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Synthesis of fragments of arginine vasopressin and oxytocin containing a cystine residue in position 6

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Abstract. The synthesis is described of several fragments of the neurohypophyseal nonapeptide hormones arginine vasopressin and oxytocin. One group of fragments is characterized by the presence of an asymmetrical disulfide (a cystine residue in position 6), a second group consists of dimeric (symmetrical disulfides) fragments.
As common intermediates, peptides containing an *S*-tritylcysteine residue were synthesized by the fragment condensation approach. Treatment with methoxycarbonylsulfenyl chloride followed by reaction with the free thiol function of cysteine gave the asymmetrical disulfides, while treatment of the *S*-tritylcysteine containing peptide with iodine resulted in the corresponding dimers. Peptides with an *N*-terminal glutamine residue (position 4) were found to convert spontaneously into the corresponding pyroglutamic acid products.

Introduction

The pituitary peptides oxytocin (OT*) and vasopressin (either as 8-lysine vasopressin, LVP, or as in most mammals including man 8-arginine vasopressin, AVP) are long known for their peripheral, hormonal activities[1].
The main actions are uterotonic and milk-ejecting activity for oxytocin and the influence on blood pressure and diuresis for vasopressin (also called antidiuretic hormone, ADH).
After the successful first synthesis of these nonapeptides by *Du Vigneaud* in 1954[2], large numbers of analogues have been synthesized and tested for oxytocic, pressor and antidiuretic activity.
Several agonists and antagonists of these responses with improved selectivity of action are known[3].
In 1965, *de Wied* found that pituitary vasopressin is involved in behavioural processes in rats[4]. Further studies over the years have shown that in intact rats vasopressin increases resistance to extinction of active[5] and passive avoidance behaviour[6], and protects against induced amnesia[7]. Interestingly, oxytocin shows the opposite effect in several test situations[8]. It has been shown that the complete nonapeptide structure of these two molecules is essential for full endocrine activity[9]. For instance, removal of the *C*-terminal glycinamide from AVP results in a peptide with only 10^{-5} of the pressor activity of AVP and about 1% of its antidiuretic activity (unpublished results). 9-Desglycinamide oxytocin possesses only weak uterotonic activity (2.4 U/mg compared with approx. 500 U/mg for oxytocin itself)[10]. This is in contrast to the nearly intact central activities of these and other fragments of vasopressin and oxytocin[11,12,13].

When oxytocin and AVP are incubated with proteolytic enzymes of a brain synaptic membrane fraction, several peptides are formed, in different amounts; they can be isolated by high-performance liquid chromatography (HPLC)[14]. By a combination of techniques the primary structures of these fragments were found to be the sequences 2–9, 3–9, 4–9 and 5–9 in the case of oxytocin and 2–9, 3–9, 4–9 and 4–8 in the case of AVP, indicating an aminopeptidase type of enzyme[14]. These fragments all contain the cystine (Cyt) residue, *i.e.* an intact disulfide bridge.

* Standard abbreviations are used for amino acids and protecting groups [IUPAC-IUB Commission on Biochemical Nomenclature, Eur. J. Biochem. **53**, 1 (1975); **27**, 201 (1972)].
Other abbreviations are:
Acm - acetamidomethyl; AVP - arginine vasopressin; Cyt - cystine; Mbs - 4-methoxybenzenesulfonyl; MSA - methanesulfonic acid; NEM - *N*-ethylmorpholine; OT - oxytocin; Scm - methoxycarbonylsulfenyl; TMAH - tetramethyl ammonium hydroxide; Trt - triphenylmethyl, trityl.

