

# Synthesis of a Putative Subtype Specific Antigenic Heptapeptide from *Escherichia Coli K88 ad* Protein Fimbriae

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The heptapeptide methyl ester Phe-Asn-Glu-Asn-Met-Ala-Tyr-OMe covering the amino acid sequence of the region 213–219 of *Escherichia Coli K88 ad* protein fimbriae is synthesized using *N*<sup>9</sup>-*t*-butyloxycarbonyl-protection and benzyl groups for side-chain-protection. All condensation reactions are performed in 84–97 % yield by preactivation of the protected amino acids by dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt), and reaction of the resulting active ester with amine in the presence of 4-methylmorpholine (NMM). A mechanism is proposed for the nitrile formation in the side-chain of activated asparagine, and the suppression of this side-reaction is investigated. The repetitive deprotection is performed in a mixture of trifluoroacetic acid (TFA), phenol and *p*-cresol to give the TFA salts in virtually quantitatively yields. The final deprotection of the heptapeptide is carried out in a mixture of 25 % hydrogen fluoride (HF) and dimethyl sulfide (DMS) in an overall yield of 48 %. The serological and conformational properties of the synthetic peptide are under investigation.

## Introduction

The protein subunits of *Escherichia Coli K88 ab, ac* and *ad* fimbriae have recently been sequenced by Klemm and colleagues<sup>1,2</sup> and Josephsen *et al.*<sup>3</sup> The fimbriae have a diameter of 2.1 nm and consist of more than 100 subunits existing in 3 variants, *K88 ab, ac* and *ad*, where *b, c* and *d* indicate subtype specific determinants expressed serologically. The subunits of *ab* and *ad* variants consist of 264 amino acids. Hydrophilicity calculations<sup>4</sup> and secondary structural prediction<sup>5</sup> were performed by Klemm *et al.*<sup>6</sup> to assess the peptide sequences which are most likely to be linear determinants of *E. Coli K88 ab* protein fimbriae. We have recently published the preparation of the sequences 19–24<sup>7</sup> and 213–219,<sup>8</sup> proposed to be respectively a common and a *K88 ab* subtype specific antigen.<sup>6</sup> The latter has the structure 213-Tyr-Arg-Glu-Asp-Met-Glu-Tyr and four amino acid patterns are known within this region for the three subtypes. Hence, the corresponding se-

quence in *K88 ad*, Phe-Asn-Glu-Asn-Met-Ala-Tyr-OMe can be expected to be a subtype specific determinant. We set out to prepare this sequence in order to carry out immunization experiments to clarify whether the sequence is actually expressed as an antigen on the surface of the *K88 ad* fimbriae and, if so, to perform inhibition experiments to measure the degree of cross reaction associated with this region.

## Results and discussion

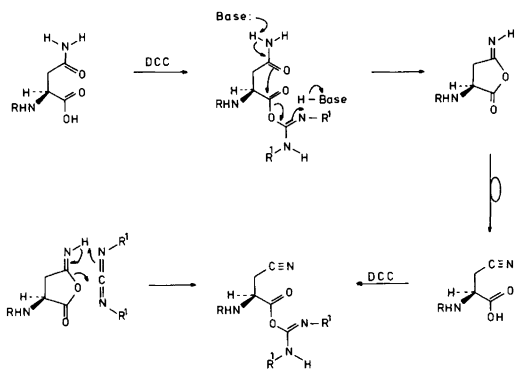
The present synthesis, outlined in Scheme 1, is based on methodology described recently in Bodanszky and Bodanszky<sup>9</sup> and by Meldal.<sup>8</sup> The hydrochloride (1) of Tyr(Bzl)-OMe was reacted with the activated mixture of 1-hydroxybenzotriazolyl ester (2) and *N*-acylated rearrangement product,<sup>10</sup> obtained by reaction of Boc-Ala-OH with DCC and HOBt, to give a 92 % yield of the dipeptide (3) in the presence of NMM. Removal of the Boc-group by treatment with a mixture of

