Synthesis of linear and cyclic peptides related to Met- and Leu-enkephalin*

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Abstract. The synthesis is described of several peptides containing the sequence Phe-Met. In order to limit enzymatic degradation and to restrict the number of possible conformations they were cyclized with variable ring size. Some unexpected side-reactions were encountered during the synthesis of the linear precursors: partial cleavage of the active ester during removal of the Boc--protecting group, partial cleavage of the Gly–Gly peptide bond during hydrazinolysis of an ester function and failure to obtain the *p*-nitrophenyl ester of Boc-Phe-Met-Gly-Gly-OH via the DCC method. Diphenyl phosphoroazidate was finally used for the cyclization of several linear peptides. Some results of behavioural activity and metabolic stability are mentioned.

Introduction

From studies by *de Wied* and coworkers on the influence of peptides related to ACTH** and β-LPH on active avoidance behaviour in rats, the common sequence H-Met-Glu-His--Phe-Arg-Trp-Gly-OH, *i.e.* ACTH-(4–10), emerged as a key fragment. The results of structure–activity studies with a large series of ACTH-derived peptides together with information from circular dichroism studies and conformational calculations according to the method of *Chou* and *Fasman*, suggested that the sequence 4–10 may assume an α-helical structure at the receptor site[1]. In this conformation the Phe approaches the *N*-terminal Met residue via a helical loop.

The finding that Met-enkephalin, *i.e.* H-Tyr-Gly-Gly-Phe--Met-OH, where Phe and Met are intra-chain neighbours appeared to be as active as ACTH-(4–10) in the pole--jumping test, and that oxidation of Met to the corresponding sulfoxide increased the potency of both ACTH-(4–10) and Met-enkephalin to the same extent[2], suggested that a common essential feature for behavioural activity in the pole-jumping test is the close proximity of the Phe and Met residues[2].

Cyclic analogues of biologically active peptide hormones have been synthesized in order to provide evidence for proposed conformations derived either from spectroscopic or theoretical studies. For example, the synthesis and properties of cyclic analogues of a modified ACTH-(5–10) sequence[3], *cyclo*-Leu-enkephalin[4], cyclic analogues of Met- and Leu-enkephalin[5,6], *cyclo*-oxytocin[7], cyclic somatostatin analogues[8] and cyclic LHRF analogues[9] have been reported.

Cyclic peptides with a variable ring size and containing Phe and Met were planned in this study. It was considered that the smallest cyclic peptide possible, the piperazinedione of Phe-Met, may be too constrained to give a good fit at the receptor site.

Therefore, we decided to increase the ring size either by incorporation of the dipeptide sequence Gly-Gly resulting in the cyclic analogue of des-tyrosine-Met-enkephalin, or by using an ω-aminoalkanoic acid as a connecting link, in this case ε-aminohexanoic acid[10,11].

Since the sulfoxide and sulfone analogues of Met-containing ACTH peptides are more active in the pole--jumping assay than the non-oxidized peptides[1,12] we also decided to synthesize some Met(O)- and Met(O_2)-containing cyclic peptides. When in ACTH-(4–10) the Phe[7] residue is exchanged for its D-isomer, a reversal of action (*i.e.* acceleration instead of delay of extinction of pole--jumping avoidance behaviour) is found[12]. We also included D-Phe- and, for the sake of completeness, D-Met--containing cyclic peptides in our series. Some side--reactions observed during the course of this work and some results of behavioural studies will be mentioned briefly.

Strategy and discussion of the synthesis

The classical approach of solution synthesis was followed for the preparation of the linear peptides using Boc groups for protection of the α-amino functions.

Methods frequently used for cyclization include the use of *p*-nitrophenyl (-ONp) or other active esters, azide reactions or large excesses of DCC (plus additive)[13]. Previous expe-

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* Part V in the series: Synthesis of fragments of human β-lipotropin, β-LPH. For part IV see This Journal **99**, 284 (1980).

** Standard abbreviations are used for amino acids and protecting groups [IUPAC-IUB Commission on Biochemical Nomenclature, Biochem. J. **126**, 773 (1972)]. The sequence of cyclic peptides is not given in alphabetical order but as the sequence that was cyclized; for example we will not write *cyclo*(-εAhx--Phe-Met-) but write *cyclo*(-Phe-Met-εAhx-) since activation took place at the εAhx residue.

Abbreviations: ACTH, adrenocorticotropic hormone; εAhx, ε-aminohexanoic acid; DPPA, diphenyl phosphoroazidate, $(PhO)_2P(O)N_3$; EEDQ, N-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline. For other abbreviations see part I of this series[15].

1 *H. M. Greven* and *D. de Wied* in Frontiers of Hormone Research Vol. 4, ed. *Tj. B. van Wimersma Greidanus*, S. Karger, Basel, 1977, p. 140.

