Synthesis of a Putative Antigenic Heptapeptide from *Escherichia Coli K88 ab* Protein Fimbriae

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The heptapeptide Tyr-Arg-Glu-Asp-Met-Glu-Tyr-OMe, spanning region 213–219 of *Escherichia coli K88 ab* protein fimbriae, was synthesized with an overall yield of 37 % using dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) preactivation in all condensation reactions. The *C*-terminal was protected as the methyl ester. The protection scheme of N_{α}-tert-butyloxycarbonyl-(Boc) and benzyl-(Bzl) or benzyloxycarbonyl (Z) groups for side chain protection was found to be orthogonal when a mixture of trifluoroacetic acid (TFA), phenol (PhOH) and *p*-cresol (CrOH) was used for repetitive deprotection. The final deprotection of Boc-Tyr(Bzl)-Arg(Z_2)-Glu(Bzl)-Asp(Bzl)-Met-Glu(Bzl)-Tyr(Bzl)-OMe (*17*) was accomplished in 80 % yield by prolonged treatment with hydrogen fluoride, dimethyl sulfide, *p*-cresol and *p*-thiocresol. The BSA-linked synthetic peptide was used in immunisation experiments on rabbits.

The protein subunits of *Escherichia coli K88 ab, ac* and *ad* fimbriae have recently been sequenced by Klemm and colleagues,[1,2] and by Josephsen *et al.*[3] The fimbriae consisting of more than 100 subunits have a diameter of 2.1 nm and exist in three variants, *K88 ab, K88 ac* and *K88 ad.* Based on this nomenclature, "a" indicates a common antigenic determinant, whereas "b", "c", and "d" denote the subtype specific determinants expressed serologically.

Klemm and Mikkelsen[4] applied hydrophilicity calculations according to Hop and Woods,[5] combined with secondary structure prediction based on the method of Chou and Fasman[6] to the *E. coli K88 ab* protein, in order to assess the sequences likely to be potential linear antigenic determinants. According to these calculations, the three most potential determinants were[4]:

A: 19-Asp-Asp-Tyr-Arg-Glu-Lys

B: 96-Arg-Asn-Thr-Asp-Gly-Glu-Thr-Asn-Lys

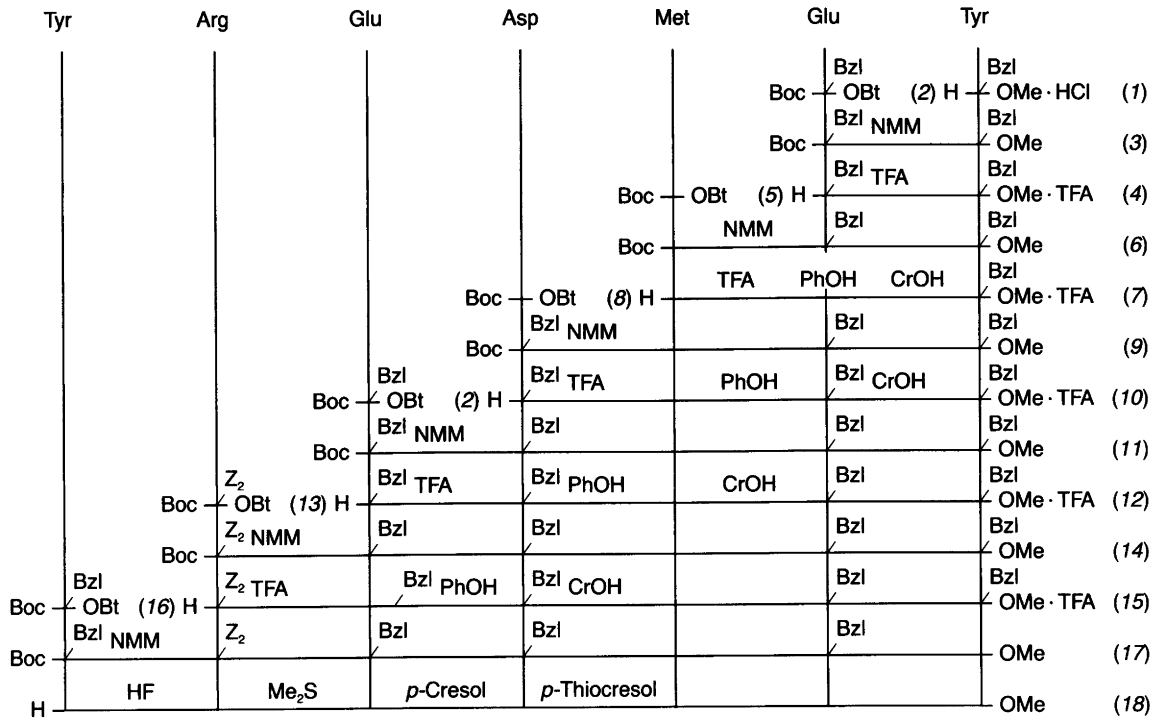
C: 213-Tyr-Arg-Glu-Asp-Met-Glu-Tyr.

