Synthesis of a Proposed Antigenic Hexapeptide from *Escherichia Coli K88* Protein Fimbriae

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The hexapeptide Boc-Asp-Asp-Tyr-Arg-Gln-Lys-OMe is assembled by stepwise synthesis in solution with an overall yield of 44 %. N_α-boc-amino acids, protected with benzyl or benzyloxycarbonyl groups in the side-chains, are coupled as active estes of 1-hydroxybenzotriazole in mixtures of dichloromethane and *N,N*-dimethylformamide. N_α-deprotection is accomplished with trifluoroacetic acid. Finally, hydrogenation with palladium on charcoal and ammonium formate produces the pure hexapeptide.
A new one-pot synthesis of Boc-Arg(Z_2) is described, and the use of this derivative in peptide coupling is studied. The synthetic peptide was coupled to BSA and used in direct immunication of rabbits.

Introduction

The fimbriae of *Escherichia coli K88,* by which the bacterium adheres to mannose-containing, porcine, small intestinal epithelium, are polymers of more than 100 subunit proteins, held together by non-covalent binding. The monomeric proteins have been sequenced by Klemm *et al.*[1,2,3] and found to consist of 264 amino acid residues. The thread-like fimbriae with a diameter of 2.1 nm exist as three variants, K88 ab, K88 ac and K88 ad. As to the indexes, "a" designates at least one common antigenic determinant, whereas "b", "c" and "d" refer to populations of subtype specific determinants arising from deletions or substitutions in the amino acid sequences.

The immunochemistry of the fimbriae is of considerable practical interest, in view of the large number of piglets succumbing to *K88* infections. Hydrophilicity calculations[4] have been carried out by Klemm and coworkers[5] in an attempt to locate potential continuous antigens within the fimbrial proteins. The sequence most likely to be a continuous antigen according to these calculations was a hexapeptide covering the region 19–25, Asp-Asp-Tyr-Arg-Gln-Lys, common for the variants ab, ac, and ad.

We set out to prepare this hexapeptide by solution synthesis to investigate whether it was a determinant, but also to improve the synthesis of peptides containing Gln, Arg and Tyr. Our method involved a reinvestigation of the 1-hydroxybenzotriazole/dicyclohexylcarbodiimide coupling reaction,[6] and the use of N_α-Boc-amino acids, side-chain protected exclusively by benzyl (Bzl) and benzyloxycarbonyl (Z) groups.

Results and discussion

The synthetic approach to the hexapeptide, Asp-Asp-Tyr-Arg-Gln-Lys-OMe is outlined in Scheme 1. Direct reaction of the dicyclohexylamine (DCHA) salt of Boc-Gln-OH (*1*) with *p*-nitrophenyl trifluoroacetate,[7] afforded a 78 % yield of Boc-Gln-ONp (*2*), higher than that obtained by the traditional DCC/HONp method.[8]

The hydrochloride (*3*) derived from L-lysine, was condensed with *2* in the presence of 4-methylmorpholine (NMM) to give a 90 % yield of the dipeptide (*4*). The N_α-deprotection of *4* was attempted with TFA, but the product did not crystallize. However, the hygroscopic hydrochloride

