standard (++); *Streptococcus* M24 extract and ovalbumin (OVA) were used as antigens.

More detailed data on biological activities of the here described compounds are given elsewhere[18-21].

EXPERIMENTAL

The melting points were determined on a Kofler block and are not corrected. The optical activity was measured on a Perkin–Elmer 141 polarimeter, the obtained values are corrected according to nitrogen content in the lyophilisate. The infrared spectra were measured on a Zeiss (Jena, G.D.R.) UR-20 spectrophotometer. ^{1}H and ^{13}C NMR spectra were obtained in the FT mode on a Varian XL-200 spectrometer at 200 MHz and 50·3 MHz, respectively. The free acid *V* was measured in a mixture of $^{2}H_2O + C^{2}H_3COO^{2}H$ (9 : 1), using the $^{2}H_2O$ residual signal (δ 4·80) as standard for the ^{1}H NMR spectrum and the residual methyl signal in $C^{2}H_3COO^{2}H$ (δ 20·0) for the ^{13}C NMR spectrum. ^{1}H NMR spectrum of the ester *VIII* was measured in $C^{2}H_3SOC^{2}H_3$ with tetramethylsilane as internal standard and the NH protons were exchanged by addition of $^{2}H_2O$. Structural assignment of the signals was done by comparison with the published ^{1}H and ^{13}C NMR data for the corresponding peptide-bonded amino acid residues[22]. The preparative electrophoreses were carried out in a modified apparatus of Hannig[23], manufactured in the workshops of this Institute. The purity was checked by chromatography on Silufol sheets (Kavalier, Votice) in the following systems: 1-butanol–acetic acid–water (4 : 1 : 1) (A), 1-butanol–acetic acid–water (4 : 1 : 5) (B), ethanol–water (7 : 3) (C), 1-butanol–acetic acid–water–pyridine (15 : 3 : 12 : 10) (D), pyridine–acetic acid–water (10 : 6 : 3) (E), chloroform–methanol (17 : 3) (F), tert-butanol–dimethylformamide–acetic acid–water (2 : 1 : 3 : 2) (G), chloroform–methanol (1 : 3) (H). The detection was effected by ninhydrin or chlorination[24,25]. Purity of the free peptides was checked by paper electrophoresis on a Whatman No. 3 MM paper at 15–20 V/cm in pyridine–acetic acid buffer (pH 5·7) or in aqueous acetic acid (pH 2·5). Samples for amino acid analysis were hydrolyzed for 8–48 h in 6 mol l^{-1} HCl at 110°C. The hydrolyzates were analyzed on a Beckman–Spinco 120 B amino acid analyzer. When hydrolyzates containing muramic and glutamic acid were analyzed, the first buffer was adjusted to pH 3 in order to obtain a good resolution of these two acids. A colour value of 3·08 was used for muramic acid. The values obtained were corrected for 25% decomposition of muramic acid during the 8 h hydrolysis[11].

Preparative separation of crude peptides, as well as analysis of the starting mixtures and the obtained fractions, was carried out on a chromatograph, consisting of a ternary gradient delivery system SP 8700, a variable wavelength UV detector LC 871 and an integrator SP 4 200 (all from Spectra-Physics, San Jose, U.S.A.). Compounds were detected at 220 or 225 nm. Preparative separations were performed on a 250 mm long column of internal diameter 9 mm, packed with Partisil ODS 2 (10 μm; Whatman, Clinton, U.S.A.); flow rate 4 ml/min. The elution was performed under gradient conditions with solvent systems prepared by mixing a 0·1% aqueous trifluoroacetic acid (system I) or an aqueous 0·05 mol l^{-1} NaH_2PO_4/0·05 mol l^{-1} H_3PO_4 (system II) with methanol. The methanol concentration increase was 1%/min. The maximum preparative column load was 30–50 mg of the crude mixture. Analytical HPLC was carried out in the same mobile phase on a 250 × 4·6 mm (internal diameter) column packed with Spherisorb ODS (5 μm; Phase Separations, Queensferry, Great Britain).

All yields of the free peptides and glycopeptides are corrected for the content of the compounds in the lyophilizate, determined by nitrogen analysis. For compounds prepared by the solid phase method the yields relate to the first amino acid bound to the carrier (unless stated otherwise). Analytical samples were dried over phosphorus pentoxide at 50–120°C/10 Pa for 8–24 h.

Solid phase syntheses were performed on a semiautomatic synthesizer (Development Workshops of Czechoslovak Academy of Sciences) using chloromethylated polystyrene resins cross-linked with 1—2% of divinylbenzene (Calbiochem, Los Angeles, U.S.A.; Merck-Schuchardt, Hohenbrunn, F.R.G.; or a copolymer prepared in our own laboratories). The first Boc-amino acid was bound to the resin by the potassium fluoride method[26] and its content was determined by the picric acid test[27], amino acid analysis and elemental analysis. The Boc-residue was used as the mobile protecting group, lysine was condensed as Boc-Lys(Z), Z-Lys(Boc) or Boc-Lys(Ac). In some branched peptides Tos-L-Ala was used at the amino-terminus. The syntheses were performed either with protected amino acids using dicyclohexylcarbodiimide according to ref.[11] (method *A*) or with symmetrical anhydrides (ref.[28], method *B*), in both cases in three-fold molar excess. The condensation of Boc-D-iGln was carried out in the presence of equimolar amount of N-hydroxybenzotriazole in both methods. Benzyloxycarbonyl groups were cleaved by repeated treatment (5 + 15 min) with 10% hydrogen bromide in glacial acetic acid. Muramic acid was condensed in the form of its benzyl 2-acetamido-2-deoxy-4,6-O-benzylidene-3-O-[(*R*)-1-carboxyethyl]-α-D-glucopyranoside[11] according to the method *A*; method *B* presented difficulties because of low solubility of the symmetrical anhydride. The completeness of all the condensations was checked by the Kaiser ninhydrin test[29].