We recently synthesized the proposed determinant, *A,* common to all subtypes.[7] In order to synthesize a determinant which is specific for subtype *K88 ab,* we set out to prepare the proposed antigen 3 which is located in an area where four amino acid substitutions occur amongst the three subtypes.[1–3]

Immunisation with the synthetic peptide linked to bovine serum albumin will indicate whether the region 213-Tyr to 219-Tyr is actually expressed on the surface of the fimbriae as a linear antigenic determinant, and inhibition experiments with subtype *ac* and *ad* fimbriae will indicate the degree of cross reaction associated with this region.

Results and discussion

The present syntheses, which are outlined in Scheme 1, are based on methodology described recently by Meldal and Kindtler,[7] and by Bodanszky and Bodanszky.[8] The hydrogen chloride salt (*1*) of Tyr(Bzl)-OMe was reacted with Boc-Glu(Bzl)-OBt (*2*) in the presence of *N*-methyl morpholine to give a 92 % yield of the dipeptide (*3*). The mixture[9] of the ester (*2*) and the *N*-acylated rearrangement product, both active as acylating agents, was formed by preactivation with less than one equivalent of DCC. The compound (*3*) was transformed into its TFA-salt (*4*) in 88 % yield by reaction with TFA. Reaction of *4* with Boc-Met-OBt (*5*) gave an 87 % yield of *6.*



Scheme 1. Synthesis of the heptapeptide *18*.

It is well documented[10] that methionine is easily alkylated and oxidized during the conventional treatment with neat TFA used for repetitive N_α deprotection. Recently, Bodanszky and Bodanszky[8] published a method to suppress these side reactions by using a mixture of TFA, *p*-cresol, and phenol. When the tripeptide was treated with neat TFA only 70 % of the TFA-salt (*7*) was obtained, and NMR spectroscopy of the residual material showed that this consisted mainly of decomposition products. On the other hand, treatment of *6* with a 4:3:3 mixture of TFA, *p*-cresol, and phenol gave a 92 % yield of *7*. Reaction of *7* and Boc-Asp(Bzl)-OBt (*8*) afforded 92 % of the tetrapeptide (*9*), which was transformed into *10* in 95 % yield by the TFA-*p*-cresol-phenol procedure. Compound *10* was acylated with *2* in 97 % yield, and the product (*11*) was quantitatively N_α deprotected to give *12*.

For the incorporation of arginine, we used the easily available compound (*13*), prepared as recently described by Meldal and Kindtler.[7] The condensation produced, in 96 % yield, a hexapeptide (*14*), which was converted by the TFA-*p*-cresol-phenol procedure the TFA-salt (*15*) in 94 % yield. Reaction of *15* with Boc-Tyr(Bzl)-OBt finally gave the protected heptapeptide (*17*) in 90 % yield. Deprotection of *17* was attempted by two routes. Reductive cleavage with ammonium formate and palladium on charcoal according to Anwer and Spatola[11] and treatment with TFA gave only 37 % yield of the deprotected product *18*. The low yield of this reaction was probably caused by deactivation of the palladium catalyst by the sulfur-containing peptide, possibly in combination with low solubility of *18* and intermediate products in polar solvents. Attempts to enhance the solubility by previous N_α deprotection with TFA gave unsatisfactory results. Recently Tam *et al.*[12] published a mild procedure for deprotection of tyrosine- and methionine-containing peptides using HF/dimethyl sulfide (DMS) mixtures. The method was shown to be very effective in suppressing the alkylation and oxidation side reactions often observed during the final HF treatment of synthetic peptides. Thus, treatment of *17* with a mixture of 25 % HF in DMS containing 5 % *p*-cresol and *p*-thiocresol