2 *H. M. Greven* and *D. de Wied* in Hormones and the Brain, eds. *D. de Wied* and *A. van Keep*, MTP Press Ltd., Lancaster, England, 1980, p. 115.

3 *P. J. Romanovskis, I. V. Siskov, I. K. Liepkaula, E. A. Porunkevich, M. P. Ratkevich, A. A. Skujins* and *G. I. Chipens* in Peptides, Synthesis – Structure – Function, Proceedings of the 7th American Peptide Symposium, Wisconsin 1981, eds. *D. H. Rich* and *E. Gross*, Pierce Chem. Co., Rockford, Illinois., p. 229.

4 *H. Kessler* and *G. Hölzemann*, Angew. Chem. **93**, 90 (1981).

5 *D. Hudson, G. W. Kenner, R. Sharpe* and *M. Szelke*, Int. J. Pept. Protein Res. **14**, 177 (1979).

6 *J. DiMaio* and *P. W. Schiller*, Proc. Natl. Acad. Sci. USA **77**, 7162 (1980).

7 *M. Zaoral* and *V. Krchňák*, Collect. Czech. Chem. Commun. **42**, 3500 (1977).

8 *D. F. Veber, F. W. Holly, W. J. Paleveda, R. F. Nutt, S. J. Bergstrand, M. Torchiana, M. S. Glitzer, R. Saperstein* and *R. Hirschmann*, Proc. Natl. Acad. Sci. USA **75**, 2636 (1978).

9 *J. Seprodi, D. H. Coy, J. A. Vilchez-Martinez, E. Pedroza, W.-Y. Huang* and *A. V. Schally*, J. Med. Chem. **21**, 993 (1978).

10 *R. Deslauriers, S. J. Leach, F. R. Maxfield, E. Minasian, J. R. McQuie, Y. C. Meinwald, G. Némethy, M. S. Pottle, I. D. Rae, H. A. Scheraga, E. R. Stimson* and *J. W. Van Nispen*, Proc. Natl. Acad. Sci. USA **76**, 2512 (1979).

11 *G. Némethy, J. R. McQuie, M. S. Pottle* and *H. A. Scheraga*, Macromolecules **14**, 975 (1981).

12 *H. M. Greven* and *D. de Wied* in Progress in Brain Research Vol. 39, eds. *E. Zimmermann, W. H. Gispen, B. H. Marks* and *D. de Wied*, Elsevier Scientific Publ. Co., Amsterdam, 1973, p. 429.

13 See e.g. *K. D. Kopple*, J. Pharm. Sci. **61**, 1345 (1972).

rience in the cyclization of H-Ala-Gly-εAhx-OH had shown the *p*-nitrophenyl ester approach to give the best results[10,14]; a 48% yield, after purification, of *cyclo* (-Ala-Gly-εAhx-) was obtained.

With the exception of the cyclization of H-Phe-Met-OH, which was carried out by treating H-Phe-Met-OMe with Et_3N/MeOH, our first attempts at cyclization of longer peptides were therefore via ONp esters. Since activation of εAhx and Gly residues proceeds without racemization, whereas racemization is possible upon activation of the *C*-termini in Boc-εAhx-Phe-Met-OH or Boc-Gly-Gly-Phe--Met-OH, we elongated the dipeptide Boc-Phe-Met-OH, obtained by saponification of the methyl ester[15], with H-εAhx-OMe and H-Gly-Gly-OMe. Saponification of the resulting esters followed by conversion into the ONp esters and removal of the α-amino protecting Boc group would then provide the desired compounds for cyclization (see Fig. 1).

Boc-Phe-Met-εAhx-ONp was synthesized without problems (see Experimental section). However, removal of the Boc function as described for Boc-Ala-Gly-εAhx-ONp[10,14] was not satisfactory in the present case.

Even the use of a small excess of HCl/EtOAc at a low temperature for a short period of time resulted in the partial cleavage (approx. 15%) of the ONp ester bond. This is in contrast to the apparent acid stability of ONp ester bonds in other peptides when *e.g.* HBr/TFA is used to remove the α-amino protecting Z function (see *e.g.* refs. 13, 16). Since TFA treatment of Boc-peptide-ONp esters at room temperature has also been reported to be successful (*e.g.* refs. 13, 17), we treated Boc-Phe-Met-εAhx-ONp with TFA for 45 min at 0–4°C. After working up we also noticed the formation of the free peptide (we observed a similar side-reaction upon treatment of Boc-Phe-Met-ONp with cold acid). Cyclization of H-Phe-Met-εAhx-ONp·HCl, containing some free tripeptide, in pyridine at high dilution gave a good yield of *cyclo*(-Phe-Met-εAhx-), leaving the contaminant H-Phe-Met-εAhx-OH unchanged.