H-L-Ala-D-iGln-L-Lys-D-Ala-NH_2 (*I*)

The synthesis was carried out on a resin containing 1% of divinylbenzene. Boc-Lys(Z)-OH was condensed by the method *B*, Boc-D-iGln-OH and Tos-L-Ala-OH by the method *A*. From 10 g (5·2 mmol) of the resin we obtained 12·6 g (97%) of the resin-bound protected tetrapeptide.

The resin-bound protected peptide (2·8 g; theory 1·16 mmol) was ammonolyzed with methanolic ammonia (200 ml; saturated solution at 0°C). After stirring at room temperature for 36 h, ammonia was evaporated and the product was extracted successively with hot methanol (3 × × 100 ml) and hot dimethylformamide (3 × 50 ml). The combined extracts were concentrated *in vacuo* and the product was precipitated with ether; yield 0·71 g (87%) of Tos-L-Ala-D-iGln-L-Lys(Z)-D-Ala-NH_2. This protected tetrapeptide (704 mg; 1 mmol) was reduced with sodium in liquid ammonia (0·5 l) for 60 s, ammonia was evaporated and the product dissolved in 5% acetic acid (55 ml). The solution was adjusted to pH 4, desalted on an Amberlite IRC 50 XE 64 column (50 ml) and freeze-dried to give 620 mg of the product which was purified by HPLC in the system II (gradient 0—60% methanol). After adjusting to pH 9 with sodium hydroxide and immediately to pH 4 with acetic acid and desalting on Amberlite IRC 50 XE 64, 415 mg of the product was obtained (65% from the protected tetrapeptide or 56·6% from Boc-D-Ala-O$\mathscr{R}$). For $C_{17}H_{33}N_7O_5$ (415·5) calculated: 23·60% N; for the undried lyophilizate found: 15·34% N; peptide content 65%. $[\alpha]_D^{20}$ +8·1° (*c* 1; 1 mol l^{-1} AcOH); amino acid analysis: Ala 1·95, Glu 0·99, Lys 0·98, NH_3 2·08. For $C_{17}H_{33}N_7O_5$. 2 1/2AcOH (565·6) calculated: 46·72% C, 7·66% H, 17·33% N; for the dried compound found: 46·54% C, 7·68% H, 17·05% N.

H-L-Ala-D-iGln-L-Lys-D-Ala-OH (*II*)

Tos-L-Ala-D-iGln-L-Lys(Z)-D-Ala-O-$\mathscr{R}$ (for preparation see *I*; 3·1 g; theory 1·28 mmol) was cleaved for 45 min at 0°C in a mixture of anisole (10 ml) and liquid hydrogen fluoride (60 ml). The hydrogen fluoride was removed by a stream of nitrogen and the peptide with the resin were washed with ethyl acetate (3 × 60 ml, 0°C, 5 min) and ether (2 × 50 ml). The peptide was taken up in hot dimethylformamide (5 × 30 ml) and hot 50% aqueous dimethylformamide (2 × 40 ml). After concentration *in vacuo*, Tos-L-Ala-D-iGln-L-Lys-D-Ala-OH.HF was precipitated with ether; yield 650 mg (86%). The compound was homogeneous on Silufol in the system A.

The tosyl tetrapeptide (650 mg) was reduced with sodium in liquid ammonia (500 ml) for 1 min. Ammonia was evaporated, the residue dissolved in 5% acetic acid (50 ml) and the solution adjusted to pH 4. Desalting on a column of Amberlite IRC 50 XE 64 (50 ml) and freeze-drying furnished 290 mg (40%) of the product. For $C_{17}H_{32}N_6O_6$ (416·5) calculated: 20·18% N; for undried sample found: 14·76% N, peptide content in the lyophilizate 73%. HPLC purity 95% (220 nm). Preparative HPLC purification of the product (230 mg), followed by desalting and lyophilization, afforded 37 mg of HPLC-homogeneous compound. In this case isocratic elution with system I (2% methanol) was used. $[\alpha]_D^{22}$ 0° (*c* 0·5; acetic acid). Amino acid analysis: Ala 2·0, Glu 1·05, Lys 0·98, NH_3 0·95. For $C_{17}H_{32}N_6O_6.2$ AcOH. 1/2 H_2O (545·6) calculated: 46·23% C, 7·57% H, 15·40% N; for dried compound found: 46·40% C, 7·28% H, 15·13% N.

Z-L-Ala-D-iGln-L-Lys(Tos)-D-Ala-D-Ala-OMe (*III*)

A solution of Z-L-Ala-D-iGln (ref.[11]; 650 mg; 1·85 mmol), N-hydroxybenzotriazole hydrate (312 mg; 2·03 mmol), the tripeptide ester hydrobromide (996 mg; 1·85 mmol; prepared from Z-L-Lys(Tos)-D-Ala-D-Ala-OMe (ref.[30]) by treatment with HBr/AcOH) and N-methylmorpholine (0·207 ml; 1·85 mmol) in dimethylformamide (20 ml) was mixed with a solution of dicyclohexylcarbodiimide (420 mg; 2·03 mmol) in dimethylformamide (3 ml) under cooling with ice. After standing at 0°C for 2 h and at room temperature for 16 h, the solution was concentrated *in vacuo* and the product was precipitated with water and isolated as neutral compound: yield 700 mg (48%), m.p. 220—226°C; after repeated crystallization from aqueous dimethylformamide m.p. 236—239°C. The product was chromatographically homogeneous in systems B and D. $[\alpha]_D^{23}$ −12·8° (*c* 0·5; dimethylformamide). For $C_{36}H_{51}N_7O_{11}S$ (789·9) calculated: 54·74% C, 6·51% H, 12·41% N, 4·06% S; found: 54·64% C, 6·30% H, 12·66% N, 3·92% S.