gave an 80 % yield of *18*. The reaction was followed by HPLC and found to pass through a stable intermediate. After 25 h the mixture still contained 17 % of this intermediate. Upon separation by preparative reversed phase liquid chromatography, the intermediate was analyzed by NMR spectroscopy and found to contain a benzyl group. Raising the amount of HF to 50 % did not enhance the rate but rather led to considerable amounts of byproducts. On the other hand, *18* was found to be perfectly stable under the condition with low HF concentration described above.

The peptide was linked to bovine serum albumin (BSA) by reaction with glutaraldehyde,[13] and rabbits were immunized with the dialyzed conjugate. Antisera raised in six weeks against this conjugate was applied to enzyme-linked immunosorbent assay (ELISA), and was found to recognize *E. coli K88 ab* fimbriae, when antisera raised against BSA was used as a negative control. No cross reaction with *E. coli K88 ad* fimbriae could be detected. These results will be described in further detail in a forthcoming publication.[14]

Experimental

Melting points were uncorrected. Optical rotations were measured on a Perkin Elmer 241 polarimeter. NMR spectra were obtained on Bruker WH 90 and HX 500 instruments. The ^{1}H NMR spectra were measured in $CDCl_3$ relative to TMS as an internal reference or in D_2O solutions with acetone 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). Microanalyses 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 TMD procedure.[15]

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 products upon acidification with potassium hydrogen sulfate, extraction with ethyl acetate, and evaporation, either directly (Boc-Asp(Bzl), Boc-Arg(Z_2)), or as the dicyclohexyl ammonium (DCHA) salts (Boc-Tyr(Bzl), Boc-Glu(Bzl), Boc-Met) by crystallization from suitable solvents.

Boc-Glu(Bzl)-Tyr(Bzl)-OMe (3). Boc-Glu (Bzl)-OH (1.02 g, 3.0 mmol) was dissolved in dichloromethane (10 ml) and HOBt (405 mg, 3.0 mmol) in DMF (1.5 ml) was added. The mixture was cooled and DCC (665 µl, 2.93 mmol) was added. The mixture was stirred for 75 min at 0 °C and the solution of *2* was filtered into a flask containing Tyr(Bzl)-OMe · HCl[16] (*1*), giving 92 % of the theoretical amount of *N,N'*-dicyclohexylurea. The mixture of *1* and *2* was neutralized (moist pH paper) by addition of *N*-methylmorpholine (500 µl). The mixture was stirred for 1 h at 0 °C and for 3 h at 20 °C. After filtration and dilution with dichloromethane (20 ml) the mixture was extracted with water and with sodium hydrogen carbonate. Drying with magnesium sulfate overnight, filtration and evaporation gave a syrup. This was triturated with ether (10 ml) and allowed to crystallize for 5 h at 0 °C giving 1.31 g of *3* (92 % yield), m.p. 102.5–104 °C; $[\alpha]_D^{20}$ + 23.3° (*c* 0.8, $CHCl_3$), anal. $C_{34}H_{40}N_2O_8$: C,H,N. ^{1}H NMR data are presented in Table 1.