Conversion of Boc-Phe-Met-Gly-Gly-OH into the corresponding ONp ester using the same conditions as for the εAhx-containing peptide, failed to give the Boc-tetrapeptide-ONp ester (when the reaction was carried out at 0°C, the same negative results were obtained). In view of this and the partial cleavage of the active ester bond we decided to use Boc-Gly-Gly-Phe-Met-OMe[15] in order to make *cyclo* (-Gly-Gly-Phe-Met)≡*cyclo*(-Phe-Met-Gly-Gly-). Since activation of the free Boc-tetrapeptide-acid with DCC/HONp may lead to racemization we decided to convert the ester into the corresponding hydrazide and to perform an azide reaction (safer from a racemization-point-of-view) for the ring closure. When the peptide ester was treated with hydrazine hydrate at room temperature overnight a mixture of products was obtained. Although on one occasion an almost 70% yield of a reasonably pure product was obtained, this approach was not considered to be very successful*.

Milder hydrazinolysis conditions (lower temperature, less excess of reagent) also did not yield a homogeneous product. Isolation and amino acid analysis of the main by--product showed only one Gly residue per Phe and Met. Cleavage of Gly–Gly bonds with hydrazine, albeit under more severe conditions, has been described in the literature[18].

* In a very recent study, *Honegger* et al.[19] treated Leu-enkephalin, γ-endorphin and dynorphin-(1–13) with 50% hydrazine at 75°C for 30 min and found a 10–50% cleavage of only the Gly–Gly bond in these three peptides.

[14] *R. Deslauriers, D. J. Evans, S. J. Leach, Y. C. Meinwald, E. Minasian, G. Némethy, I. D. Rae, H. A. Scheraga, R. Somorjai, E. R. Stimson, J. W. van Nispen* and *R. W. Woody*, Macromolecules **14**, 985 (1981).

[15] *W. A. A. J. Bijl, J. W. van Nispen* and *H. M. Greven*, Recl. Trav. Chim. Pays-Bas **98**, 571 (1979).

[16] *K. D. Kopple* and *A. Go*, J. Am. Chem. Soc. **99**, 7698 (1977).

[17] *U. Ludescher* and *R. Schwyzer*, Helv. Chim. Acta **55**, 2052 (1972).

[18] *J. H. Bradbury*, Biochem. J. **68**, 475 (1958).