H-L-Ala-D-iGln-L-Lys-D-Ala-D-Ala-OMe (*IV*)

The title compound was prepared by reduction of the protected compound *III* (300 mg; 0·38 mmol) with sodium in liquid ammonia. After evaporation of ammonia the residue was dissolved in 1% acetic acid (200 ml), the peptide was bound on a column of Ostion KM (Spolek pro chemickou a hutní výrobu, Ústí n. L., Czechoslovakia) and eluted with 50% acetic acid. After dilution with water the eluate was freeze-dried. The product was chromatographically not homogeneous (system C) and therefore was further separated on a column of Sephadex G-10 (30 × 1·5 cm; 40—120 μm). Elution with water afforded 131 mg of the pure product as lyophilizate. For $C_{21}H_{39}N_7O_7$ (501·6) calculated: 19·55% N; for undried sample found: 15·05% N. Peptide content in the lyophilizate 77%, yield 53%. $[\alpha]_D^{23}$ +15·1° (*c* 0·4; water). Amino acid analysis: Ala 3·06, Glu 1·10, Lys 0·85. For $C_{21}H_{39}N_7O_7.2$ AcOH (621·7) calculated: 48·30% C, 7·62% H, 15·77% N; found: 48·44% C, 7·71% H, 16·04% N.

H-L-Ala-D-iGln-L-Lys-D-Ala-L-Ala-L-Ala-OH (*V*)

Boc-L-Ala-O-ℛ (4·94 g; 4 mmol) was converted into the resin-bound peptide (6·7 g; 88%) using method *B*. The peptide was cleaved off with hydrogen fluoride in the presence of anisole, analogously as described for *II*. The free peptide was washed out from the resin with 20% acetic acid (4 × 60 ml) and the eluate was freeze-dried. The residue was dissolved in water, the solution adjusted to pH 9 with 1 mol l^{-1} sodium hydroxide and immediately to pH 3·9 with acetic acid. The desalting was carried out on an Amberlite IRC 50 XE 64 column (240 ml). The impurities were removed by washing with 0·25% acetic acid (1 000 ml) and the peptide was eluted with 50% acetic acid (720 ml). Freeze-drying gave 2 040 mg (60·7%) of the product. For $C_{23}H_{42}N_8O_8$ (558·6) calculated: 20·06% N; for undried lyophilizate found: 13·33% N, peptide content in

lyophilizate 66·5%. HPLC purity 96% (220 nm). $[\alpha]_D^{22}$ −24·5° (*c* 0·5; 1 mol l^{-1} acetic acid), −16·5° (*c* 1·2; water). Amino acid analysis: Ala 3·9, Glu 0·99, Lys 0·98, NH_3 1·03. For $C_{23}H_{42}$·.N_8O_8.3 AcOH (738·8) calculated: 47·15% C, 7·37% H, 15·17% N. For dried compound found: 46·82% C, 7·23% H, 15·28% N. IR spectrum (KBr pellet) (ν, cm^{-1}): COOH 1 716, $CONH_2$ 1 697, —CONH— 1 635, 1 560, 1 549. ^{1}H NMR spectrum (δ, ppm): 1·24 d, 6 H; 1·28 d, 3 H; 1·41 d, 3 H (4 × CH_3 (Ala), J = 7 Hz); 1·50—2·05 m, 8 H ($C^\beta H_2$, $C^\gamma H_2$, $C^\delta H_2$ (Lys), $C^\beta H_2$ (iGln)); 2·28 bt, 2 H ($C^\gamma H_2$(iGln), J = 7 Hz); 2·86 bt, 2 H ($C^\varepsilon H_2$(Lys), J = 7 Hz); 4·01 q, 1 H; 4·18 m, 5 H (6 × C^αH). ^{13}C NMR spectrum (δ, ppm): 16·8 q (3 C), 16·6 q (4 × CH_3(Ala)); 22·2 t (C^γ(Lys)); 26·5 t (C^β(Lys)); 27·1 t (C^β(iGln)); 30·5 t (C^δ(Lys)); 31·4 t (C^γ(iGln)); 39·2 t (C^ε(Lys)); 49·3 d, 49·5 d, 49·7 d, 49·9 d (4 × C^α(Ala)); 53·1 d (C^α(iGln)); 54·0 d (C^α(Lys)); 171·0 s, 174·1 s, 174·2 s, 174·6 s, 174·9 s, 175·6 s, 176·9 s (6 × C=O).

Z-L-Lys(Tos)-D-Ala-L-Ala-L-Ala-OMe (*VI*)

Hydrazide of Z-L-Lys(Tos)-D-Ala-OH (ref.[30]; 5·2 g; 10 mmol) was dissolved in dimethylformamide (60 ml) and conc. hydrochloric acid (3 ml) was added. After cooling to −10°C, a solution of potassium nitrite (1·55 g) in water (2 ml) was added and the temperature was kept at −10°C for 5 min. The mixture was adjusted to pH 6—7 with N-methylmorpholine and a solution of the amino ester (1·74 g; 10 mmol; prepared from Z-L-Ala-L-Ala-OMe (ref.[31]) by reaction with hydrogen bromide in acetic acid and deionization on Ostion AT) in dimethylformamide (20 ml) was added. The mixture was cooled for 1 h at −10°C and then set aside in a refrigerator for 20 h. The product was precipitated with water and worked up as neutral compound; yield 6·05 g (91·5%), m.p. 181—184°C, after recrystallization from ethanol–ethyl acetate–n-hexane, m.p. 187—189°C; homogeneous in systems A and D. $[\alpha]_D^{25}$ −16·3° (*c* 1·0; methanol). For $C_{31}H_{43}$·.N_5O_9S (661·8) calculated: 56·26% C, 6·55% H, 10·58% N, 4·85% S; found: 56·07% C, 6·80% H, 10·36% N, 4·99% S.