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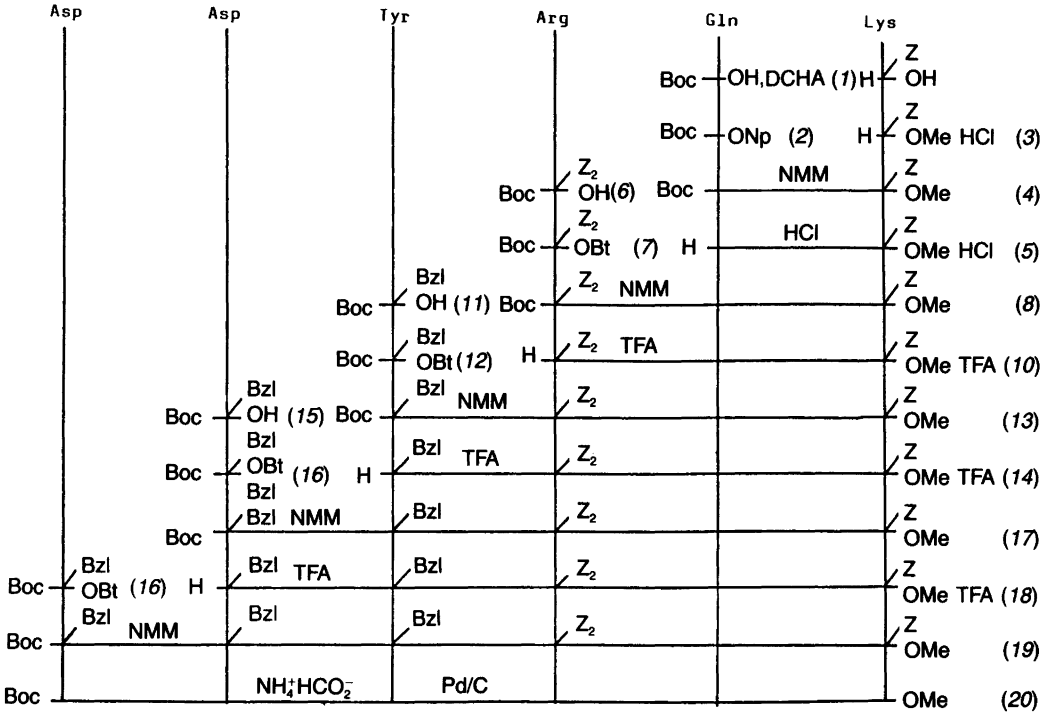

Scheme 1. The syntheses of the hexapeptide, *20*.

(*5*) was isolated in 81 % yield on treatment of *4* with an ethereal solution of hydrogen chloride. To introduce the arginine, a novel "one-pot" synthesis of Boc-Arg (Z_2)-OH (*6*) was developed. The cumbersome preparation of this compound,[9,10] has resulted in extensive use of the less favourable protection of arginine as Arg(NO_2) and Arg(Tos) derivatives in connection with benzyl based side-chain protection. The reaction of arginine with Boc_2O at pH = 9.7, followed by reaction with Z-Cl at pH > 14 gave the crystalline *6* in a much better yield (15 %) than reported[9,10] (3 %).

The acid (*6*) reacted with dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) under acidic conditions to give a mixture of two isomeric 1-hydroxybenzotriazole esters (*7*) as reported.[6] The esters were brought into reaction with *5* by slow addition of NMM to give 90 % of the tripeptide, *8*. When the syrupy trifluoroacetate was used rather than the hydrochloride *5*, compound *8* was isolated in 70 % yield. The reactions of *6* and *7* are slow due to sterical crowding, both in the activation and the condensation step. When the usual procedure of mixing the trifluoroacetate, compound *6*, and NMM, followed by addition of DCC, was applied, only 20 % of *8* could be isolated along with a 40 % yield of CF_3CO-Gln-Lys(Z)-OMe (*9*) and some diketopiperazine.

Deprotection of (*8*) with TFA gave a 99 % yield of (*10*) which was reacted with NMM and Boc-Tyr(Bzl)-OBt (*12*), prepared from *11* by the procedure described above, to give a 96 % yield of the tetrapeptide (*13*). This was converted quantitatively into the TFA salt (*14*), which on reaction with Boc-Asp(Bzl)-OBt (*16*), prepared from *15* by the conditions described above, and NMM, gave an 85 % yield of the pentapeptide (*17*).

Quantitative removal of the Boc group, followed by reaction with *16* and NMM, gave the hexapeptide (*19*) in 84 % yield. The less than quantitative yield of *17* and *19* is believed to arise from difficulties in removing residual dicyclohexylurea by recrystallization. Throughout the syn-

theses, the condensations were monitored by ^1H NMR spectroscopy at 500 MHz, a technique that was found superior to both elemental analysis and ^{13}C NMR spectroscopy for detecting the presence of minor impurities.