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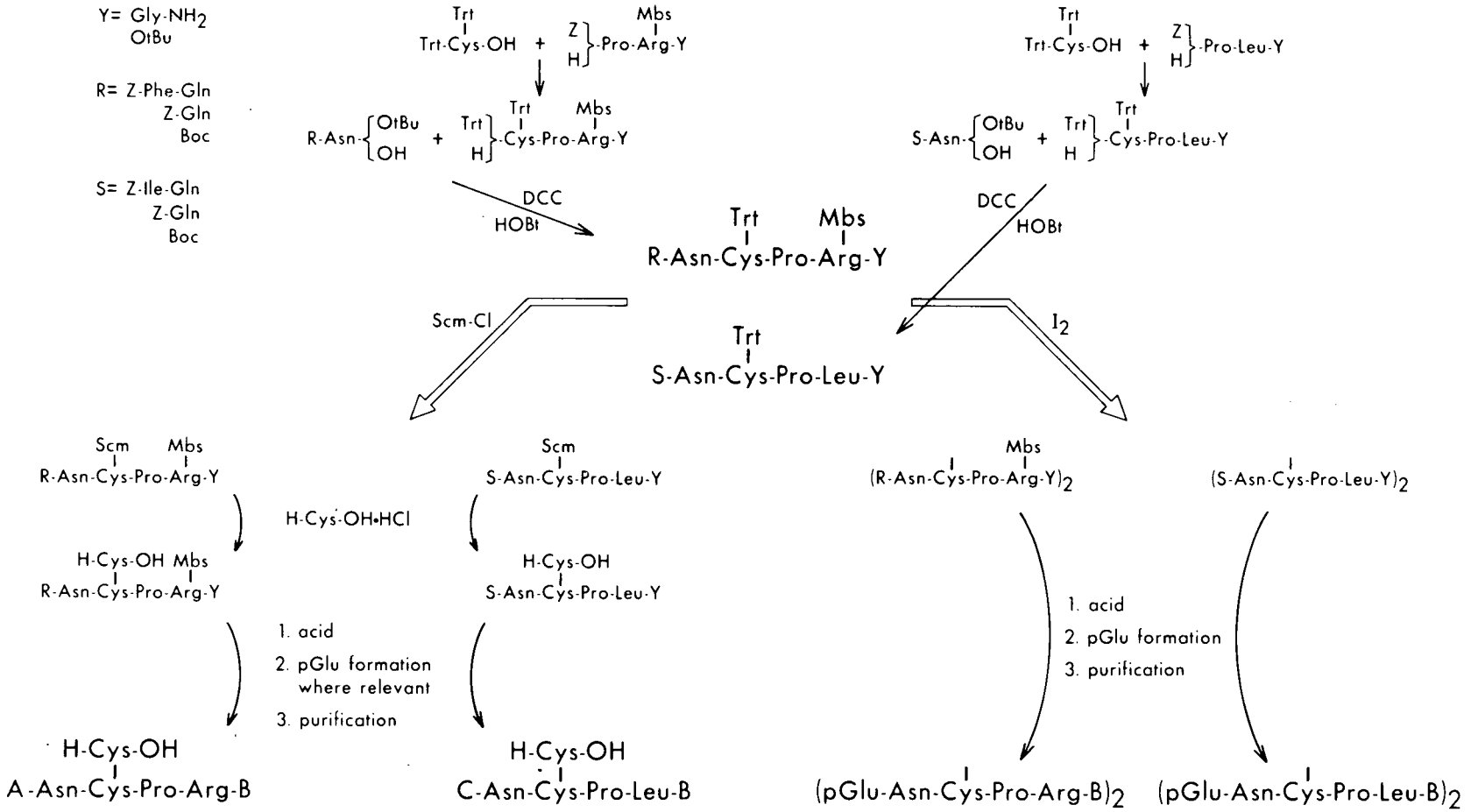

1	A = H-Phe-Gln ; B = $Gly-NH_2$	**7**	C = H-Ile-Gln ; B = $Gly-NH_2$	**5**	B = $Gly-NH_2$	**11**	B = $Gly-NH_2$
2	A = pGlu ; B = $Gly-NH_2$	**8**	C = pGlu ; B = $Gly-NH_2$	**6**	B = OH	**12**	B = OH
3	A = pGlu ; B = OH	**9**	C = pGlu ; B = OH				
4	A = H ; B = $Gly-NH_2$	**10**	C = H ; B = $Gly-NH_2$				

Fig. 1. Scheme of synthesis of fragments of AVP and OT containing a cystine residue in position 6, both asymmetrical disulfides and dimers. (Reproduced from the Proceedings of the Eight American Peptide Symposium with permission of Pierce Chemical Co., U.S.A.).

Interestingly, the 4–9 and 4–8 fragments had a pyroglutamic acid residue as the *N*-terminal end[14]. Most of the peptides found in these studies were synthesized and were found to co-elute in HPLC with their isolated counterparts. When these fragments were tested in a passive avoidance situation in rats, they were found to be more potent than the parent molecules[15,16].