Phe	Asn	Glu	Asn	Met	Ala	Tyr			
					Boc	OBt (2)	H	Bzl OMe·HCl (1)	
					Boc	NMM		Bzl OMe (3)	
				Boc	OBt (5)	H		Bzl OMe·TFA (4)	
			Boc	OH (8)	Boc	NMM		Bzl OMe (6)	
			Boc	OBt (9)	H	TFA	PhOH	p-Cresol	Bzl OMe·TFA (7)
			Boc	NMM				Bzl OMe (10)	
		Boc	OBt (12)	H	TFA	PhOH	p-Cresol	Bzl OMe·TFA (11)	
		Boc	Bzl	NMM				Bzl OMe (13)	
	Boc	OBt (9)	H	Bzl	TFA	PhOH	p-Cresol	Bzl OMe·TFA (14)	
	Boc	NMM		Bzl				Bzl OMe (15)	
Boc	OBt (17)	H	TFA	Bzl	PhOH	p-Cresol		Bzl OMe·TFA (16)	
Boc	NMM		Bzl					Bzl OMe (18)	
H	HF	Me <sub>2</sub> S						OMe (19)	

Scheme 1. Synthesis of the antigenic heptapeptide 19.

TFA, phenol and *p*-cresol gave a 94% yield of the TFA salt (4). This was brought to react with Boc-Met-OBt (5)<sup>8</sup> in the presence of NMM affording a 91% yield of the tripeptide (6). The Boc-group in compound 6 was removed in a mixture of TFA, phenol and *p*-cresol in order to suppress alkylation of methionine,<sup>8,9</sup> yielding 95% of the TFA-salt (7).

In order to minimize the formation of nitrile in the side-chain of asparagine during the activation and condensation the role of HOBT was investigated. It is well documented<sup>11</sup> that nitrile formation in the side-chain of asparagine occurs during the activation; hence, the reaction must involve the activated center. It is also known<sup>12</sup> that active esters do not induce nitrile formation; thus, *p*-nitrophenyl esters have often been used for the introduction of asparagine. Based on these observations and the results of Kashelkar and Ressler<sup>11</sup> the mechanism outlined in Scheme 2 is suggested for the dehydration of the amido group. In order to elucidate the effect of HOBT addition on this reaction we studied the reaction of DCC with HOBT in an NMR tube, following the course of reaction by the appearance of resonance signals. On addition of DCC to an insoluble suspension of HOBT in chloroform a solution of two products appear as characterized in Table 2, corresponding to the products 20 and 21 respectively (*cf.* Scheme 3). Both products are formed instantaneously and the equilibrium is dependent on concentration and solvent polarity, the more polar solvents favouring the formation of 20. After standing for 24 h 20 and 21 were sup-

Scheme 2. Proposed mechanism for the DCC-mediated dehydration of *N*<sup>α</sup>-protected asparagine.

## MELDAL

Table 1. <sup>1</sup>H NMR data of synthetic peptides from *E. Coli K88 ad* protein fimbriae.<sup>a</sup> CDCl<sub>3</sub> was used as a solvent.<sup>b</sup> DMSO-*d*<sub>6</sub> was used as a solvent.