The protective groups of the side-chains of *19* were removed by reductive cleavage[11] to give the hexapeptide (*20*) in almost quantitative yield. Since the product after gel filtration was homogeneous according to amino acid analysis, HPLC and NMR spectroscopy, no further purification was deemed necessary. The peptide was linked to bovine serum albumin by reaction with glutaraldehyde,[12] and rabbits were immunized with the dialyzed conjugate. Antisera raised in 6 weeks against this conjugate was applied to enzyme-linked immunosorbent assay (ELISA) and was found to recognize fimbriae from both *E. Coli K88* ab and *E. Coli K88* ad, when antisera raised against bovine serum albumin was used as a negative control. These results will be described in further detail in a forthcoming publication.[13]

Experimental

Melting points are uncorrected. Optical rotations were measured on a Perkin Elmer 241 polarimeter. NMR spectra were obtained on Bruker WH 90, HX 270, HX 400, and HX 500 instruments. The ^1H NMR spectra were measured in $CDCl_3$ relative to TMS as internal reference. In D_2O solution acetone served as an internal reference (2.22 ppm), unless otherwise indicated. ^{13}C NMR spectra were measured in D_2O, with dioxane as an internal reference (67.4 ppm). Micro analyses and amino acid analyses were performed by Novo Microanalytical Laboratory. TLC was performed on silica gel-coated aluminum plates and amides were made visible by the *N,N,N′,N′*-tetramethyl-4,4′-diaminodiphenylmethane color yielding reaction.[14] The elution solvents were A: ethyl acetate, B: ethyl acetate/petroleum ether/acetic acid (75/12/1). N_α-Boc amino acids were prepared by reaction of amino acids with di-*t*-butyl pyrocarbonate at pH 9.5–10.0 maintained by a pH-stat, in mixtures of *t*-butanol and water. The Boc-amino acids were isolated as crystalline material upon acidification with potassium hydrogen sulfate, extraction with ethyl acetate, and evaporation of the solvent, either directly as the acids (Boc-Asp(Bzl)) or as the dicyclohexylammonium salts (Boc-Gln, Boc-Tyr(Bzl)) by crystallization from a suitable solvent.

Boc-Gln-ONp. (2) The DCHA-salt (*1*) (1.5 g, 3.5 mmol) of Boc-Gln was dissolved in anhydrous pyridine (3.5 ml) and the mixture was stirred at 20 °C for 5 min. *p*-Nitrophenyl trifluoroacetate (1.25 g, 5.3 mmol) was added and the mixture was stirred for 18 min. During this period the mixture crystalized. Water (2 ml) was added with stirring, and after 5 min the mixture was diluted to 25 ml with water. After 10 min of stirring the product was filtered and washed with water, dried *in vacuo* over phosphorus pentoxide, and recrystallized from butanone (15 ml) at 50 °C by addition of petroleum ether (18 ml) and cooling to 0 °C. The product was filtered and washed in a 3:5 mixture of butanone and petroleum ether (15 ml). Drying over phosphorus pentoxide gave 1.005 g (78 % yield) of *2*. $[\alpha]_D^{20}$ −37° (*c* 0.2, DMF); m.p. 148–150 °C (reported[7] $[\alpha]_D^{20}$ −35° (*c* 1, DMF); m.p. 154–155 °C); ^1H NMR: δ 4.49 (H^α); 5.40 ($N^\alpha H$); 1.88–2.53 (H^β,H^γ); 5.35 ($N^\delta H$); 5.70 ($N^\delta H$); 7.25 (H2−Np); 8.21 (H3−Np). $J_N\alpha_{HH}\alpha$ 7.5 Hz; $J_H\alpha_H\beta$ 4.2 and 8.3; $J_{H2H3-Np}$ 9.0.

Boc-Arg(Z$_2$)-OH (6). Arginine (15 g, 85 mmol) was suspended in a mixture of water (22 ml) and *t*-butanol (45 ml) and stirred mechanically at 0 °C. pH was adjusted to 9.7 with solid carbon dioxide, and di-*t*-butyl pyrocarbonate (20.7 g, 95 mmol) was added dropwise at 0 °C over a period of 20 min, keeping the pH value at 9.7 by addition of sodium hydroxide solution (2M). The mixture was stirred for 1.5 h at 20 °C until the base consumption ceased. After extraction of the aqueous phase with petroleum ether (3×300 ml), dioxane (60 ml) was added and the mixture was cooled to −5 °C with mechanical stirring. Sodium hydroxide solution (33 %, 68 ml, 666 mmol) was added over a period of 2 min, and benzyl chloroformate (48 ml, 336 mmol) was added with vigorous stirring at such a rate that the temperature did not rise above 0 °C. The mixture was stirred at −5 °C until pH=9.0 and the product was then extracted with chloroform (4×400 ml). The extract was dried over sodium sulfate, filtered and evaporated *in vacuo* at 30 °C. The residual oil was dissolved in ethyl acetate (50 ml) with stirring and acidified to pH=1–2 (pH paper) with saturated potassium hydrogen sulfate. The aqueous

Table 1. ^{1}H NMR data of peptide sequences corresponding to region 19–24 of *E. Coli K88* fimbrial protein.