Z-L-Ala-D-iGln-L-Lys(Tos)-D-Ala-L-Ala-L-Ala-OMe (*VII*)

A solution of the tetrapeptide ester hydrobromide (3·65 g; 6 mmol; prepared from compound *VI* with hydrogen bromide in acetic acid) and N-methylmorpholine (0·67 ml; 6 mmol) in dimethylformamide (10 ml) was added to Z-L-Ala-D-iGln (ref.[11]; 2·1 g; 6 mmol) and N-hydroxybenzotriazole hydrate (0·92 g; 6 mmol) in dimethylformamide (10 ml). Dicyclohexylcarbodiimide (1·24 g; 6 mmol) in dimethylformamide (5 ml) was added under cooling with ice. After 1 h at 0°C the mixture was stirred at room temperature for 20 h, concentrated and the product was precipitated with water and processed as neutral compound; m.p. 150—165°C. Crystallization from aqueous methanol and acetic acid and precipitation with ether afforded 2·9 g (56%) of the product, m.p. 165—167°C, chromatographically homogeneous (system F); $[\alpha]_D^{24}$ −6·2° (*c* 1·05; dimethylformamide). Amino acid analysis: Ala 4·16, Glu 0·91, Lys 0·94. For $C_{39}H_{56}N_8O_{12}S$ (861·0) calculated: 54·41% C, 6·56% H, 13·01% N, 3·72% S; found: 54·24% C, 6·80% H, 12·83% N, 3·95% S.

H-L-Ala-D-iGln-L-Lys-D-Ala-L-Ala-L-Ala-OMe (*VIII*)

a) The protected hexapeptide *VII* (430 mg; 0·5 mmol) was reduced with sodium in liquid ammonia. Ammonia was evaporated, the residue was dissolved in 1% acetic acid (100 ml), the solution was extracted with ether and the peptide sorbed on a column of Ostion KM. After elution from the column, the solution of the peptide was evaporated *in vacuo* and the residue was dissolved in 0·2% acetic acid and applied on a column of Sephadex G 10 (90 × 1·6 cm). Elution with 0·2% acetic acid gave 140 mg of the product which was electrophoretically (at pH 5·7) and

chromatographically (system E) homogeneous. For $C_{24}H_{44}N_8O_8$ (572·7) calculated: 19·57% N; for undried sample found: 15·32% N. Peptide content in lyophilizate 78%, yield of the peptide 38%. $[\alpha]_D^{25}$ −30·5° (*c* 0·3; water). Amino acid analysis: Ala 4·11, Glu 1·01, Lys 0·87. For $C_{24}H_{44}$·N_8O_8.2 AcOH.H_2O (710·8) calculated: 47·32% C, 7·66% H, 15·76% N; found: 47·66% C, 7·47% H, 15·57% N.

b) The hexapeptide acid *V* (840 mg; 1 mmol; peptide content in the sample 66·5%) was dissolved in 0·1M hydrogen chloride in absolute methanol. After standing at room temperature for 24 h, the mixture was concentrated *in vacuo* and the evaporation was repeated after addition of methanol (2 × 50 ml) and water (2 × 50 ml). The residue was dissolved in water and freeze-dried; yield 820 mg. The compound was purified by preparative HPLC in the system I (gradient 0—25% methanol). The product-containing fractions were concentrated *in vacuo*, dissolved in water, adjusted to pH 9 with 1 mol l^{-1} sodium hydroxide and immediately to pH 3·9 with acetic acid. Desalting on Amberlite IRC 50 XE 64 (analogously as described for the hexapeptide acid *V*) followed by freeze-drying afforded 410 mg (52%) of the product. For $C_{24}H_{44}N_8O_8$(572·7) calculated: 19·57% N; for undried lyophilizate found: 14·36% N; peptide content in lyophilizate 73%. The compound was homogeneous according to HPLC and electrophoresis at pH 5·7 or pH 2·5. $[\alpha]_D^{22}$ −28° (*c* 0·5; water). For $C_{24}H_{44}N_8O_8$.2 AcOH.H_2O (710·8) calculated: 47·32% C, 7·66% H, 15·76% N; for dried substance found: 47·23% C, 7·38% H, 15·32% N. IR spectrum (KBr pellet) (ν, cm^{-1}): $COOCH_3$ 1 749, $CONH_2$ 1 697, —CONH— 1 660, 1 555. ^1H NMR spectrum (δ, ppm): 1·21 d, 6 H; 1·30 d, 3 H; 1·40 d, 3 H (4 × CH_3(Ala), J = 7 Hz); 1·58 m, 6 H ($C^\beta H_2$, $C^\gamma H_2$, $C^\delta H_2$(Lys)); 1·77 m, 1 H; 1·98 m, 1 H ($C^\beta H_2$(iGln)); 2·20 m, 2 H ($C^\gamma H_2$ (iGln)); 2·73 m, 2 H ($C^\varepsilon H_2$(Lys)); 3·61 s, 3 H (COOMe); 3·93 m, 1 H; 4·23 m, 5 H (6 × $C^\alpha H$).

Boc-L-Ala-D-iGln-L-Lys(Ac)-D-Ala-L-Ala-L-Ala-OMe (*IX*)

Boc-L-Ala-O-ℛ (3·0 g, 3·12 mmol) was converted into the resin-bound peptide (4·27 g; 72%) by method *A*. The obtained resin-bound peptide (2·13 g) was reesterified with methanol (100 ml), containing 1% of triethylamine, for 24 h. Extraction with hot dimethylformamide and crystallization from aqueous methanol gave 695 mg (62%) of the product, m.p. 220—224°C, homogeneous in systems A and G; $[\alpha]_D^{22}$ −6·7° (*c* 0·4; dimethylformamide). Amino acid analysis: Ala 4·15, Glu 1·03, Lys 0·86. For $C_{31}H_{54}N_8O_{11}$ (714·8) calculated: 52·09% C, 7·61% H, 15·68% N; found: 51·87% C, 7·43% H, 15·53% N.