Compound	3 <sup>a</sup>	6 <sup>a</sup>	10 <sup>b</sup>	13 <sup>b</sup>	15 <sup>b</sup>	18 <sup>b</sup>	19 <sup>b</sup>
Phe							
N <sup>α</sup> H						6.840	
H <sup>α</sup>						4.197	3.701
H <sup>β</sup>						2.714	2.655
						2.973	2.979
H2,6						7.26	7.224
H3,5						7.26	7.287
H4						7.19	7.224
Asn							
N <sup>α</sup> H					6.927	8.174	7.282
N <sup>ε</sup>					4.299	4.548	4.404
H <sup>β</sup>					2.388	2.483	2.514
					2.511	2.514	2.590
N <sup>δ</sup> H					6.866	6.966	6.922
					7.358	(7.344)	7.425
							7.50
Glu							
N <sup>α</sup> H			6.924	8.212	8.184	8.653	
H <sup>α</sup>			3.964	4.236	4.238	4.001	
H <sup>β</sup>			1.758	1.797	1.811	1.808	
			1.951	1.976	1.989		
H <sup>γ</sup>			2.500	2.475	2.469	2.134	2.217
Asn							
N <sup>α</sup> H			6.980	8.071	7.964	8.111	8.199
H <sup>α</sup>			4.228	4.502	4.504	4.503	4.542
H <sup>β</sup>			2.499	2.440	2.431	2.567	2.555
			2.542	2.566	2.611	2.602	2.638
H <sup>δ</sup> H			6.885	6.931	6.923	6.924	6.965
			7.333	7.347	7.344	7.474	7.392
Met							
N <sup>α</sup> H		5.198	8.044	7.946	7.875	7.840	7.799
H <sup>α</sup>		4.270	4.334	4.279	4.279	4.305	4.279
H <sup>β</sup>		1.912	1.783	1.758	1.797	1.786	1.785
		2.074	1.939	1.927	1.947	1.955	1.945
H <sup>γ</sup>		2.542	2.146	2.388	2.388	2.395	2.376
		2.574	2.472				2.465
H <sup>ε</sup>		2.106	2.003	1.974	1.983	1.976	1.994
Ala							
N <sup>α</sup> H	4.863	6.775	7.904	7.960	7.938	7.884	7.820
H <sup>α</sup>	4.113	4.431	4.289	4.254	4.256	4.254	4.229
H <sup>β</sup>	1.305	1.368	1.198	1.188	1.191	1.191	1.188
Tyr							
N <sup>α</sup> H	6.417	6.457	8.027	8.017	7.974	7.985	7.979
H <sup>α</sup>	4.797	4.810	4.397	4.383	4.392	4.393	4.322
H <sup>β</sup>	3.028	3.058	2.881	2.870	2.877	2.877	2.819
	3.091	3.099	2.949	2.930	2.936	2.931	2.859
H2,6	7.013	7.043	7.127	7.116	7.127	7.133	6.975
H3,5	6.890	6.587	6.930	6.924	6.930	6.933	6.654

ANTIGENIC HEPTAPEPTIDE SYNTHESIS

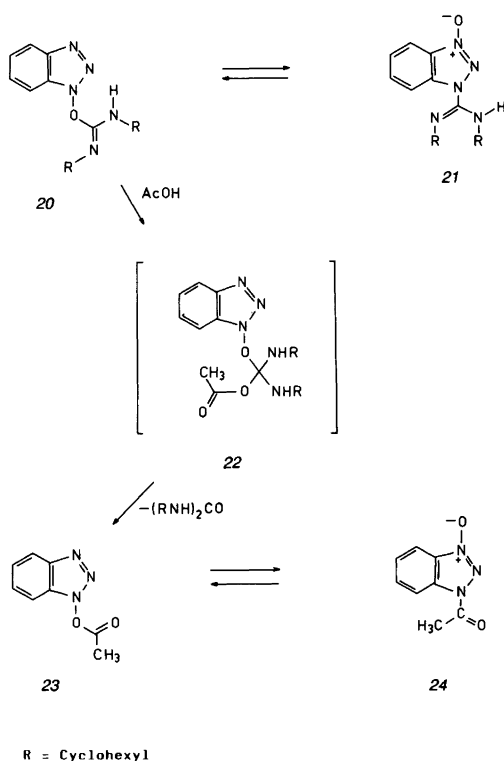
	3	6	10	13	15	18	19
JH <sup>α</sup> HH <sup>α</sup>						5.3	
JH <sup>α</sup> H <sup>β</sup>						5.0	7.2
JH <sup>β</sup> H <sup>β'</sup>						8.5	7.2
						14.4	14.5
JH <sup>α</sup> HH <sup>α</sup>					(7.0)	6.8	
JH <sup>α</sup> H <sup>β</sup>						5.3	
						6.0	(8.0)
JH <sup>α</sup> HH <sup>α</sup>					7.7	7.0	
JH <sup>α</sup> H <sup>β</sup>					3.0		
					10.2		
JH <sup>α</sup> HH <sup>α</sup>			7.7	6.4	6.3	6.3	7.2
JH <sup>α</sup> H <sup>β</sup>			7.5	7.5	7.2	6.3	6.6
JH <sup>β</sup> H <sup>β'</sup>			6.0	5.0	6.8	6.3	7.2
				16.3	16.1	16.1	
JH <sup>α</sup> HH <sup>α</sup>		8.2	6.9	6.5	7.7	7.7	7.0
JH <sup>α</sup> H <sup>β</sup>		5.2	5.0	5.3	5.0	5.1	4.5
JH <sup>β</sup> H <sup>β'</sup>		9.0	9.4	9.2	9.5	9.2	8.5
JH <sup>β</sup> H <sup>γ</sup>		14.0	13.7				
JH <sup>γ</sup> H <sup>γ'</sup>		9.5, 8.0	9.5, 6.3	7.1			9.5, 6.0
JH <sup>γ</sup> H <sup>γ'</sup>		9.0, 8.0	9.0, 4.7				9.0, 5.9
JH <sup>α</sup> HH <sup>α</sup>		7.7	7.8	7.8	8.3	7.9	6.5
JH <sup>α</sup> H <sup>β</sup>	7.2	7.4	7.4	7.2	7.3	7.0	8.0
JH <sup>α</sup> HH <sup>α</sup>	7.2	7.3	7.3	6.9	7.5	7.2	7.2
JH <sup>α</sup> H <sup>β</sup>	6.0	6.0	5.9	6.3	6.4	6.3	6.5
JH <sup>β</sup> H <sup>β'</sup>	6.2	8.0	8.4	8.2	8.1	7.8	9.0
JH <sup>β</sup> H <sup>β'</sup>	14.4	13.9	13.8	13.8	13.8	13.8	14.9
JH2H3, H5,H6	8.7	8.7	8.7	8.7	8.7	8.5	8.8