Compound							Coupling constants						
	4[a]	*8*[b]	*13*[b]	*17*[b]	*19*[b]	*20*[c]		*4*	*8*	*13*	*17*	*19*	*20*
Asp													
$N^{\alpha}H$						7.941							
H^{α}					4.245	4.122	$JH^{\alpha}H^{\beta}$					5.1	6.0
H^{β}					2.594	2.670						8.0	7.7
					2.732	2.715	$JH^{\beta}H^{\beta-}$					16.5	16.5
Asp													
$N^{\alpha}H$						8.270							
H^{α}				4.263	4.616	4.280	$JH^{\alpha}H^{\beta}$				4.9	5.2	6.4
H^{β}				2.518	2.585	2.600					9.0	8.8	8.0
				2.683	2.746	2.690	$JH^{\beta}H^{\beta\prime}$				16.1	16.5	16.5
Tyr													
$N^{\alpha}H$						8.222							
H^{α}			4.101	4.482	4.422	4.362	$JH^{\alpha}H^{\beta}$			3.8	4.0	4.0	6.0
H^{β}			2.652	2.794	2.724	3.003				10.0	8.4	8.9	7.5
			2.906	2.949	2.951	3.007	$JH^{\beta}H^{\beta\prime}$			14.5	13.9		14.0
H2,6			7.140	7.084	7.091	7.046	JH2H3,			8.5	8.5	8.3	8.5
H3,5			6.889	6.843	6.847	6.763	H5H6						
Arg													
$N^{\alpha}H$						8.141							
H^{α}		3.899	4.284	4.307	4.370	4.320	$JH^{\alpha}H^{\beta}$		8.5				4.8
H^{β}		1.574	1.563	1.567	1.569	1.724							9.5
		1.654	1.652	1.640	1.661	1.871	$JH^{\gamma}H^{\delta}$		7.0	6.1, 7.0	7.1, 7.1	6.9, 6.9	6.3, 6.3
H^{γ}		1.378	1.354	1.343	1.343	1.371				7.0, 7.0	7.1, 7.1	6.9, 6.9	6.3, 6.3
						1.459	$JH^{\delta}H^{\delta\prime}$			13.6	13.2	14.0	13.2
H^{δ}		3.874	3.837	3.822	3.800	3.085							
			3.900	3.896		3.135							
$N^{\delta}H$						7.138							
$N^{\epsilon}H$						7.189							
						7.263							
						7.360							
Gln													
$N^{\alpha}H$	5.610					8.035							
H^{α}	4.172	4.299	4.034	4.300	4.291	4.182	$JH^{\alpha}H^{\beta}$	5.0	5.5	5.5	5.6	5.6	5.6
H^{β}	1.943	1.736	1.746	1.759	1.748	1.931		6.8	7.5	8.5	8.1	8.1	9.2
	2.048	1.881	1.944	1.910	1.906	2.060	$JH^{\beta}H^{\beta\prime}$		14.0	15.0	13.9	13.5	14.4
H^{γ}	2.385	2.109	2.089	2.113	2.119	2.317	$JH^{\beta}H^{\gamma}$		7.5	8.0, 8.0	8.0, 8.1	7.2, 7.2	8.3, 8.3
			2.139						8.5	8.0, 7.0	8.0, 8.1	7.2, 7.2	8.3, 8.3