H-L-Ala-D-iGln-L-Lys(Ac)-D-Ala-L-Ala-L-Ala-OMe (*X*)

Compound *IX* (367 mg; 0·5 mmol) was dissolved in a mixture of trifluoroacetic acid (8 ml) and dichloromethane (2 ml). After standing at room temperature for 30 min, the solution was evaporated *in vacuo* and the residue dissolved in isopropyl alcohol (3 ml) and precipitated with ether. The precipitate was dissolved in water (2 ml) and the trifluoroacetate ions were removed on Ostion AT (60 ml) which was then washed with water. After concentration, the product was purified on a column of Sephadex G 10 (98 × 1·5 cm) in 0·1 mol l^{-1} acetic acid; yield 247 mg (66%) of lyophilizate. For $C_{26}H_{46}N_8O_9$ (614·7) calculated: 18·23% N; for undried sample found: 14·95% N; peptide content in lyophilizate 82%. Chromatographically homogeneous in systems A and E; $[\alpha]_D^{25}$ −40·6° (*c* 0·2; water). Amino acid analysis: Ala 3·89, Glu 1·05, Lys 1·05. For $C_{26}H_{46}N_8O_9$.1 1/2 AcOH (704·8) calculated: 49·42% C, 7·44% H, 15·90% N; found: 49·20% C, 7·69% H, 15·73% N.

Boc-L-Ala-D-iGln-L-Lys(Ac-L-Ala-L-Ala)-D-Ala-OMe (*XI*)

Boc-D-Ala-O-ℛ (2·03 g; 2·0 mmol) was converted into the resin-bound peptide (2·83 g; 78%) by method *A*. The obtained material (1·40 g) was reesterified by stirring for 24 h in methanol (80 ml)

containing 1% of triethylamine. The solvent was evaporated and the peptide extracted with boiling dimethylformamide. The extract was evaporated and the dry residue crystallized from methanol–ether; yield 430 mg (60%), m.p. 232–235°C. The product was homogeneous in the system F; $[\alpha]_D^{25}$ −10·8° (*c* 0·2; dimethylformamide). For $C_{31}H_{54}N_8O_{11}$ (714·8) calculated: 52·09% C, 7·61% H, 15·68% N; found: 51·97% C, 7·83% H, 15·42% N.

H-L-Ala-D-iGln-L-Lys(Ac-L-Ala-L-Ala)-D-Ala-OMe (*XII*)

Compound *XI* (143 mg; 0·2 mmol) was converted into the title derivative as described for the preparation of *X*; yield 105 mg (71%). For $C_{26}H_{46}N_8O_9$ (614·7) calculated: 18·23% N; for undried sample found: 15·21% N; peptide content in lyophilizate 83%. The product was homogeneous in the systems A and E; $[\alpha]_D^{25}$ −26·5° (*c* 0·2; water). Amino acid analysis: Ala 4·10, Glu 0·95, Lys 0·93. For $C_{25}H_{46}N_8O_9.AcOH.2\,H_2O$ (710·8) calculated: 47·32% C, 7·66% H, 15·76% N; found: 47·50% C, 7·58% H, 15·81% N.

H-L-Ala-D-iGln-$(Gly)_5$-NH_2 (*XIII*)

The synthesis was carried out on a resin containing 1% of divinylbenzene. Boc-Gly-O-ℛ (4 g; 2 mmol) was converted into the resin-bound protected heptapeptide (4·84 g; 98·2%) by the method *B*. This material (3 g; theory 1·24 mmol) was ammonolyzed with methanolic ammonia (200 ml; saturated at 0°C). After stirring at room temperature for 24 h, the mixture was concentrated *in vacuo* and the product was washed out successively with methanol (2 × 50 ml), hot methanol (4 × 50 ml), hot 80% methanol (4 × 50 ml) and hot dimethylformamide (3 × × 50 ml). The combined filtrates were concentrated *in vacuo* and the product was precipitated with dry ether; yield 0·44 g (59%) of Boc-L-Ala-D-iGln-$(Gly)_5$-NH_2.

The Boc group was removed by treatment with 55% trifluoroacetic acid in dichloromethane for 30 min. Evaporation *in vacuo* and precipitation with ether gave 0·44 g (98%) of the heptapeptide amide trifluoroacetate which was purified by preparative continuous free-flow electrophoresis at 2 600 V and 0°C; carrier buffer 1 mol l^{-1} acetic acid, electrode buffer 1·5 mol l^{-1} acetic acid. Freeze-drying yielded 132 mg (15·5%) of product. For $C_{18}H_{31}N_9O_8$ (501·5) calculated: 25·14% N; for undried lyophilizate found: 18·40% N; peptide content in lyophilizate 73%. The compound was chromatographically (system A) as well as electrophoretically (at pH 2·5) homogeneous; HPLC purity 95% (220 nm); $[\alpha]_D^{22}$ −15·1° (*c* 0·2; water). Amino acid analysis: Ala 1·00, Glu 1·01, Gly 5·35. For $C_{18}H_{31}N_9O_8.3\,AcOH$ (690·7) calculated: 41·74% C, 6·28% H, 18·25% N; for undried sample found: 41·70% C, 6·00% H, 18·40% N.