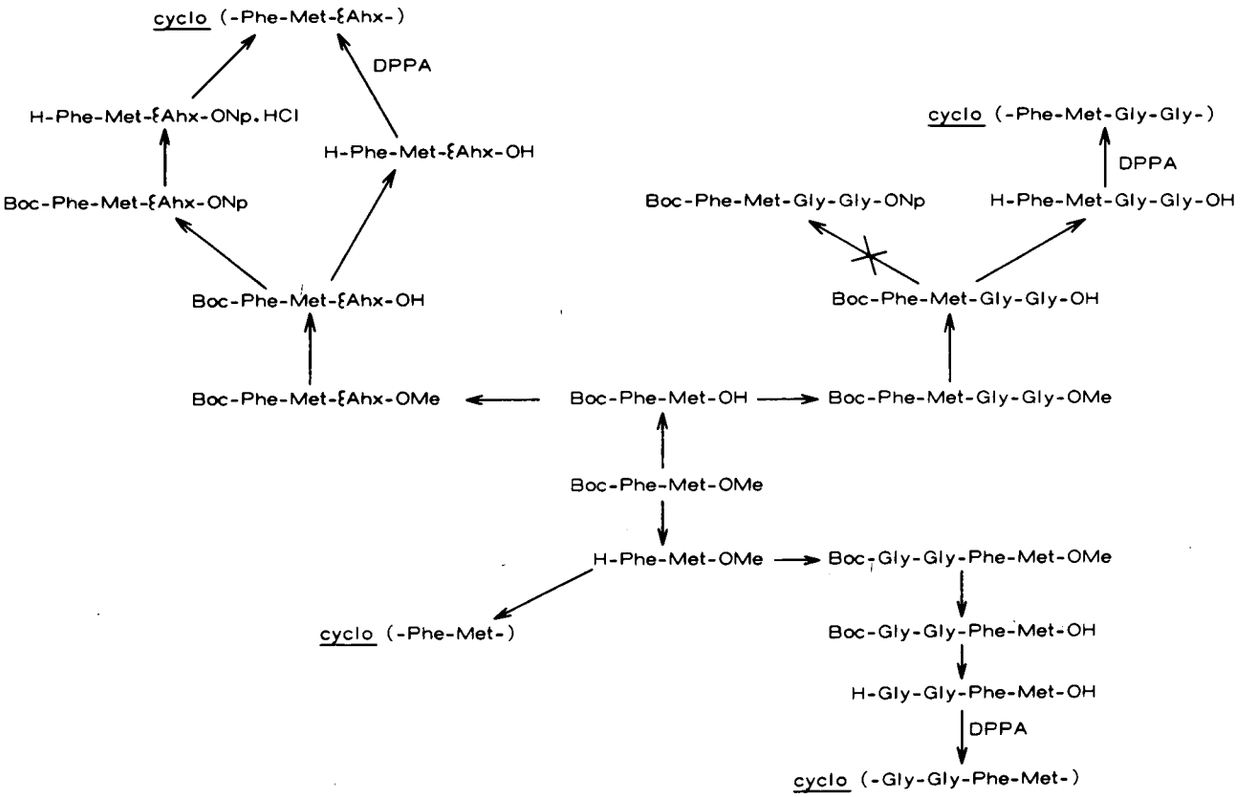

Fig. 1. Schematic representation of the synthesis of several linear and cyclic peptides containing -Phe-Met-.

After we had observed the above-mentioned side-reactions, a practical procedure for the synthesis of cyclic peptides was reported by *Varga* et al.[20]. They used diphenyl phosphoroazidate[21], DPPA, at a low temperature in high dilution for the cyclization of free peptides; good yields were obtained and little or no racemization was found[20]. We have now used this DPPA method for the cyclization of several free peptides containing εAhx or Gly at the *C*-terminus and isolated the desired cyclic peptides in 13–45% yields (after purification). These yields were comparable with those reported by *Varga* et al.[20] but somewhat lower than with ONp ester cyclizations. An advantage of this approach is that we did not experience any problems in the synthesis of the free, linear precursor peptides*.

Behavioural studies

When *cyclo*(-Phe-Met-εAhx-) was tested in the pole-jumping assay it was found to be as active as ACTH-(4–10)[22] in delaying extinction of pole-jumping avoidance behaviour[23].
This supports the hypothesis of *Greven* and *de Wied*[1,2] that the spatial proximity of Phe and Met is essential for inducing a delay in extinction in this test.
For acceleration of extinction of avoidance behaviour in the pole-jumping test different structure–activity relationships exist. Introduction of a D-Phe residue in ACTH-(4–10) results in acceleration of extinction whereas the same modification in the endorphin sequence H-Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-OH leads to behavioural inactivity. When *cyclo*(-D-Phe-Met-εAhx-) was tested in the pole-jumping test, it *delayed* the extinction of the avoidance response, with a potency 3–10 times that of ACTH-(4–10) (*De Wied, van Nispen* and *Greven*, unpublished results), perhaps a reflection of a further increase of metabolic stability.
The effect of *cyclo*(-Phe-Met-εAhx-) and several other cyclic (and linear) peptides on acquisition of the step-up active avoidance response[24] has also been examined[25]. It was found that *cyclo*(-Phe-Met-εAhx-) decreased acquisition but with a longer time course of efficacy than for ACTH-(4–10) or Met-enkephalin. In contrast to these last two peptides, the cyclic analogue was active after oral administration in this step-up test[25].

Experimental

The purity of the amino acid derivatives and peptides was checked by thin-layer chromatography (TLC) on Merck silicagel plates (F.254, 0.25 mm) using the following solvent systems (ratios are v/v):
a) toluene/ethanol = 4/1
b) chloroform/methanol = 4/1
c) chloroform/methanol/water = 70/30/5
d) *n*-butanol/acetic acid/water = 4/1/1
e) *n*-butanol/acetic acid/water = 8/1/1
f) *n*-butanol/pyridine/acetic acid/water = 8/3/1/4
g) *n*-butanol/pyridine/acetic acid/water = 16/3/1/4
h) chloroform/methanol = 9/1
i) chloroform/trifluoroethanol = 4/1
j) methylene chloride/methanol = 4/1

UV (254 nm), fluorescamine and chlorine/*o*-tolidine were used for the detection of components on TLC plates.
The description of other methods is given in part I[15].
High-performance liquid chromatography (HPLC) of some of the end-products was carried out on a Spectra Physics (SP 8000) apparatus[15]. Several linear and cyclic peptides had been synthesized before the introduction of HPLC in our laboratories; in

* The applicability of DPPA in cyclization reactions was recently[6] demonstrated in the cyclization of Tos-A_2bu-Gly-Phe-Leu-OH (A_2bu standing for L-α,γ-diaminobutyric acid). While DCC, EEDQ and the classical azide reaction were unsuccessful in formation of the ring, a 50% yield of the desired cyclic peptide could be obtained using DPPA.