Compound	4[a]	8[b]	13[b]	17[b]	19[b]	20[c]	Coupling constants	4	8	13	17	19	20
$N^{\alpha}H$						6.763							
						6.892							
Lys													
$N^{\alpha}H$	7.726					7.853							
H^{α}	4.517	4.147	4.127	4.129	4.120	4.105	$JH^{\alpha}H^{\beta}$	5.5	5.6	5.6	5.4	6.0	4.9
H^{β}	1.756	1.465	1.503	1.498	1.507	1.656		5.5	8.5	8.4	8.7	8.8	9.8
		1.605	1.616	1.536	1.585	1.715	$JH^{\delta}H^{\varepsilon}$	6.4, 6.4	7.0, 7.0	7.1, 7.1	7.0, 7.0		7.2
H^{γ}	1.407	1.183	1.235	1.235	1.227	1.368							
H^{δ}	1.504	1.615	1.615	1.588–1.610	1.615	1.616							
H^{ε}	3.173	2.956	2.941	2.938	2.935	2.913							
$N^{\varepsilon}H$	5.296												

[a]$CDCl_3$ was used as a solvent. [b]DMSO-d_6 was used as a solvent. [c]D_2O-H_2O 1:10 was used as a solvent at pH 3.8.

phase was separated and washed twice with ethyl acetate (80 ml). The organic phases were combined and dried with sodium sulfate. Filtration and evaporation gave an oil (36 g), which was dissolved in dichloromethane (40 ml) and fractionated by flash chromatography on a silica gel column (kiselgel 60, Merck, 1000 g) (eluent: Solvent B). Fractions containing the major product (TLC:R_f=0.48 in the same solvent) were pooled and evaporated. The product was crystallized twice from a mixture of chloroform and petroleum ether giving 6.9 g of *6* (15 % yield) $[\alpha]_D^{20}$+13° (*c* 1, $CHCl_3$); m.p. 138–140 °C (reported [9]m.p. 141–142 °C. ^1H NMR: δ 4.30 (H^{α}); 5.34 ($N^{\alpha}H$); 1.71 (H^{β}, $2H^{\gamma}$); 1.82 (H^{β}); 4.05 ($2H^{\delta}$); 1.42 (Boc); 5.16 (Z); 5.26 (Z′); 7.35 (Z,Z′); 9.30 (CO_2H); 9.45 ($N^{\varepsilon}H$). Coupling patterns could not be resolved.

Boc-Gln-Lys(Z)-OMe (4). Boc-Gln-ONp (*2*) (1.00 g, 2.7 mmol) and Lys (Z)-OMe · HCL, (*3*)[15] (0.92 g, 2.7 mmol) were suspended in dichloromethane (20 ml). 1-Hydroxybenzotriazole (HOBt) (40 mg, 0.3 mmol) in *N,N*-dimethylformamide (DMF) (1.0 ml) and *N*-methyl morpholine (NMM) (306 µl, 2.7 mmol) were added. The mixture was stirred at 20 °C for 20 h and then diluted with dichloromethane (20 ml), and washed with water (20 ml), cold sodium carbonate solution (10 %, 2×15 ml), and with water. The organic solution was dried (magnesium sulfate), filtered and evaporated to give 1.45 g of product. This was dissolved in dichloromethane (10 ml) and made crystalline by addition of diethyl ether (10 ml). Filtration and washing with diethyl ether gave 1.30 g of *4* (90 % yield). Compound *4* gave only one spot on TLC (solvent A). $[\alpha]_D^{20}$−3.2 (*c* 0.7, $CHCl_3$); m.p. 133–135 °C; ^1H NMR data are given in Table 1. Anal. $C_{25}H_{38}N_4O_8$: C,H,N.

Boc-Arg(Z_2)-Gln-Lys(Z)-OMe (8). The compound *4* (427 mg, 0.81 mmol) was dissolved in lukewarm dichloromethane (6 ml) and the solution was added to a saturated solution of hydrogen chloride in diethyl ether (25 ml) with stirring. After 2 min the crystallisation began. The mixture was stirred for 10 min and the supernatant decanted. The crystals were washed three times with diethyl ether (10 ml) and dried over phosphorus pentoxide at 1 mm Hg for 2 h giving 300 mg (81 % yield) of the hydroscopic Gln-Lyz(Z)-OMe · HCl (*5*). Compound *6* (660 mg, 1.22