In this paper we will describe the synthesis of thirteen fragments of AVP and oxytocin, including those with a pyroglutamic acid residue in position 4. A preliminary report has been presented at the 8th American Peptide Symposium[17].

Strategy and description of the synthesis

The synthesis of asymmetrical disulfides via sulfenyl thiocarbonates, R–S–S–$COOCH_3$, has been reported by *Brois* et al.[18]. *Kamber* has applied the thiol-induced fragmentation of sulfenyl thiocarbonates, resulting in the formation of mixed disulfides, to the synthesis of open-chain asymmetrical cystine peptides[19]. He found that the methoxythiocarbonate derivatives could easily be obtained by treatment of *S*-Trt- or *S*-Acm-cysteine-containing peptides with methoxycarbonylsulfenyl chloride, Scm-Cl[20].

The formation of the disulfide bond was then effected by reaction with the free thiol function of cysteine-containing peptides. Using this approach various insulin fragments have been synthesized by *Kamber*[19] and later also by *Kullmann*[21]. Other successful syntheses of asymmetrical disulfides have been reported *e.g.* by *Hiskey*[22] and *Weber* and *Schmid*[23] *.

The presence of a trityl function also makes possible the formation of symmetrical disulfides by a one-step reaction with iodine[29]. Since we were also interested in symmetrical disulfide fragments of AVP and oxytocin, taken together with the good experience of one of us with the Scm approach for the synthesis of asymmetrical disulfides[30], we decided to use the Trt group for *S* protection.

The fragment condensation approach was used to synthesize the key fragments R-Asn-Cys(Trt)-Pro-Arg(Mbs)-Y and S-Asn-Cys(Trt)-Pro-Leu-Y (see Fig. 1). Benzyloxycarbonyl (Z) functions were used for α-amino protection with the exception of a triphenylmethyl(Trt) group for Trt-Cys(Trt)-OH, and *tert*-butyl esters for *C*-terminal blocking. The 4-methoxybenzenesulfonyl (Mbs) group was chosen for the side-chain protection of arginine. The dipeptide Z-Pro-Arg(Mbs)-O*t*Bu was hydrogenated and coupled with Trt-Cys(Trt)-OH in DMF using *N,N'*-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt)[31]. It was also used, after removal of the O*t*Bu ester with 90% TFA, for the DCC/HOBt mediated coupling with H-Gly-NH_2. The obtained product behaved identically with the reaction product of Z-Pro-Arg(Mbs)-Gly-OH (via O*t*Bu ester) with NH_3 using the mixed anhydride method; this latter approach results in more reproducible results.

Applying the same approach, the corresponding oxytocin fragments 6–8 and 6–9 were obtained. After selective removal of the N^{α}-Trt group in 80% aqueous acetic acid[32], the resulting product was acylated (using DCC and HOBt at 0–4°C) with Z-Phe-Gln-Asn-OH, Z-Gln-Asn-OH or Z-Asn-OH in case of the AVP fragments and with Z-Ile-Gln-Asn-OH, Z-Gln-Asn-OH or Boc-Asn-OH in the case of the oxytocin 6–8 or 6–9 sequence to give the key intermediates (see Fig. 1). The protected peptides corresponding to the sequences 4–8 and 4–9 of both AVP and oxytocin were then treated with iodine (0.005 M in acetic acid) for approx. 1 h at room temperature to give the corresponding dimers. In order to obtain the Cys(Scm)-containing peptides, the corresponding Cys(Trt) intermediates were treated with Scm-Cl in CH_2Cl_2 or MeOH/CH_2Cl_2 (1/1, v/v) for 5–60 min at room temperature. Afterwards, the precipitate (spontaneously formed or after the addition of ether) was filtered, washed with ether and dried. In some cases an oil was obtained; decanting of the supernatant was then followed by trituration of the residue with CH_2Cl_2. The Cys(Scm)-containing peptides were treated with H-Cys-OH·HCl in MeOH, MeOH/CH_2Cl_2 or DMF* depending on the solubility of the Scm-peptide. An excess of 0.5–1 equivs. of H-Cys-OH·HCl was used and a reaction time of 10–60 min. Ether was then added and the formed precipitate filtered and washed. In some cases chromatography on Sephadex LH-20 (using MeOH as the eluent) was performed to remove excess cysteine·HCl, otherwise an extraction from 2-BuOH/CH_2Cl_2 (3/2, v/v) with water was used. When the reaction product without prior (partial) purification was treated with acid to remove the protecting

* Other sulfenyl chlorides that have been used more recently to form an activated mercapto group of cysteine preliminary to disulfide bond formation, include 2-pyridinesulfenyl chloride[24] and 3-nitro-2-pyridinesulfenyl chloride[25]. The use of dialkyl azodicarboxylates for the preparation of asymmetrical disulfides has been reported by *Mukaiyama* and *Takahashi*[26] and introduced into peptide chemistry by *Arold* and *Eule*[27]; a clear improvement in this method has been presented recently by *Wünsch* and *Romani*[28].