19 *A. Honegger, G. J. Hughes* and *K. J. Wilson*, Biochem. J. **199**, 53 (1981).
20 *S. L. Varga, S. F. Brady, R. M. Freidinger, R. Hirschmann, F. W. Holly* and *D. F. Veber* in Peptides 1978, Proceedings of the XVth European Peptide Symposium, Gdánsk, Poland, eds. *I. Z. Siemion* and *G. Kupryszewski*, Wroclaw Univ. Press, Wroclaw, Poland 1979, p. 183.
21 *T. Shioiri, K. Ninomiya* and *S. Yamada*, J. Am. Chem. Soc. **94**, 6203 (1972).
22 *J. W. van Nispen* and *H. M. Greven*, Pharmacol. Ther. **16**, 67 (1982).
23 *D. de Wied*, Proc. Soc. Exp. Biol. Med. **122**, 28 (1966).
24 *H. Rigter, I. Dekker* and *J. L. Martinez, Jr.*, Regulatory Peptides **2**, 317 (1981).
25 *H. Rigter, H. M. Greven, J. W. van Nispen* and *J. L. Martinez*, Biochem. Biophys. Res. Commun. **98**, 424 (1981).
26 *W. Danho* and *C. H. Li*, Int. J. Protein Res. **3**, 99 (1971).

Table I Data on protected intermediates in the synthesis of cyclic peptides (see also Fig. 1).

Peptide	Method; yield after purification	TLC, R_f value (system)	$[\alpha]_D^{21}$ (*c* 1, DMF)
Boc-Gly-Gly-Phe-Met-OMe	DCC/HOBt; 89% (ref. 15)	0.44 (a)	−22.5°
Boc-Gly-Gly-Phe-Met-OH	aqueous NaOH/dioxane; 91%	0.73 (g)	−13.8°
Boc-Phe-Met-Gly-Gly-OMe	DCC/HOBt; 76%	0.43[a] (a)	
Boc-Phe-Met-Gly-Gly-OH	aqueous NaOH/dioxane; 72%	0.86 (c)	−10.9°
Boc-D-Phe-Met-OH	-ONp ester with free Met; 62%	0.70 (j)	−13.1° (MeOH)
Boc-D-Phe-Met-εAhx-OMe	DCC/HOBt; 81%	0.50 (a)	−24.4°
Boc-D-Phe-Met-εAhx-OH	aqueous NaOH/dioxane; 90%	0.30[a] (a)	−17.9°
Boc-Phe-D-Met-OH	-ONp ester with free D-Met; oil	0.67 (j)	
Boc-Phe-D-Met-εAhx-OMe	DCC/HOBt; 60%[b]	0.52 (a)	+23.5°
Boc-Phe-D-Met-εAhx-OH	aqueous NaOH/dioxane; 90%	0.32 (a)	+23.6°
Boc-Phe-Leu-OMe	-ONp ester; 73%	0.70 (a)	−16.5°
Boc-Phe-Leu-OH	aqueous NaOH/dioxane; 71%	0.35 (a)	−8.7°; −15.2° (MeOH)[d]
Boc-Phe-Leu-εAhx-OMe	DCC/HOBt; 70%	0.50 (a)	−26.7°
Boc-Phe-Leu-εAhx-OH	aqueous NaOH/dioxane; 83%	0.46 (c)	−14.4°
Boc-Phe-Leu-εAhx-ONp	DCC/HONp; −[c]	0.56[c] (a)	

[a] Not completely homogeneous; two faint spots of by-products were seen. [b] This 60% is an overall yield of the two reactions, including purification on a SiO_2 column. [c] A more than theoretical yield was obtained; TLC indicated however, that the product contained residual DCU and HONp. [d] A value of −11.5° in MeOH has been reported[26].

Table II Some data on cyclic and linear end-products (see also Fig. 1).

Peptide	Amino acid analysis[a]				Mass spectrum (M^+ peak)	$[\alpha]_D^{21}$	HPLC (main component in %)	TLC R_f value (system)
	Phe	Met or Met(O) or Met(O_2)	εAhx	Gly				
cyclo (-Phe-Met-)[b]	1.03	0.97				−57.1° (*c* 1, DMF)		0.76 (c)
H-Gly-Gly-Phe-Met-OH	1.02	0.96		2.02		−18.6° (*c* 1, 10% HOAc)	95.6[c]	0.30 (g)
H-Phe-Met-Gly-Gly-OH	1.04	1.01		1.95		+14.9° (*c* 1, 10% HOAc)	98.1[c]	0.41 (f)
cyclo (-Phe-Met-Gly-Gly-)[d]	0.99	1.02		1.98	392	−41.6° (*c* 0.5, DMF)		0.77 (d)
H-Phe-Met(O)-Gly-Gly-OH	0.92	0.90		2.08		+17.4° (*c* 1, 10% HOAc)	97.0[c]	0.17 (f)
cyclo (-Phe-Met(O)-Gly-Gly)[e]	0.98	0.95		2.02		− 6.7° (*c* 0.5, DMF)		0.54 (c)
cyclo (-Phe-Met(O_2)-εAhx-)[f]	0.90	0.89[e]	1.10					
H-D-Phe-Met-εAhx-OH	1.02	1.01	0.96			− 108° (*c* 1, 10% HOAc)	99.4[g]	0.61 (g)
cyclo (-D-Phe-Met-εAhx-)[h]	1.07	1.00	0.93		391	−[i]	90.0[j]	0.83 (e)
H-Phe-D-Met-εAhx-OH	1.01	1.00	0.86			+ 108° (*c* 1, 10% HOAc)	94.2[g]	0.61 (g)
cyclo (-Phe-D-Met-εAhx-)[k]	1.04	1.00	0.97		391	+ 45° (*c* 0.5, DMF)	92.2[j]	0.84 (e)
cyclo (-Phe-Leu-εAhx-)[l]	1.02	1.00 (Leu)	0.98		373	−[i]		0.88 (b)