Boc-Met-Glu(Bzl)-Tyr(Bzl)-OMe (6).
Compound *3* (1.27 g, 2.07 mmol) was dissolved in TFA (5 ml). After 10 min, the mixture was evaporated with diethyl ether, and the product was crystallized by addition of diethyl ether giving 1.14 g of *4* (88 % yield). Boc-Met-OH (635 mg, 2.55 mmol) and HOBt (350 mg, 2.59 mmol) in DMF (1 ml) were dissolved in dichloromethane (6 ml) and DCC (557 µl, 2.46 mmol) was added at 0 °C. Stirring was continued at 0 °C for 75 min and the solution of *5* was filtered into a flask containing *4* (1.14 g, 1.82 mmol). The mixture was neutralized with NMM (400 µl) and stirred for 1 h at 0 °C and 3 h at 20 °C. The reaction mixture was filtered and evaporated. The resulting syrup was dissolved in methanol (15 ml), water (5 ml) was added, and the mixture was allowed to crystallize at 0 °C. Filtering, washing with water and drying over phosphorus pentoxide gave 1.21 g of *6* (87 % yield), m.p. 115–116 °C; $[\alpha]_D^{20}$ + 8.5 (*c* 0.5 $CHCl_3$), anal. $C_{39}H_{49}N_3O_9S$: C,H,N. ^{1}H NMR data are presented in Table 1.

Boc-Asp(Bzl)-Met-Glu(Bzl)-Tyr(Bzl)-OMe (9).
Compound *6* (1.10 g, 1.45 mmol) was dissolved

in a mixture of phenol and *p*-cresol (1:1, 8 ml), and TFA (5.3 ml) was added. The mixture was stirred for 50 min and volatile material was removed at 28 °C and 1 mmHg. Diethyl ether (35 ml) was added and the mixture was cooled for 1 h. Filtration and washing with diethyl ether gave 1.03 g of *7* (92 % yield), m.p. 177–178 °C. Boc-Asp(Bzl)-OBt (*8*), prepared from Boc-Asp(Bzl)-OH (641 mg, 1.98 mmol) as described previously[7], was added to *7* in dichloromethane (10 ml) and DMF (0.6 ml). The mixture was neutralized with NMM to pH 8 (moist pH paper) and stirred for 1 h at 0 °C and 3 h at 20 °C. The dichloromethane was removed *in vacuo* and the product crystallized twice from DMF and water (5:3, 10 ml) and from dichloromethane and diethyl ether (1:5, 15 ml) to give 1.215 g of *9*, m.p. 103–105 °C; $[\alpha]_D^{20}$ − 5.0° (*c* 0.8, $CHCl_3$), anal. $C_{50}H_{60}N_4O_{12}S$: C,H,N. ^1H NMR data are given in Table 1.

Boc-Glu(Bzl)-Asp(Bzl)-Met-Glu(Bzl)-Tyr(Bzl)-OMe (12). Compound *9* (1.145 g, 1.22 mmol) was treated with the TFA/phenol/*p*-cresol mixture (10 ml) as described above giving 1.10 g (95 % yield) of *10*, m.p. 160–165 °C. Compound *10* (1.07 g, 1.12 mmol) was reacted with *2*, prepared from Boc-Glu(Bzl)-OH (566 mg, 1.68 mmol) as described above and NMM (200 µl) in dichloromethane (15 ml) and DMF (1 ml) solution for 2 h at 0 °C and 2 h at 20 °C. The mixture was filtered and dichloromethane removed *in vacuo*. The product was crystallized from DMF and water giving 1.4 g of material. This was recrystallized from dichloromethane and diethyl ether resulting in 1.216 g (97 % yield) of *12*, m.p. 154–157 °C; $[\alpha]_D^{20}$ − 3.8 (*c* 1.4, $CHCl_3$), anal. $C_{62}H_{73}N_5O_{15}S$: C,H,N. ^1H NMR data are given in Table 1.

Boc-Arg(Z_2)-Glu(Bzl)-Asp(Bzl)-Met-Glu(Bzl)-Tyr(Bzl)-OMe (14). Compound *11* (1.150 g, 1.02 mmol) was treated with TFA/phenol/*p*-cresol mixture (10 ml), and the product was precipitated with diethyl ether as described above to give 1.164 g (100 % yield) of crystalline *12*, m.p. 144–145 °C. Reaction of *12* (571 mg, 0.5 mmol) with Boc-Arg(Z_2)-OBt (*13*) (prepared from Boc-Arg (Z_2)-OH (400 mg, 0.738 mmol) as recently described[7]) in dichloromethane/DMF solution (15:1, 8 ml) with addition of NMM (162 µl) for 30 min at 0 °C and 3 h at 20 °C gave, after filtration through charcoal and evaporation of dichloromethane, by repeated crystallization from DMF (5 ml) and water (3 ml) 740 mg (96 % yield) of *14*, m.p. 154–157 °C; $[\alpha]_D^{20}$ − 6.2° (*c* 1.6, $CHCl_3$), anal. $C_{84}H_{97}N_9O_{20}S$: C,H,N. ^1H NMR data are given in Table 1.