H-L-Ala-D-iGln-L-Lys(H-$(Gly)_5$)-D-Ala-NH_2 (*XIV*)

The synthesis was carried out from Tos-L-Ala-D-iGln-L-Lys(Z)-D-Ala-O-ℛ (6·3 g; theory 2·6 mmol; described in the preparation of *I*). Boc-Gly-OH was condensed using the method *B*. After the synthesis, the Boc group was removed and the mixture neutralized; yield 6·2 g of the resin-bound tosyl nonapeptide. The thus-obtained product (2·7 g; theory 1·13 mmol) was ammonolyzed with methanolic ammonia (150 ml; saturated at 0°C). After stirring at room temperature for 36 h, ammonia was evaporated and the product extracted with hot methanol (3 × 100 ml) and hot dimethylformamide (3 × 50 ml). The combined extracts were concentrated *in vacuo* and the tosyl nonapeptide was precipitated with ether; yield 0·5 g (52%). The compound was reduced with sodium in liquid ammonia (400 ml) for 60 s and ammonia was evaporated. The residue was dissolved in 5% acetic acid (50 ml), adjusted to pH 4, desalted on Amberlite IRC 50 XE 64 (50 ml) and freeze-dried to afford 432 mg (43%) of the product. For $C_{27}H_{48}N_{12}O_{10}$ (700·8) calculated: 23·98% N; for undried lyophilizate found: 18·81% N; peptide content in

lyophilizate 78%. The preparative separation was performed in the system II (gradient 0—30% methanol). The product fraction was desalted on a column of Amberlite IRC 50 XE 64 (50 ml) and freeze-dried to afford 73 mg (7·2%) of compound *XIV*; $[\alpha]_D^{22}$ +14·8° (*c* 0·5; 1 mol l^{-1} acetic acid). Amino acid analysis: Ala 2·12, Glu 1·05, Lys 1·02, Gly 4·71, NH_3 2·14. For $C_{27}H_{48}N_{12}O_{10}$.2 AcOH.H_2O (838·9) calculated: 44·39% C, 6·97% H, 20·04% N; for dried compound found: 44·56% C, 6·79% H, 20·08% N.

H-L-Ala-D-iGln-L-Lys(H-(Gly)$_5$)-D-Ala-OH (*XV*)

Tos-L-Ala-D-iGln-L-Lys(H-(Gly)$_5$)-D-Ala-O-ℛ (3·3 g; theory 1·38 mmol; for preparation see *XIV*) was cleaved with a mixture of anisole (10 ml) and liquid hydrogen fluoride (60 ml) at 0°C for 45 min. Hydrogen fluoride was driven off by a stream of nitrogen and the resin with the peptide were washed with ethyl acetate (3 × 60 ml; 0°C, 5 min) and ether (2 × 50 ml). The peptide was extracted with hot dimethylformamide (3 × 50 ml) and hot 50% aqueous dimethylformamide (3 × 50 ml). After concentration *in vacuo*, the tosyl nonapeptide hydrofluoride was precipitated with dry ether; yield 0·6 g (50%). The compound was reduced for 1 min with sodium in liquid ammonia (500 ml), and ammonia was evaporated. The product was dissolved in 5% acetic acid (50 ml) and the solution adjusted to pH 4, desalted on an Amberlite IRC 50 XE64 column (50 ml) and freeze-dried to give 447 mg (47%; from the tosyl nonapeptide 93%) of the product which was purified by preparative HPLC in the system II (gradient 0—30% methanol). The product-containing fractions were desalted and freeze-dried; yield 152 mg (12·5%) of *XV*. For $C_{27}H_{47}N_{11}O_{11}$ (701·7) calculated: 21·96% N; for undried sample found: 17·59% N; peptide content in the lyophilizate 80%. $[\alpha]_D^{22}$ +5·0° (*c* 0·4; 1 mol l^{-1} acetic acid). Amino acid analysis: Ala 2·00, Glu 1·05, Lys 0·99, Gly 4·99, NH_3 0·90. For $C_{27}H_{47}N_{11}O_{11}$.2 AcOH (821·8) calculated: 45·30% C, 6·75% H, 18·75% N; for dried substance found: 45·21% C, 6·62% H, 18·73% N.

Protected Branched Decapeptide *XVI*

Boc-D-Ala-O-ℛ (3·96 g; 2·46 mmol) was converted to the resin-bound peptide (6·48 g; 88%) using method *A*. Reesterification and crystallization from 50% aqueous methanol afforded 1·9 g (57%) of the product, m.p. 220—225°C, after reprecipitation from dimethylformamide–water m.p. 227—229°C. The compound was chromatographically homogeneous in the system A; $[\alpha]_D^{24}$ −26·2° (*c* 0·3; dimethylformamide). Amino acid analysis: Ala 6·11, Glu 2·06, Lys 1·83. For $C_{57}H_{88}N_{14}O_{18}S_2.H_2O$ (1 339·6) calculated: 51·11% C, 6·77% H, 14·64% N, 4·79% S; found: 51·32% C, 6·89% H, 14·39% N, 4·83% S.

Branched Decapeptide *XVII*

The protected decapeptide *XVI* (250 mg; 0·19 mmol) was reduced with sodium in liquid ammonia. After evaporation of ammonia, the residue was dissolved in 1% acetic acid (100 ml), the solution was washed with ether and the peptide from the aqueous phase was desalted on an Ostion KM column. The product was purified in an acetate buffer by continuous free-flow electrophoresis. Freeze-drying gave 139 mg of the peptide, homogeneous in the systems A and C. For $C_{43}H_{76}N_{14}O_{14}$ (1 013·2) calculated: 19·35% N; for undried sample found: 14·75% N; yield 55%. $[\alpha]_D^{23}$ −42·4° (*c* 0·2; water). Amino acid analysis: Ala 5·89, Glu 1·97, Lys 2·10. For $C_{43}H_{76}N_{14}O_{14}$.3 AcOH.2 H_2O (1 229·4) calculated: 47·87% C, 7·54% H, 15·95% N; found: 47·62% C, 7·79% H, 15·82% N.