[a] After acid hydrolysis, LAO digestions were also performed. No racemization could be detected. [b] Obtained after treatment of H-Phe-Met-OMe in MeOH containing Et_3N. [c] Stationary phase: Nucleosil 10C-18. Mobile phase: A, 0.1% H_3PO_4, pH 2.1; B, MeOH/H_2O 50/50 with the addition of 0.05 mol/l TMAH and H_3PO_4 till pH 2.8. Gradient: of 80% A +20% B in 35 min to 0% A +100% B, then 5 min at this composition. [d] Cyclization using DPPA; after working up with a mixed bed resin and recrystallization from *n*-butanol, approx. 45% of the cyclic peptide were obtained. [e] Oxidation of the linear peptide with H_2O_2 in aqueous HOAc resulted in a mixture of Met(O)- and Met(O_2)-containing tetrapeptide. Cyclization using DPPA followed by chromatography on SiO_2 using system e yielded approx. 23% of *cyclo* (-Phe-Met(O)-Gly-Gly-). [f] According to the procedure given under d. Chromatography on SiO_2 (system e) gave approx. 25% of the sulfone; a small amount of the corresponding Met(O) analogue was still present. [g] Conditions as for H-Phe-Met-εAhx-OH. [h] Cyclization using DPPA; overall yield after purification by chromatography on SiO_2 was 13% (solvent system i). [i] The peptide was not soluble enough to measure the rotation. [j] HPLC conditions as in ref. 15; linear gradient from 30% A +70% B to 100% B in 15 min, then 5 min with 100% B. [k] Obtained in an overall yield of 20% as for the corresponding D-Phe cyclic peptide. [l] Removal of the Boc group from Boc-Phe-Leu-εAhx-ONp with cold HCl/EtOAc also results in the partial cleavage of the ONp ester. Cyclization of the resulting product was in pyridine as described for H-Phe-Met-εAhx-ONp. Trituration of the isolated reaction product with EtOH gave TLC-homogeneous cyclic peptide in approx. 40% yield.

some other cases the cyclic peptides showed a too low solubility in the mobile phase to give meaningful data. Mass spectrometry was performed on the cyclic peptides in order to establish their ring size.

The synthesis of the key compound *cyclo*(-Phe-Met-εAhx-) will be given in detail below. The important data on intermediates are summarized in Table I while in Table II some properties of the linear and cyclic end-products are presented.

Boc-Phe-Met-εAhx-OMe

10 mmol (3.96 g) of Boc-Phe-Met-OH [obtained in 94% from the corresponding methyl ester[15] with aqueous NaOH in dioxane; m.p. 142–143°C; $[\alpha]_D^{21}$ −10.1° (*c* 1, DMF)] was dissolved in DMF (1 mmol in 1–1.2 ml) and 1 equiv. of H-εAhx-OMe·HCl[10,14] was added. After the addition of *N*-ethylmorpholine (1 equiv.) the solution was cooled to 0°C, HOBt (1.5 equiv.) and DCC (1 equiv.) were added and the solution was stirred for 2 h at 0°C and overnight at room temperature under a N_2 blanket. The solution was then cooled, the precipitated DCU filtered and the filtrate evaporated. The residue was dissolved in EtOAc and the organic phase washed successively with H_2O, 5% $NaHCO_3$ solution, H_2O, 5% $KHSO_4$ solution, H_2O and saturated NaCl solution. After drying on anhydrous Na_2SO_4, the filtered solution was concentrated and pet. ether was added. The precipitate was filtered after cooling, washed and dried. Yield 4.66 g (89%); m.p. 84–85.5°C; $[\alpha]_D^{21}$ −30.0° (*c* 1, DMF). TLC: R_f 0.50 (b), 0.95 (a).