Table 2.  $^1\text{H}$  NMR and kinetic data on HOBt derivatives. <sup>a</sup>Conditions: HOBt, DCC, AcOH 1:1:1 in  $\text{DMSO-}d_6$ ,  $\text{CDCl}_3$ , 1:9.

Compound	Aromatic and Other Characteristic Resonances						Percentage at Various Times of Reaction				
	H4	H5	H6	H7	$\text{R}_2\text{N}-\text{CH}$	$\text{R}=\text{N}-\text{CH}$	$\text{CH}_3$	5 min	15 min	1 h	24 h
20	7.593 (8.1)	7.312	7.293	7.730 (7.5)	3.472	3.202		69	69	69	Decomp.
21	7.950 (8.4)	7.625 (8.4, 6.5)	7.375 (8.0, 6.5)	7.860 (8.0)	3.472	3.202		31	31	31	
23	7.428 (8.7)	7.554 (8.7, 6.0)	7.431 (8.8, 6.0)	8.074 (8.8)			2.546	37 <sup>a</sup>	53	50	50
24	8.409 (8.8)	7.784 (8.8, 7.2)	7.567 (8.1, 7.2)	8.014 (8.1)			2.779	9	28	48	50
AcOH							2.010	54	19	2	0

plemented by two additional products in the solution.

Upon reaction of **20** and **21** with acetic acid NMR signals appeared corresponding to **23** and



Scheme 3. Formation of complexes **20** and **21** by reaction of HOBt with DCC and their conversion into 1-hydroxybenzotriazolyl acetates.

**24** (cf. Scheme 3), both previously isolated and characterized by Horeki.<sup>13</sup> Compound **23** isolated as a crystalline product upon treatment of HOBt with acetyl chloride according to Kim *et al.*,<sup>14</sup> was formed initially and then rearranged in a base-catalyzed reaction to the equilibrium mixture containing 35% of **23** and 65% of **24** in chloroform solution, cf. Table 1. This mixture was stable for weeks. On addition of  $\text{DMSO-}d_6$  the equilibrium shifted towards **23**. Since DCC is not likely to be formed from **20** and **21** we suggest that the reaction of **20** with acetic acid occurs via the adduct **22**, cf. Scheme 3. Compound **23** was recognized through its characteristic carbonyl absorption at  $1825\text{ cm}^{-1}$ . The  $^1\text{H}$  NMR spectra of **20**, **21**, **23** and **24** were assigned by decoupling experiments, and comparisons. There are numerous compounds of the type  $(\text{RO})_2\text{C}(\text{NR}_2)_2$ , where R and R' or alkyl or aryl groups are known as stable compounds.<sup>15</sup> According to Scheme 2 the removal of DCC from the reaction mixture by reaction with HOBt prior to the addition of *N*<sup>α</sup>-protected asparagine or glutamine should suppress the formation of nitrile.