* *Hiskey* et al.[22] noted the formation of only a small amount of asymmetrical disulfide and a substantial amount of dimer when DMF was used in the reaction of Z-Cys(Scm)-Gly-OTmb and Z-Cys-OCH_3 (30 h). Therefore we dedided, as order of addition, to add H-Cys-OH·HCl to a peptide solution in DMF and to use short reaction times in order to try to prevent dimer formation as much as possible.

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groups, the overall yield (only first crop) in the final reaction step (*i.e.* deprotection, pGlu formation where relevant and one or two purification steps) was generally 20–25% lower than after purification in the previous stage. Removal of all the protecting groups was performed with TFA and methanesulfonic acid (MSA) (about 20 equiv. per Z and Mbs function[33]) in the presence of thioanisole*. In the early experiments with oxytocin fragments, TFA and thioanisole alone were used (for about 4 h)[34] but later results with MSA/TFA/thioanisole (for 2 h) were just as satisfactory. After the exchange of strong acid ions for acetate (with Dowex 2 X-8 in the acetate form) the crude peptides were purified by counter current distribution in 1-butanol/acetic acid/water (4/1/5, by volume) or by chromatography on silica gel (Merck Fertigsäule) using solvent systems based on 1-butanol/pyridine/acetic acid/water. In some cases a second purification step was carried out in order to further increase the purity. pGlu formation was effected by dissolving the lyophilized peptide·acetate salt in 50% aqueous acetic acid and keeping this solution at approx. 50°C for 4–5 h**. Attempts to purify peptides with an *N*-terminal glutamine residue, possessing a strong or weak acid as counterion for the α-amino group, failed; pGlu formation took place spontaneously during purification or simply during storage.
The final products **1**–**13** were checked for purity by HPLC, TLC, amino acid analysis (including ammonia determination), electrophoresis at two pH values and isotachophoresis.

Behavioural studies

Preliminary testing of some of the mentioned compounds in a passive avoidance behaviour assay has been reported[15,16]. As is the case with the complete nonapeptide, the AVP fragments **2** and **3** improve memory consolidation in a passive avoidance situation. The oxytocin fragments **8** and **9**, like oxytocin, show an opposite effect.
Potency ratios for the AVP fragments **2** and **3** are approx. 3000 and 1000 with AVP as a reference, while the corresponding oxytocin fragments **8** and **9** are 100 and 1–10, respectively, as potent as oxytocin itself (all data are after intracerebroventricular administration).

Experimental

The purity of the amino acid derivatives and peptides was checked by thin-layer chromatography on Merck silica gel plates (F.254 nm, 0.25 mm) and in some cases (end-products) on Woelm TLC plates (silica gel F.254/366; 0.25 mm) from ICN Pharmaceuticals GmbH & Co., West-Germany; the abbreviation W will be used here. The following solvent systems were used (ratios are v/v):
(a) toluene/EtOH = 8/2
(b) CH_2Cl_2/MeOH = 8/2
(c) $CHCl_3$/MeOH/H_2O = 70/30/5
(d) CH_2Cl_2/MeOH/H_2O = 70/30/5
(e) 1-BuOH/HOAc/H_2O = 4/1/1
(f) 1-BuOH/HOAc/H_2O = 2/1/1
(g) 1-BuOH/HOAc/H_2O = 1/1/1
(h) 1-BuOH/HOAc/H_2O = 1/2/1
(i) 2-BuOH/HOAc/H_2O = 67/10/23
(j) 1-BuOH/pyridine/HOAc/H_2O = 16/3/1/4
(k) 1-BuOH/pyridine/HOAc/H_2O = 8/3/1/4
(l) 2-BuOH/pyridine/25% NH_4OH/H_2O = 20/20/3/15