Protected Branched Tridecapeptide *XVIII*

Resin-bound Boc-D-Ala (3·13 g; 2 mmol; Calbiochem, 2% of divinylbenzene) was converted to the resin-bound protected tridecapeptide (4·87 g; 64%) which was reesterified (3·6 g) by treat-

ment with absolute methanol (200 ml) containing triethylamine (2 ml) at room temperature for 24 h. The mixture was evaporated to dryness and the product was taken up in hot dimethylformamide (3 ×). The extracts were concentrated, the product was precipitated with ether, filtered and dried; yield 1·15 g (50%) of compound melting at 232–244°C. On crystallization from aqueous acetic acid the m.p. increased to 245–247°C. The compound was chromatographically homogeneous in the systems A and D; $[\alpha]_D^{20}$ −15·5° (*c* 0·5; dimethylformamide). Amino acid analysis: Ala 3·95, Glu 2·02, Gly 5·14, Lys 1·89. For $C_{67}H_{97}N_{17}O_{22}S_2$.AcOH (1 616·8) calculated: 51·26% C, 6·30% H, 14·73% N, 3·97% S; found: 51·01% C, 6·47% H, 14·54% N, 3·69% S.

Branched Tridecapeptide *XIX*

The protected tridecapeptide *XVIII* (376 mg; 0·23 mmol) was reduced with sodium in liquid ammonia, and the mixture was processed and desalted as described in the preparation of *IV*. Freeze-drying afforded 209 mg (67%) of the product which was purified by HPLC on a 300 × × 25 mm column packed with Separon SiC_{18} (10 μm), mobile phase methanol – 0·2% aqueous trifluoroacetic acid (12:88); detection at 230 nm, flow rate 6 ml/min. The product (retention time t_R = 52 min) was desalted on a column of Ostion KM and freeze-dried; yield 148 mg. For $C_{45}H_{79}N_{17}O_{16}$ (1 114·2) calculated: 21·37% N; for undried sample found: 17·13% N; yield of lyophilizate 46%. $[\alpha]_D^{25}$ +15·3° (*c* 0·3; water). Amino acid analysis: Ala 3·92, Glu 2·0, Gly 5·1, Lys 1·98. For $C_{45}H_{79}N_{17}O_{16}$.3 AcOH.2 H_2O (1 330·4) calculated: 46·04% C, 7·20% H, 17·90% N; found: 45·73% C, 7·42% H, 17·68% N.

Protected Branched Octadecapeptide *XX*

Boc-L-Ala-O-ℛ (8·9 g; 2 mmol) was converted to the resin-bound protected octadecapeptide (12·47 g; 89%) using the method *A*. The obtained material (6·23 g) was reesterified in methanol with 1% triethylamine and extracted with hot dimethylformamide. Precipitation with ether and three crystallizations from a water–methanol–ether mixture gave 1·45 g (73%) of the product, m.p. 291–295°C, homogeneous in the systems A and G; $[\alpha]_D^{25}$ −19·3° (*c* 0·2; dimethylformamide). Amino acid analysis: Ala 11·81, Glu 2·94, Lys 3·27. For $C_{87}H_{150}N_{24}O_{29}.H_2O$ (2 014·3) calculated: 51·88% C, 7·61% H, 16·69% N; found: 51·65% C, 7·67% H, 16·48% N.

Branched Octadecapeptide *XXI*

The protected compound *XX* (232 mg; 0·11 mmol) was dissolved in 80% trifluoroacetic acid (10 ml) in dichloromethane. After standing at room temperature for 30 min, the solution was concentrated and the residue precipitated from isopropyl alcohol–ether. The obtained octadecapeptide trifluoroacetate (198 mg) was purified by continual free-flow electrophoresis in an acetate buffer. The product-containing fractions were combined and freeze-dried, yield 44 mg. For $C_{72}H_{126}N_{24}O_{23}$ (1 696) calculated: 19·82% N; in undried product found: 17·21% N; yield 20%. The compound was electrophoretically homogeneous (at pH 2·5 and 5·7). $[\alpha]_D^{26}$ −32·7° (*c* 0·5; water). Amino acid analysis: Ala 11·85, Glu 2·82, Lys 3·3. For $C_{72}H_{126}N_{24}O_{23}$.3 AcOH.2 H_2O (1 912) calculated: 49·00% C, 7·49% H, 17·58% N; found: 48·75% C, 7·32% H, 17·34% N.

BBM-L-Ala-D-iGln-L-Lys(Tos)-D-Ala-(L-Ala)$_2$-OMe (*XXII*)

N-Hydroxysuccinimide ester of the protected muramic acid was prepared from 1-α-O-benzyl--4,6-O-benzylidene-N-acetylmuramic acid[11] (943 mg; 2 mmol), N-hydroxysuccinimide (290 mg; 2·5 mmol) and dicyclohexylcarbodiimide (410 mg; 2 mmol) in tetrahydrofuran (20 ml) according

to ref.[32]. Dicyclohexylurea was filtered off and a solution of the hexapeptide amino ester (1·45 g; 2 mmol) in dimethylformamide (prepared from compound *VII*) was added. After 20 h the product was isolated as neutral compound, m.p. 191–198°C, yield 1·55 g (73%). The m.p. increased to 204–207°C upon two precipitations from dimethylformamide–water; yield 1·10 g (52%) of compound, homogeneous in systems A and D; $[\alpha]_D^{23}$ +61·4° (*c* 2·0; dimethylformamide). Amino acid analysis: Ala 4·19, Glu 0·90, Lys 0·91, Mur not determined. For $C_{56}H_{77}N_9O_{17}S$ (1 180·4) calculated: 56·98% C, 6·58% H, 10·68% N, 2·72% S; found: 57·23% C, 6·81% H, 10·83% N, 2·56% S.