In fact the reaction Boc-Asn-OH (**8**) with the mixture of **20** and **21** produced the 1-hydroxybenzotriazole ester (**9**) which reacted with compound **7** in the presence of NMM, giving an 84% yield of **10**, which by HPLC analysis and NMR spectroscopy was found to be devoid of nitrile contaminants. Quantitative *N*<sup>α</sup>-deprotection of **10** with a mixture of TFA, phenol and *p*-cresol gave compound **11**. Reaction of **11** with Boc-Glu(OBzl)-OBt (**12**)<sup>8</sup> in the presence of NMM afforded compound **13** in 87% yield, which could

be quantitatively  $N^{\alpha}$ -deprotected. The resulting TFA salt (14) was made to react with Boc-Asn-OBt (9) in the presence of NMM to afford a 97% yield of 15, which by HPLC and NMR spectroscopy was found to be devoid of nitrile-containing compounds.  $N^{\alpha}$ -deprotection gave a 94% yield of the TFA salt (16), which by reaction with Boc-Phe-OBt (17), obtained by reaction of Boc-Phe-OH with HOBt and DCC, produced a 94% yield of compound 18. Finally, deprotection of 18 was achieved in a 25% (v/v) mixture of hydrogen fluoride in dimethyl sulfide according to Tam *et al.*<sup>16</sup> and the pure crystalline heptapeptide methyl ester (19) was isolated in 81% yield after two recrystallisations. Attempts at deprotection of the methionine-containing heptapeptide (18) by catalytic transfer hydrogenation with ammonium formate, as described by Anwer and Spatola,<sup>17</sup> proved of no avail.

The peptide was linked to bovine serum albumin (BSA) by reaction with glutaraldehyde,<sup>18</sup> 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 ad fimbriae, when antisera raised against BSA was used as a negative control. No crossreaction with *E. coli* K88 ab fimbriae could be detected. These results will be described in further detail in a forthcoming publication.<sup>19</sup>

## Experimental

Melting points were uncorrected. Optical rotations were measured on a Perkin Elmer 241 polarimeter. NMR spectra were recorded on Bruker WH 90 or HX 500 instruments. <sup>1</sup>H NMR spectra were measured in DMSO-*d*<sub>6</sub> where D<sub>3</sub>CSOCD<sub>2</sub>H served as an internal reference (2.50 ppm) or in CDCl<sub>3</sub> relative to TMS as an internal reference. <sup>13</sup>C NMR spectra were measured in CDCl<sub>3</sub> relative to CDCl<sub>3</sub> as an internal reference (77.7 ppm) or in DMSO-*d*<sub>6</sub> relative to DMSO-*d*<sub>6</sub> as an internal reference (39.6 ppm). IR spectra were measured in KBr tablets on a Perkin-Elmer 700 Infrared Spectrophotometer. Micro analyses and amino acid analyses were performed by the Novo Microanalytical Laboratory. TLC was performed on silica gel-coated aluminum plates and amides were made visible by the TMD color yielding reaction.<sup>20</sup> HPLC was performed on a 12×4 mm

nucleosil 5 C<sub>18</sub> column with UV detection at 280 nm.  $N^{\alpha}$ -Boc-amino acids were prepared as described in recent publications.<sup>7,8</sup>

*N<sup>α</sup>-Boc-amino acid-1-hydroxybenzotriazole esters. General procedure.* 1-Hydroxybenzotriazole (135 mg, 1 mmol) was dissolved in DMF (0.2 ml) and  $N^{\alpha}$ -Boc-amino acid (1 mmol) was added. The mixture was diluted with dichloromethane (2.5 ml) and cooled to 0°C. DCC (210 μl, 0.95 mmol) was added and the mixture was stirred at 0°C for 1.5–2 h. The resulting solution of active ester was filtered directly into a flask containing the  $N^{\alpha}$ -deprotected peptide.

*Removal of N<sup>α</sup>-Boc-groups. General procedure.* The  $N^{\alpha}$ -Boc-protected peptide (0.5–1.5 mmol) was suspended in a mixture of TFA (2 ml), *p*-cresol (1.5 ml) and phenol (1.5 ml) with stirring; when all material had dissolved the mixture was stirred for 30 min at 20°C. Volatile material was removed on a rotatory evaporator at 1 mmHg and 30°C and the resulting TFA-salt was precipitated by addition of diethyl ether (20 ml). The precipitate was washed several times with diethyl ether and dried over phosphorus pentoxide *in vacuo*.