Boc-Phe-Met-εAhx-OH

3.48 g (6.65 mmol) of Boc-Phe-Met-εAhx-OMe were dissolved in 50 ml of dioxane/water (9/1 v/v) and 1.4 equiv. of NaOH (as 2 N solution) were added. After 45 min the solution was acidified with $KHSO_4$ solution, evaporated to dryness and the residue dissolved in EtOAc; after washing with H_2O and saturated NaCl solution and drying on anhydrous Na_2SO_4 ether/pet. ether (1/3) was added to the concentrated EtOAc solution. Yield after washing and drying 3.08 g (91%); m.p. 79–81°C; $[\alpha]_D^{21}$ −14.3° (*c* 1.2, DMF). TLC: R_f 0.75 (b), 0.80 (c).

Boc-Phe-Met-εAhx-ONp

To a cooled solution of 2.48 g (4.87 mmol) of Boc-Phe-Met-εAhx-OH in 25 ml of DMF were added 1.1 equiv. of DCC and HONp. After stirring for 2 h at 0–4°C and overnight at room temperature under N_2, the precipitated DCU was removed by filtration and the filtrate was evaporated to dryness. The yellow, oily residue was dissolved in ether to which a few drops of MeOH had been added; pet. ether was then added and the solution cooled. Crystalline material was formed slowly; after filtration and washing with pet. ether, ether and again pet. ether, the compound was dried (86%). TLC showed that some DCU and traces of unknown by-products were still present. Therefore, the product was dissolved in a few ml of MeOH, and ether and cold pet. ether were added; 62% of the tripeptide derivative were obtained with m.p. 126–128°C and $[\alpha]_D^{21}$ −12.3° (*c* 1.5, DMF). TLC: R_f 0.60 (a), 0.90 (b).

H-Phe-Met-εAhx-ONp·HCl

Treatment of the Boc-tripeptide active ester with HCl/EtOAc as described for Boc-Ala-Gly-εAhx-ONp[14] did not result in a pure product. The best results were obtained as follows: 770 mg (1.22 mmol) of Boc-Phe-Met-εAhx-ONp were suspended in EtOAc (3.2 ml) and the suspension cooled to 0°C.

With stirring 2.84 ml of a cold 4.29 N HCl/EtOAc solution were added (final conc. 2 N HCl/EtOAc). Dissolution took place and after 30 min at 0–4°C, EtOAc was added and the solution concentrated (rotatory evaporator) at diminished pressure. A precipitate was formed during the concentration step, filtered and thoroughly washed with EtOAc; 340 mg were obtained after drying. TLC showed that not all of the starting material was converted; therefore, the mother liquor was evaporated to dryness and the HCl treatment repeated. A second crop of 290 mg (total of 90%) was obtained. TLC of both crops showed a main component (R_f value 0.30 system a, 0.67 system h) that was UV- and fluorescamine-positive as well as positive in the chlorine/*o*-tolidine reaction. A second component with R_f value 0.23 (a) or 0.61 (h) was present in amounts of approx. 15%, negative with regard to UV quenching indicating the absence of the *p*-nitrophenyl group. Later it was confirmed that this was H-Phe-Met-εAhx-OH·HCl.

H-Phe-Met-εAhx-ONp·TFA

When instead of HCl, cold TFA was used for the removal of the Boc group from the tripeptide active ester (45 min reaction time

at 0°C in the presence of anisole; working up was with ether), the presence of a spot with lower R_f value was observed indicating removal of the ONp group had also taken place here.

H-Phe-Met-εAhx-OH

Boc-Phe-Met-εAhx-OH was treated with TFA/H_2O (9/1, v/v) in the presence of anisole for 1 h at room temperature. The solution was then concentrated and ether added. The isolated oily product was dissolved in *tert*-butanol/water and the solution treated with an ion-exchange resin in the acetate form, and lyophilized. The peptide was freed from traces of by-products by chromatography on silica gel using solvent system g.
$[\alpha]_D^{21}$ +11.8° (*c* 1, 10% aqueous HOAc). TLC: R_f 0.70 (g), 0.37 (d). Amino acid analysis: Phe 1.03, Met 1.04, εAhx 0.94. LAO digestion: no racemization of Phe and Met was found.
HPLC: main component 94% (mobile phase as in ref. 15; 25 min linear gradient of 100%A–0%B to 30%A–70%B and then 5 min at this composition).