Boc-Tyr(Bzl)-Arg(Z_2)-Glu(Bzl)-Asp(Bzl)-Met-Glu(Bzl)-Tyr(Bzl)-OMe (17). Compound *14* (690 mg, 0.445 mmol) was N_α-deprotected as described for compound *11* giving 650 mg (94 % yield) of *15*, m.p. 130–132 °C. Reaction of *15* (636 mg, 0.406 mmol) with Boc-Tyr(Bzl)-OBt (*16*), prepared from Boc-Tyr(Bzl)-OH (226 mg, 0.609 mmol) as described previously,[7] was performed in dichloromethane/DMF solution (15: 1,8 ml) with addition of NMM (100 µl) at 0 °C. The mixture was stirred at 0 °C for 30 min and at 20 °C for 2.5 h, filtered and evaporated. The product was crystallized from DMF (5 ml) and water giving 784 mg of material. This was recrystallized twice from dichloromethane and diethyl ether giving 660 mg (90 % yield) of *17*, m.p. 184–188 °C; $[\alpha]_D^{20}$ − 6.1° (*c* 0.8, $CHCl_3$), anal. $C_{100}H_{112}N_{10}O_{22}S$: C,H,N. ^1H NMR data are given in Table 1.

Tyr-Arg-Glu-Asp-Met-Glu-Tyr-OMe (18).

1) *Reductive removal of protective groups.* Compound *17* (150 mg 0.083 mmol) was suspended in methanol (4.5 ml), and palladium on charcoal (5 %, 150 mg) and ammonium formate (300 mg, 4.8 mmol) were added. The mixture was stirred for 30 min at 25 °C, and after short heating to 50 °C, was filtered through Celite® The filtrate was evaporated and purified by gel filtration on a Sephadex® G15 column by elution with methanol/water (1:1). The product was dissolved in TFA. After 15 min, the TFA was removed *in vacuo*. The product was again purified by gel filtration to give 30 mg (37 % yield) of *18* · TFA. ^{13}C NMR: σ 170.1 (C'-1); 54.1 (C_α-1); 36.2 (C_β-1); 126.5 (C1-1); 131.7 (C2,C6-1); 118.6 (C3,C5-1); 156.4 (C4-1); 173.5 (C'-2); 55.4 (C_α-2); 29.6 (C_β-2); 25.4 (C_γ-2); 41.7 (C_σ-2); 157.9 (C_ε-2); 173.9 (C'-3); 55.2 (C_α-3); 27.1 (C_β-3); 30.9 (C_γ-3); 177.8 (C_σ-3); 174.4 (C'-4); 51.4 (C_α-4); 37.1 (C_β-4); 175.0 (C_γ-4); 173.7 (C'-5); 54.1 (C_α-5); 31.2 (C_β-5); 30.4 (C_γ-5); 15.3 (C_ε-5); 173.9 (C'-6); 54.4 (C_α-6); 27.3 (C_β-6); 31.0 (C_γ-6); 178.0 (C_σ-6); 173.1 (C'-7); 55.4 (C_α-7); 36.9 (C_β-7); 129.3 (C1-7); 132.0 (C2,C6-7); 117.1 (C3,C5-7); 155.7 (C4-7); 53.8 (−OMe); 117.4, 163.8 (TFA-salt).

Table 1. NMR data on antigenic peptides from *Eschericia Coli K88* protein fimbriae.