*Boc-Ala-Tyr(Bzl)-OMe (3).* The hydrochloride salt (1)<sup>21</sup> (0.750 g, 2.34 mmol) of Tyr(Bzl)-OMe and Boc-Ala-OBt (3.04 mmol) dissolved in dichloromethane was cooled to 0°C and the mixture was neutralized by addition of NMM (370 μl, 3.3 mmol). The mixture was stirred for 1 h at 0°C and 2 h at 20°C, diluted with dichloromethane (10 ml) and washed successively with water (10 ml) and sodium carbonate solution (2×10 ml). The mixture was dried with magnesium sulfate, filtered and evaporated affording a syrup, which was crystallized twice from diethyl ether and petroleum ether yielding 1.00 g (94%) of 3. M.p. 100–101°C;  $[\alpha]_D^{20}$  32° (c 1.0, CHCl<sub>3</sub>); Anal. C<sub>25</sub>H<sub>32</sub>N<sub>2</sub>O<sub>6</sub>: C, H, N; <sup>1</sup>H NMR data are presented in Table 1.

*Boc-Met-Ala-Tyr(Bzl)-OMe (6).* Compound 3 (800 mg, 1.75 mmol) was  $N^{\alpha}$ -deprotected as described above giving 780 mg of 4 (95% yield), m.p. 260°C. Compound 4 was reacted with Boc-Met-OBt (5) (2.6 mmol) dissolved in dichloromethane (10 ml) at 0°C for 1 h and at 20°C for 2 h in the presence of NMM (460 μl 4.10 mmol).

The solvent was removed *in vacuo* and the resulting syrup made crystalline by addition of DMF (5 ml) and water (3 ml). The product was filtered off, washed with water, dried, dissolved in chloroform (5 ml) and treated with charcoal. Filtration through Celite, and addition of diethyl ether (10 ml) afforded 900 mg (91 % yield) of crystalline **6**. M.p. 133–134 °C;  $[\alpha]_D^{20}$  19.6 (*c* 0.4, CHCl<sub>3</sub>); Anal. C<sub>30</sub>H<sub>41</sub>N<sub>5</sub>O<sub>7</sub>S: C, H, N; <sup>1</sup>H NMR data are given in Table 1.

**Boc-Asn-OBt (9)**. 1-Hydroxybenzotriazole (293 mg, 2.1 mmol) was dissolved in DMF (1 ml) and DCC (453 μl, 2.0 mmol) dissolved in dichloromethane (5 ml) was added at 0 °C. The mixture was stirred at 0 °C for 40 min and Boc-Asn-OH (**8**) (496 mg, 2 mmol) dissolved in DMF (2 ml) was added. Stirring was continued for 2.5 h at 0 °C and the solution containing Boc-Asn-OBt (**9**) was separated from precipitated dicyclohexylurea by filtration.

**Boc-Asn-Met-Ala-Tyr(Bzl)-OMe (10)**. Compound **6** (850 mg, 1.44 mmol) was *N*<sup>α</sup>-deprotected yielding 837 mg (96 %) of **7**, m.p. 211–213 °C, which was reacted with **9** (2 mmol) dissolved in dichloromethane (7 ml) and DMF (5 ml) at 0 °C for 1 h with addition of NMM (360 μl, 3.2 mmol) in small portions during the first 15 min. The mixture was stirred for 2 h at 20 °C and dichloromethane was removed *in vacuo*. The product was precipitated by dilution with DMF (5 ml) and addition of water (5 ml) and the precipitate was removed by filtration and dried over phosphorus pentoxide. Recrystallisation from a mixture of DMF (4 ml) and chloroform (20 ml) by addition of diethyl ether afforded 776 mg of **10** (84 % yield). M.p. 192–196 °C;  $[\alpha]_D^{20}$  –15.4° (*c* 0.3, DMF); Anal. C<sub>34</sub>H<sub>47</sub>N<sub>5</sub>O<sub>9</sub>S: C, H, N; <sup>1</sup>H NMR data are given in Table 1. The product was analyzed by reversed phase HPLC (eluent: methanol/water, 1:1) and only one compound could be detected.