Cyclo(*-Phe-Met-εAhx-*)

a.1 *via H-Phe-Met-εAhx-ONp·HCl*
H-Phe-Met-εAhx-ONp·HCl was dissolved in a small volume of DMF. This solution was added dropwise over a period of 5 h (with stirring) to pyridine (approx. 600 mg peptide/l pyridine) at about 50°C under a N_2 blanket. After standing overnight the solution was evaporated to dryness, the residue dissolved in MeOH and the solution evaporated again. TLC showed that the active ester had disappeared but that the lower-running fluorescamine-positive H-Phe-Met-εAhx-OH was still present.
Trituration of the residue with water and subsequent filtration was performed several times; this treatment removed the fluorescamine-positive by-products. Further washing with methanol gave the title compound in approx. 47% yield. TLC of the sparingly soluble cyclic peptide (a 0.5% solution in warm DMF or trifluoroethanol was used) showed a nearly homogeneous preparation; R_f 0.92 (b), 0.85 (d), 0.60 (i). M.p. >270°C. Amino acid analysis: Phe 0.98, Met 0.96, εAhx 1.08. LAO digestion: no racemization of Phe and Met was found. Mass spectrometry: M^+ peak 391; no di- or trimeric isomers were found. ^1H NMR spectroscopy of a dilute solution in $CDCl_3$ + 10% CD_3OD: δ 2.0 (s, CH_3S–); δ 3.0 (AB part of ABX system, $-CH_2-Ph$); δ 4.5 (t, X part of ABX system, $-CH-CH_2-Ph$); δ ca. 7.3 (m, C_6H_5–).

a.2 *via H-Phe-Met-εAhx-ONp·TFA*
Cyclization of H-Phe-Met-εAhx-ONp·TFA as described under *a*.1 gave, after recrystallization from warm DMF/ether, the cyclic peptide in a yield of approx. 34%. TLC: R_f 0.63 (a). Analytical data were in agreement with those reported under *a*. **1**.

b. via H-Phe-Met-εAhx-OH
The free peptide was dissolved in DMF (approx. 1.5 g/l) and the solution cooled to approx. −20°C. The pH was adjusted to 7.5 with Et_3N and 4 ml of DPPA (Aldrich) were added. After one day at −20°C the reaction mixture was placed at 0°C and kept for 4 more days. On days 2 and 4, 1 ml of DPPA was added while the pH was maintained at approx. 7.5 by periodic addition of triethylamine.
After 5 days, no fluorescamine-positive material could be detected on TLC plates; the reaction mixture was concentrated to about one-third of its volume and water was added. All ionic species were then removed by treatment with a mixture of acid and base ion-exchange resins. After filtration, the filtrate was evaporated to dryness and the residue triturated with methanol; yields ranged from 30 to 70%. For further purification the material was chromatographed on a silica gel column (Merck Fertigsäule) using the solvent system chloroform/trifluoroethanol (4/1, v/v); overall yields 10 to 20%. TLC showed the same R_f values as given under *a*. **1**. Correct amino acid compositions and mass spectra were obtained; no racemization was found.

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Synthesis of peptide-morphinans based on *Diels–Alder* adducts of thebaine with enkephalin moieties (Chemistry of opium alkaloids, Part XVI)*

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Abstract. The preparation of *Diels–Alder* adducts of (−)-thebaine and ethyl acrylate is described. Hydrolysis of the major adduct gave the 7α-carboxylic acid which was coupled with the ethyl esters of L-leucine, L-phenylalanyl-L-leucine and glycyl-L-phenylalanyl-L-leucine, respectively, three peptide segments derived from the endogenous opiate, leucine-enkephalin. These compounds, as well as the 7β-ethoxycarbonyl isomer, were *O*-demethylated to give the corresponding 3,6-dihydroxymorphinan derivatives. *N*-(6,14-*endo*-Etheno-6,7,8,14-tetrahydrothebaine-7α-carbonyl)-L-phenylalanyl-L-leucine ethyl ester and its 7,8-dihydromorphine analogue were reduced to the corresponding leucinols.
Pharmacological screening** showed that several of these compounds are morphine-like analgesics, notably compound **11**, *N*-(6,14-*endo*-etheno-7,8-dihydromorphine-7α-carbonyl)-L-leucine ethyl ester.

Introduction

The identification of methionine- and leucine-enkephalin, two brain pentapeptides with opiate activity, by *Hughes*, *Kosterlitz* et al.[1] has intensified the study of the structure–activity relationships of analgesic compounds. A struc-

* Part XV: *P. R. Crabbendam*, *L. Maat* and *H. C. Beyerman*, Recl. Trav. Chim. Pays-Bas **100**, 293 (1981).
** Dr. *A. E. Jacobson*, National Institutes of Health, Bethesda, Maryland, U.S.A.
[1a] *J. Hughes*, Brain Res. **88**, 295 (1975).
[b] *J. Hughes*, *T. W. Smith*, *H. W. Kosterlitz*, *L. A. Fothergill*, *B. A. Morgan* and *H. R. Morris*, Nature (London) **258**, 577 (1975).