Chemical shift Compound	*3*	*6*	*9*	*11*	*14*	*17*	*18*
Tyr							
$N_\alpha H$						4.860	
H_α						4.090	4.302
H_β						2.902	3.171
						2.722	3.142
H2,6						6.926	7.127
H3,5						6.820	6.861
Arg							
$N_\alpha H$					5.414		8.36
H_α					3.956	4.068	4.374
H_β					1.710	1.710	1.802
					1.674	1.584	1.710
H_γ					1.562	1.584	1.562
							1.526
H_σ					3.937	3.892	3.119
					3.978		
$N_\sigma H$							7.26
$N_\varepsilon H$					9.292	9.304	7.59
							7.50
Glu							
$N_\alpha H$				5.705	7.128	7.155	8.18
H_α				3.969	4.067	4.132	4.351
H_β				2.317	2.262	2.322	2.029
				1.956	2.048	2.079	1.950
H_σ				2.541	2.556	2.594	2.494
				2.499	2.482	2.507	2.446
Asp							
$N_\alpha H$			5.553	7.602	7.738	7.650	8.44
H_α			4.496	4.554	4.617	4.658	4.689
H_β			2.966	3.122	2.956	3.024	2.806
			2.790	2.733	2.898	3.006	2.744
Met							
$N_\alpha H$		5.030		7.542	7.563	7.524	8.15
H_α		4.191	4.453	4.554	4.446	4.491	4.511
H_β		2.047	2.146	2.205	2.199	2.213	2.094
		1.863	1.971	1.911	1.999	1.971	2.001
H_γ		2.496	2.528	2.429	2.414	2.435	2.429
		2.508					
H_σ		2.062	2.086	2.056	2.016	2.034	2.052
Glu							
$N_\alpha H$	5.09	7.081	7.210	7.210			8.42
H_α	4.12	4.422	4.423	4.417	4.397	4.437	4.370
H_β	2.02	2.102	2.146	2.044	1.940	2.070	1.964
	1.92	1.921	1.971		1.848		1.898
H_γ	2.37	2.518	2.473	2.464	2.439	2.457	2.338
		2.424	2.414		2.342	2.363	2.250
Tyr							
$N_\alpha H$	6.58	6.644	6.835	6.909	6.908	6.962	8.18
H_α	4.75	4.737	4.743	4.767	4.734	4.734	4.738
H_β	3.03	3.061	3.071	3.073	3.074	3.080	3.156
		2.973	2.981	2.999	2.983	2.988	2.934
H2,6	7.00	7.007	7.047	7.070	7.074	7.092	7.099
H3,5	6.83	6.874	6.888	6.891	6.874	6.876	6.822

Coupling constants	*3*	*6*	*9*	*11*	*14*	*17*	*18*
$JN_{\alpha}HH_{\alpha}$						5.3	
$JH_{\alpha}H_{\beta}$						5.0	7.2
						8.5	7.2
$JH_{\beta}H_{\beta}'$						14.4	14.5
JH2H3						8.3	8.6
$JN_{\alpha}HH_{\alpha}$						6.5	
$JH_{\alpha}H_{\beta}$						4.5	6.5
						7.5	7.8
$JN_{\alpha}HH_{\alpha}$				4.5	5.0	5.6	
$JH_{\alpha}H_{\beta}$				7.0	5.0	5.6	6.5
				7.0	8.0	7.8	8.5
$JH_{\beta}H_{\beta}'$					13.9	13.9	
$JH_{\beta}H_{\gamma}$					5.3, 6.3	5.0, 6.8	5.9, 7.5
					9.7, 8.7	9.5, 9.5	8.0, 8.5
$JH_{\gamma}H_{\gamma}'$					13.5	13.0	13.6
$JN_{\alpha}HH_{\alpha}$			7.5	7.5	5.0	5.5	
$JH_{\alpha}H_{\beta}$			4.5	4.3	6.5	6.5	6.2
			6.5	5.4	7.8	8.0	7.0
$JH_{\beta}H_{\beta}'$			17.0	17.3	17.5		17.0
$JN_{\alpha}HH_{\alpha}$		8.0	7.5	7.5	8.0	7.0	
$JH_{\alpha}H_{\beta}$		6.8	5.3		4.0	4.0	5.0
		7.0	7.5		9.5	9.4	9.0
$JH_{\beta}H_{\beta}'$		14.0	14.0		14.0		
$JH_{\beta}H_{\gamma}$		7.3	7.0	7.3	7.5		7.5
		7.0					
$JN_{\alpha}HH_{\alpha}$	7.5	8.2	7.8	7.8	8.1	8.0	
$JH_{\alpha}H_{\beta}$	5.7	5.5	5.3	4.7	5.2	5.1	6.0
	7.1	7.5	7.5	9.2	9.0	9.0	8.3
$JH_{\beta}H_{\beta}'$		14.6	14.6				
$JH_{\beta}H_{\gamma}$	6.9	7.0	6.8		5.3	6.8, 7.5	6.5, 7.3
			7.2		7.7	9.0, 8.5	7.8, 8.5
$JH_{\gamma}H_{\gamma}'$		17.0	17.3		17.7	17.7	17.7
$JN_{\alpha}HH_{\alpha}$	8.0	8.0	7.8	7.6	8.1	8.4	
$JH_{\alpha}H_{\beta}$	6.0	5.8	6.0	5.7	5.7	6.1	5.6
	6.0	6.6	7.1	6.5	7.1	7.3	9.1
$JH_{\beta}H_{\beta}'$		14.0	14.0	14.0	13.7	14.0	14.2
JH2H3	9.0	8.0	8.0	8.0	8.3	8.3	8.3