mmol) was dissolved in dichloromethane (5 ml), and HOBt (170 mg, 1.25 mmol), dissolved in DMF (0.5 ml), was added. The mixture was cooled to 0 °C, and dicyclohexyl carbodimide (DCC) (260 µl, 1.14 mmol) was added. The mixture was stirred for 2 h and 45 min at 0 °C. The solution of Boc-Arg(Z_2)-OBt (*7*) was filtered into a flask containing *5* giving 82 % of the theoretical amount of dicyclohexyl urea (DCU). The mixture was stirred at 0 °C for 1 h; during this period NMM (240 µl, 2.1 mmol) was added in 5 portions. The mixture was stirred at 20 °C for 3 h and evaporated. Dissolving the residual material in DMF and adding water resulted in the formation of a crystalline material, which was filtered off, dried over phosphorus pentoxide, and recrystallized twice from chloroform and diethyl ether to give 472 mg of *8* (90 % yield). $[\alpha]_D^{20}-7.5°$ (*c* 0.3, $CHCl_3$); m.p. 175–176 °C; ^{1}H NMR data (500 MHz in d_6-DMSO) are given in Table 1. Anal. $C_{47}H_{62}N_8O_{13}$: C,H,N.

Boc-Tyr(Bzl)-Arg(Z_2)-Gln-Lys(Z)-OMe (13). Compound *8* (350 mg, 0.37 mmol) was dissolved in trifluoroacetic acid (TFA) (3 ml) and left at 20 °C for 30 min. The TFA was removed on a rotatory evaporator. The residual oil was stirred and evaporated four times with diethyl ether, and the product was precipitated with diethyl ether (50 ml). Filtration, washing with diethyl ether and drying gave 350 mg (99 % yield) of the TFA-salt (*10*) m.p. 155–160 °C. Boc-Tyr(Bzl)-OH (*11*) (284 mg, 0.76 mmol) was dissolved in dichloromethane (5 ml), and HOBt (103 mg, 0.76 mmol) in DMF (0.5 ml) was added. The mixture was cooled to 0 °C and DCC (154 µl, 0.68 mmol) was added. The mixture was stirred for 1.5 h and the solution of *12* was filtered into a flask containing *10*. NMM (172 µl, 1.5 mmol), was added to the stirred solution at 0 °C, and the mixture was stirred at 0 °C for 1 h and at 20 °C for 2.5 h. The mixture was evaporated, and the residual oil dissolved in DMF (5 ml). The product precipitated on addition of water. Filtration, followed by drying over phosphorus pentoxide, gave 530 mg of material which was recrystallized from chloroform and diethyl ether to give 411 mg (96 % yield) of *13*. $[\alpha]_D^{20}-10.4°$ (*c* 0.6, $CHCl_3$); m.p. 143–146 °C; ^{1}H NMR data (500 MHz, d_6-DMSO) are given in Table 1.

Boc-Asp(Bzl)-Tyr(Bzl)-Arg(Z_2)-Gln-Lys(Z)-OMe (17). Compound (*13*) (350 mg, 0.29 mmol) was dissolved in TFA, and after standing 25 min at 20 °C the mixture was evaporated. Evaporation was repeated 4 times with addition of diethyl ether (5 ml), and the residual material was stirred with diethyl ether (10 ml) for 10 min. Filtering and drying over phosphorus pentoxide gave 354 mg (100 % yield) of the TFA salt, (*14*), m.p. 95–105 °C. Boc-Asp(Bzl)-OH (*15*) (86 mg, 0.27 mmol) was dissolved in dichloromethane (2 ml), and HOBt (36 mg, 0.27 mmol) in DMF (0.5 ml) was added. The mixture was cooled to 0 °C and DCC (58 µl, 0.255 mmol) was added. The mixture was stirred for 2 h at 0 °C, and the solution containing (*16*) was filtered into a flask, containing *14* (215 mg, 0.177 mmol). The filtrate was washed with dichloromethane (3 ml). NMM (50 µl, 0.45 mmol) was added, and the mixture was stirred for 1 h at 0 °C and for 2.5 h at 20 °C. The product was brought to crystallization by evaporation, addition of DMF (5 ml) and water (3 ml). The material was filtered and washed with water. The crystals were dissolved in DMF, and the solution was treated with charcoal. Filtration through Celite, washing with DMF (2 ml) and crystallization by addition of water (3 ml) gave, after filtration and drying, 250 mg of material, which was recrystallized from chloroform (3 ml) and diethyl ether (1 ml). The crystals were filtered off, washed with diethyl ether and dried to give 213 mg (85 % yield) of *17*. $[\alpha]_D^{20}-11.9°$ (*c* 0.6, $CHCl_3$); m.p. 148–150 °C; ^{1}H NMR data (500 MHz, d_6-DMSO) are given in Table 1. Anal. $C_{74}H_{88}N_{10}O_{18}$: C,H,N.