**Boc-Glu(OBzl)-Asn-Met-Ala-Tyr(Bzl)-OMe (13)**. Compound **10** (650 mg, 0.93 mmol) was *N*<sup>α</sup>-deprotected to yield 670 mg (100 %) of the TFA-salt (**11**), m.p. 200–203 °C which was dissolved in 2 ml DMF and reacted with Boc-Glu(Bzl)-OBt (**12**) (1.5 mmol) dissolved in dichloromethane (7 ml) and DMF (1 ml) at 0 °C by addition of NMM (220 μl). The mixture was stirred at 0 °C for 30

min and diluted with dichloromethane (10 ml) and DMF (5 ml). Stirring was continued for 2 h at 20 °C and dichloromethane was removed *in vacuo*. The precipitate formed on addition of water was filtered off and recrystallized from DMF and water. Finally the dried product was recrystallized from DMF (5 ml) and chloroform (5 ml) by addition of diethyl ether affording 750 mg of **13** (87 % yield). M.p. 208–210 °C;  $[\alpha]_D^{20}$  –8.2° (*c* 0.5, DMF); Anal. C<sub>46</sub>H<sub>60</sub>N<sub>6</sub>O<sub>12</sub>S: C, H, N; <sup>1</sup>H NMR data are given in Table 1.

**Boc-Asn-Glu(OBzl)-Asn-Met-Ala-Tyr(Bzl)-OMe (15)**. The peptide, **13** (647 mg, 0.70 mmol) was treated with the TFA mixture affording 660 mg of **14** (100 % yield), m.p. 214–216 °C, which was reacted with Boc-Asn-OBt (**9**) (1.3 mmol) dissolved in dichloromethane (3 ml) and DMF (3 ml) at 0 °C by addition of NMM (200 μl, 2 mmol) in small portions. The mixture was stirred for 30 min at 0 °C and 2 h at 20 °C. Dichloromethane was removed *in vacuo* and the product was precipitated by dilution with DMF (10 ml) and addition of water (10 ml). The product was recrystallized from DMF and water affording 720 mg of **15** (97 % yield). M.p. 236–240 °C;  $[\alpha]_D^{20}$  –19.1 (*c* 0.7, DMF); Anal. C<sub>50</sub>H<sub>66</sub>N<sub>8</sub>O<sub>14</sub>S: C, H, N; <sup>1</sup>H NMR data are given in Table 1. The product was analyzed by reversed phase HPLC and only one compound could be detected.

**Boc-Phe-Asn-Glu(OBzl)-Asn-Met-Ala-Tyr(Bzl)-OMe (18)**. Removal of the Boc-group in **15** (530 mg, 0.50 mmol) afforded 500 mg of **16** (94 % yield), m.p. 231–235 °C, which was reacted with Boc-Phe-OBt (**17**) (0.73 mmol) dissolved in dichloromethane (5 ml) and DMF (5 ml) at 0 °C by addition of NMM (125 μl, 1.38 mmol). The mixture was stirred for 30 min at 0 °C and for 2 h at 20 °C and dichloromethane was removed *in vacuo*. The product was precipitated by addition of DMF (3 ml) and water (6 ml), filtered off and dried. Recrystallization from DMF (8 ml), chloroform (5 ml) and diethyl ether (7 ml) afforded 518 mg of **18** (94 % yield). M.p. 250–255 °C;  $[\alpha]_D^{20}$  –7.5 (*c* 0.7, DMF); Anal. C<sub>59</sub>H<sub>75</sub>N<sub>9</sub>O<sub>15</sub>S: C, H, N; <sup>1</sup>H NMR data are given in Table 1.