N-Ac-Mur-L-Ala-D-iGln-L-Lys-D-Ala-(L-Ala)$_2$-OMe (*XXIII*)

Compound *XXII* (300 mg; 0·25 mmol) was reduced with sodium in liquid ammonia. The residue after evaporation of ammonia was dissolved in 2% acetic acid (100 ml), the solution was adjusted to pH 4 and the product was desalted on a column of Sephadex G 10 (98 × 1·5 cm). Elution with 0·2% acetic acid afforded 42 mg (18%) of *XXIII*, chromatographically homogeneous in systems A and D; $[\alpha]_D^{25}$ +20·1° (*c* 0·3; water). Amino acid analysis: Mur 1·11, Ala 3·96, Glu 1·03, Lys 0·90. For $C_{35}H_{61}N_9O_{15}$.AcOH.H_2O (926·0) calculated: 47·99% C, 7·29% H, 13·61%N; found: 47·68% C, 7·55% H, 13·87% N.

BBM-L-Ala-D-iGln-L-Lys(Ac-(L-Ala)$_2$)-D-Ala-OMe (*XXIV*)

Condensation of resin-bound compound *XI* (1·40 g; theory 1 mmol) with protected muramic acid afforded 1·69 g (82%) of the resin-bound product which was reesterified by treatment with methanol, containig 1% of triethylamine, for 48 h.The methanolic solution was filtered and the product was extracted with hot dimethylformamide (3 × 50 ml) and concentrated. The amorphous product (950 mg) was precipitated from 90% aqueous dimethylformamide–ether. Yield 550 mg (51%) of compound, chromatographically homogeneous in systems A and D, m.p. 267–270°C; $[\alpha]_D^{22}$ +54·8° (*c* 0·2; dimethylformamide). Amino acid analysis: Mur 1·19, Ala 3·86, Glu 1·06, Lys 0·95. For $C_{51}H_{73}N_9O_{16}$ (1 068·2) calculated: 57·35% C, 6·89% H, 11·80% N; found: 57·58% C, 7·01% H, 11·66% N.

N-Ac-Mur-L-Ala-D-iGln-L-Lys(Ac-(L-Ala)$_2$)-D-Ala-OMe (*XXV*)

Compound *XXIV* (213 mg; 0·2 mmol) was reduced with sodium in liquid ammonia and ammonia was evaporated. The residue was dissolved in water (100 ml) and stirred with Dowex 50 WX 4 (60 ml) for 1 h. The mixture was filtered, the resin was washed with water and the combined filtrates were freeze-dried. The residue was dissolved in 0·5 mol l^{-1} acetic acid (2 ml) and the solution was purified on a column of Sephadex G 15 (100 × 1·5 cm), eluent 0·5 mol l^{-1} acetic acid. Freeze-drying afforded the pure product (86 mg), homogeneous in systems A and C. For $C_{37}H_{63}N_9O_{16}$ (890·0) calculated: 14·16% N; in undried product found: 13·58% N; yield 46%. $[\alpha]_D^{23}$ +28·4° (*c* 0·2; water). Amino acid analysis: Mur 1·06, Ala 3·98, Glu 1·04, Lys 0·97. For $C_{37}H_{63}N_9O_{16}$.1 1/2 H_2O (917·0) calculated: 48·46% C, 7·25% H, 13·74% N; found: 48·60% C, 7·16% H, 13·76% N.

Protected Branched Glycooctadecapeptide *XXVI*

Resin-bound compound *XX* (6·2 g; theory 1 mmol) was deblocked and condensed with protected muramic acid; weight increase 960 mg. The product was reesterified with methanol (containing 1% of triethylamine) for 24 h, the resin was filtered off and the methanolic extract was concentrated. Another portion of the product was obtained by extraction of the resin with boiling

dimethylformamide (3 × 50 ml). The solvents were evaporated, leaving 2·55 g (84%) of the crude product which was repeatedly precipitated from dimethylformamide and water. Yield 1·18 g (39%) of *XXVI*, chromatographically homogeneous in systems A and G, m.p. 298–302°C; $[\alpha]_D^{25}$ +62·1° (*c* 0·2; dimethylformamide). Amino acid analysis: Mur 2·85, Ala 11·9, Glu 2·89, Lys 3·05. For $C_{147}H_{207}N_{27}O_{44}.H_2O$ (3 074) calculated: 57·43% C, 6·85% H, 12·30% N; found: 57·17% C, 6·93% H, 12·36% N.

Branched Glycooctadecapeptide *XXVII*

The protected compound *XXVI* (305 mg; 0·1 mmol) was reduced with sodium in liquid ammonia. After evaporation of ammonia the residue was dissolved in water (200 ml) and the solution was added to Dowex 50 W X 4 (100 ml). The mixture was stirred for 1 h, the supernatant was decanted and the resin was washed with water. The combined filtrates were freeze-dried (220 mg). The residue was dissolved in 0·5 mol l^{-1} acetic acid (2 ml) and applied on a column of Sephadex G 25 (100 × 2·5 cm). Elution with 0·5M acetic acid and freeze-drying afforded 159 mg of compound, homogeneous in systems A and H. For $C_{105}H_{177}N_{27}O_{44}$ (2 522) calculated: 15·00% N; in undried product found: 14·12% N; yield 59%. $[\alpha]_D^{23}$ +17·1° (*c* 0·2; water) (24 h). Amino acid analysis: Mur 3·12, Ala 12·19, Glu 2·91, Lys 2·82. For $C_{105}H_{177}.N_{27}O_{44}.2\,AcOH.H_2O$ (2 660) calculated: 49·22% C, 7·09% H, 14·22% N; found: 48·98% C, 6·91% H, 14·38% N.

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