Boc-Asp(Bzl)-Asp(Bzl)-Tyr(Bzl)-Arg(Z_2)-Gln-Lys(Z)-OMe (19). Compound (*17*) (325 mg, 0.23 mmol) was dissolved in TFA (2.5 ml), and after 20 min the solvent was removed *in vacuo*. The residual oil was evaporated four times with diethyl ether (5 ml). The resulting crystals were washed three times with diethyl ether, and dried over phosphorus pentoxide to give 324 mg (99 % yield) of the trifluoroacetate salt (*18*), m.p. 95–105 °C. Boc-Asp(Bzl)-OH (*15*) (57 mg, 0.178 mmol) was dissolved in dichloromethane (2 ml), and a solution of HOBt (25 mg, 0.178 mmol) in DMF (0.2 ml) was added. The mixture was cooled to 0 °C, and DCC (38 µl, 0.167 mmol) was added. After 1.6 h the cold mixture was filtered into a flask containing *18* (140 mg, 0.96 mmol).

NMM (25 µl, 0.22 mmol) was added, and the mixture was stirred at 0 °C for 1 h and at 20 °C for 2 h. Dichloromethane was removed *in vacuo*, and after dilution with DMF (2 ml) and filtration the product was brought to crystallization by addition of water. The product was filtered, dried and recrystallized twice from chloroform and diethyl ether to give 135 mg (84 % yield) of *19*. $[\alpha]_D^{20}$ −14.7° (*c* 0.5, $CHCl_3$); m.p. 153–156 °C; ^1H NMR data (500 MHz, d_6-DMSO) are given in Table 1. Anal. $C_{85}H_{99}N_{11}O_{21}$: C,H,N.

Boc-Asp-Asp-Tyr-Arg-Gln-Lys-OMe (20). Compound *19* (100 mg, 0.061 mmol) was dissolved in methanol (5 ml), and palladium on charcoal (5 %, 100 mg) and ammonium formate (370 mg) were added, and the mixture was stirred for 1 h. After dilution with water (5 ml) the mixture was stirred for 5 min and filtered through Celite. Solvents were removed on a Rotovapor (10 mmHg, 25 °C) and the product was purified on a Sephadex G15 column by elution with methanol/water (1:1). Fractions containing the peptide were pooled and lyophilized, giving 57 mg (99 % yield) of *20*. Amino acid analysis: Asp 2.08; Glu 1.03; Tyr 0.92; Lys 1.02; Arg 0.96; NH_3 1.1. HPLC on 12 cm Nucleosil $5C_{18}$ (eluant, 2 ml/min, methanol-water 1:1) with detection at 280 n.m. gave only 1 peak positioned at 2.6 min. ^1H NMR data (500 MHz, H_2O-D_2O 10:1, pH 3.8) are given in Table 1.

^{13}C NMR (67.89 MHz, H_2O-D_2O 1:1, pH 7.0): 175.4 ppm (C1-1); 52.6 (C_α-1); 39.5 (C_β-1); 178.5 (C_γ-1); 174.8 (C1-2); 54.2 (C_α-2); 40.2 (C_β-2); 178.5 (C_γ-2); 174.2 (C1-3); 56.8 (C_α-3); 36.2 (C_β-3); 129.0 (C1′-3); 131.4 (C2, C6-3); 116.7 (C3, C5-3); 155.6 (C4-3); 174.4 (C1-4); 53.9 (C_α-4); 28.8 (C_β-4); 25.4 (C_γ-4); 41.6 (C_δ-4); 158.0 (C_ε-4); 174.5 (C1-5); 53.6 (C_α-5); 27.6 (C_β-5); 32.0 (C_γ-5); 178.7 (C_δ-5); 174.8 (C1-6); 54.7 (C_α-6); 30.8 (C_β-6); 23.0 (C_γ-6); 27.2 (C_δ-6); 40.4 (C_ε-6); 54.2 (OMe); 28.5 (Boc-CH_3); 82.1 (Boc-C_{tert}); 157.3 (Boc-CO).

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