2) *Cleavage with HF/Me_2S.* Compound *17* (100 mg, 0.055 mmol), *p*-cresol (200 mg) and *p*-thiocresol (150 mg) were dissolved in a mixture of HF (1.3 ml) and dimethyl sulfide (3.3 ml) at −78 °C. The mixture was allowed to stand at 0 °C for 25 h. The reaction was monitored by HPLC of small aliquots (totally 4 % of volume) taken after 2-, 5- and 25 h. Volatile material was removed with a stream of nitrogen. The product was precipitated with diethyl ether (10 ml) and washed 3 times with diethyl ether. Separation on a RP-8 Lobar A-Column (Merck) (Eluent; methanol/water, 1:3) followed by lyophilization gave 37 mg (80 % yield) of *18* · HF. Amino acid analysis: Asp, 1.01; Arg, 0.90; Glu, 2.29; Met, 0.80; Tyr, 2.00. ^{1}H NMR data are given in Table 1. HPLC on 12 cm nucleosil $5C_{18}$ (eluent, 2 ml/min, methanol-water 3:5) gave only one peak positioned at 2.2 min. A second fraction (9 mg, 17 %) was isolated. HPLC with the same system as above (eluent, methanol-water, 1:1) gave one peak positioned at 8.0 min. ^{1}H NMR spectroscopy at 500 MHz showed this intermediate to contain one benzyl group. ^{1}H NMR (500 MHz in d_6-DMSO): σ 1.773 (3H, m); 1.878 (1H, dtri, 5.0 Hz, 9.9 Hz); 1.948 (1H, m); 1.979 (3H, s); 1.979 (1H, m); 2.000 (1H, m); 2.226 (3H, m); 2.269 (3H, m); 2.376 (1H, m); 2.441 (2H, m); 2.584 (1H, dd, 4.9 Hz, 16 Hz); 2.685 (1H, dtri, 16 Hz, 7.0 Hz); 2.850 (1H, dd, 7.5 Hz, 13 Hz); 2.860 (1H, m); 2.878 (1H, dd, 6.2 Hz, 13 Hz); 2.927 (1H, dd, 5.0 Hz, 14.5 Hz); 3.133 (2H, broad signal); 3.545 (3H, s); 4.182 (1H, dd, 5.2 Hz, 9.0 Hz); 4.213 (1H, dd, 5.2 Hz, 9.0 Hz); 4.226 (1H, m); 4.312 (1H, dd, 4.7 Hz, 9.0 Hz); 4.349 (2H, tri, 6.8 Hz); 4.367 (1H, m); 4.966 (1H, broad signal); 5.084 (1H, broad signal); 6.667 (2H, d, 8.5 Hz); 6.689 (2H, d, 8.2 Hz); 6.984 (2H, d, 8.5 Hz); 7.021 (2H, d, 8.2 Hz); 7.338 (5H, broad s).

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