**Phe-Asn-Glu-Asn-Met-Ala-Tyr-OMe (19)**. Compound **18** (150 mg, 0.127 mmol) was suspended in DMS (3.7 ml) and the mixture was cooled to –78 °C. Addition of HF (1.3 ml) at this tempera-

ture gave a clear solution which was allowed to stand at 0°C for 2 h. The volatile material was removed with a stream of nitrogen within an efficient fume cupboard, and the product was precipitated with diethyl ether (10 ml) and washed 3 times with diethyl ether. HPLC of the crude material (eluent, 2 ml/min, methanol-water-acetic acid, 15:25:1) showed only one product absorbing at 280 nm with retention time 3.4 min. The material was stirred for 3 h with lukewarm methanol and filtered yielding 120 mg of product. This material (90 mg) was recrystallized from a mixture of DMSO (3 ml), DMF (6 ML) and diethylether (20 ml) affording 70 mg (82% yield) of 19. Amino acid analysis: Ala, 1.03; Asp, 2.20; Glu, 1.25; Met, 0.60; Phe, 1.08; Tyr, 0.82. <sup>1</sup>H NMR data are given in Table 1. <sup>13</sup>C NMR at 125 MHz in DMSO-*d*<sub>6</sub>: δ 171.2 (C<sup>α</sup>-1); 55.3 (C<sup>α</sup>-1); 36.2 (C<sup>β</sup>-1); 137.4 (C1-1); 129.5 (C2,6-1); 130.1 (C3,5-1); 127.0 (C4-1); 171.9 (C<sup>α</sup>-2); 50.1 (C<sup>α</sup>-2); 40.5 (C<sup>β</sup>-2); 172.2 (C<sup>α</sup>-2); 171.7 (C<sup>α</sup>-3); 54.2 (C<sup>α</sup>-3); 26.7 (C<sup>β</sup>-3); 31.9 (C<sup>γ</sup>-3); 175.6 (C<sup>β</sup>-3); 171.9 (C<sup>α</sup>-4); 50.3 (C<sup>α</sup>-4); 36.5 (C<sup>β</sup>-4); 172.2 (C<sup>α</sup>-4); 171.4 (C<sup>α</sup>-5); 51.8 (C<sup>α</sup>-5); 31.2 (C<sup>β</sup>-5); 29.6 (C<sup>γ</sup>-5); 14.7 (C<sup>α</sup>-5); 172.2 (C<sup>α</sup>-6); 48.2 (C<sup>α</sup>-6); 17.8 (C<sup>β</sup>-6); 170.6 (C<sup>α</sup>-7); 54.13 (C<sup>α</sup>-7); 36.2 (C<sup>β</sup>-7); 126.5 (C1-7); 128.4 (C2,6-7); 115.2 (C3,5-7); 156.1 (C4-7); 51.9 (CH<sub>3</sub>-O-).

#### Investigation of the reaction of HOBt with DCC by NMR-spectroscopy

HOBt (10 mg, 0.075 mmol, 1 eqv. or 20 mg, 0.15 mmol, 2 eqv) was suspended in deuteriochloroform, DCC (15 mg, 0.074 mmol) was added and the mixture was agitated for 3 min. <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded at 500 and 125 MHz respectively and at *T* = 300 K. The <sup>1</sup>H NMR data for the instantaneously formed complexes (20) and (21) are given in Table 2. <sup>13</sup>C NMR of 20: δ 141.9; 129.6, 126.3; 125.8; 117.9; 115.9; 111.2; 56.0; 35.2; 33.6; 25.8; 25.3; 24.9; 24.8. The mixture was stable for 2 h at 300 K and then decomposition products appear in the <sup>1</sup>H NMR spectrum. When HOBt was dissolved in DMSO-*d*<sub>6</sub> (50 μl) prior to reaction with DCC the equilibrium of the mixture was shifted in favour of the compound (20).

Acetic acid (4.4 μl, 0.074 mmol) was added and the appearances of resonances corresponding to the 1-hydroxybenzotriazolyl acetates (23 and (24) were followed by <sup>1</sup>H NMR spectroscopy at 500 or 90 MHz at 300 and 290 K respectively. The

results are summarized in Table 2 together with the <sup>1</sup>H NMR data on compound 23 and 24. Crystalline 23 was prepared by reaction of HOBt with acetyl chloride<sup>14</sup> and the IR spectrum of 23 had a characteristic carbonyl absorption at 1825 cm<sup>-1</sup>.

Reaction of the mixture of 23 and 24 with benzylamine (7.8 μl 0.07 mmol) gave an initial depression of the ratio 23/24 from 1.85 to 1.56 indicating that 23 reacts faster than does 24 with amines, in agreement with hydrolysis studies performed by Horiki.<sup>13</sup>

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