UV (254 nm), fluorescamine and chlorine/*o*-tolidine were used for the detection of compounds on TLC plates. Melting points were determined with a Totolli apparatus but not corrected. Rotations were measured with a Perkin-Elmer 241 polarimeter and not corrected for the peptide content. Electrophoresis was carried out on Merck silica gel plates at pH 1.9 and 6.4 in a Pherograph Mini 68 (Frankfurt, West-Germany). Amino acid analyses were carried out on samples that had been hydrolyzed in 5.7 N HCl for 24 h at 105°C (Kontron Liquimat III machine). The presence of residual TFA and MSA as well as the quantitative determination of acetic acid was established in a single run by isotachophoresis[36]. HPLC assays were carried out on a Spectra Physics apparatus, model 8000. Stationary phase: Nucleosil 10 C-18. As mobile phase gradients of tetramethylammonium phosphate (pH 2.8) and MeOH or buffers containing NaH_2PO_4 + hexanesulfonic acid (in the case of very basic peptides) and MeOH were run; details are given in the footnotes to Table II. Detection was at 210 nm, flow rate 2 ml/min and temperature 20°C. The percentage of the main component is given.
We will describe in detail the synthesis of the 4–8 fragment of AVP with a pGlu in position 4 and cystine (Cyt) in position 6, and the corresponding pGlu[4]-containing dimer; details of the preparation of the protected fragment AVP-(6–9) will also be given. Data on other protected intermediates and on free, purified end-products are summarized in Tables I and II.

Synthesis of Trt-Cys(Trt)-Pro-Arg(Mbs)-OtBu

Z-Arg(Mbs)-OtBu. Z-Arg(Mbs)-OH·DCHA[37] (42.6 g, 64.6 mmol) still containing some Z-Arg$(Mbs)_2$-OH was liberated from its salt in the usual way and the resulting oil dissolved in CH_2Cl_2. Isobutene and H_2SO_4 were added at low temperature and the reaction mixture kept at room temperature for 4 days. After the usual extractions, the oily product was purified (mainly from small amounts of Z-Arg$(Mbs)_2$-O*t*Bu and residual starting material) by chromatography on silica gel using as solvent system $CHCl_3$/MeOH (95/5, v/v); overall yield 41%; m.p. 59°C (dec.); $[\alpha]_D^{21}$ −6.6° (*c* 1, DMF). TLC: R_f 0.84 (b).

Z-Pro-Arg(Mbs)-OtBu. After removal of the Z group by catalytic reduction, the resulting H-Arg(Mbs)-O*t*Bu was acylated with Z-Pro-OH using 1.2 equiv. of HOBt and 1.1. equiv. of DCC. After 2 h at 0°C and overnight at room temperature, the DCU was filtered off and the filtrate evaporated to dryness. The oily residue was dissolved in EtOAc and the organic solution washed successively with 5% $KHSO_4$ solution, 5% $NaHCO_3$ solution, water and saturated NaCl solution. After drying on Na_2SO_4 and removal of the solvent, the residue was isolated after treatment with MeOH/H_2O as an oil in 86% yield. $[\alpha]_D^{20}$ −23.5° (*c* 1, DMF). TLC: R_f 0.40 (a), 0.96 (d).

Trt-Cys(Trt)-Pro-Arg(Mbs)-OtBu. 3.96 mmol of the previous preparation (2.50 g) were hydrogenated in MeOH as usual. After 3 h the catalyst was removed by filtration, the solvent evaporated and the residue dissolved in 20 ml of DMF. After cooling to −10°C, 3.68 mmol (2.17 g) of Trt-Cys(Trt)-OH[38], 4.00 mmol (541 mg) of HOBt and 4.00 mmol (828 mg) of DCC were added with stirring. After 45 min at this temperature, the reaction mixture was allowed to warm up to room temperature. After 16 h, the protected tripeptide was isolated after extractions as described above. Repetition of the extraction procedure (with toluene as the organic phase *vs.* ether in the first case) was followed by concentration of the toluene solution and addition of three volumes of pet.

* Methyl phenyl sulfide, $C_6H_5SCH_3$.
** At room temperature in 50–100% (aqueous) acetic acid only a slow conversion took place (about 30–85% in 3–4 days). A more rapid pGlu formation took place, in 50% aqueous acetic acid, when the temperature was increased to about 50°C (*cf.* ref. 35).

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Table 1 Data on protected intermediates in the synthesis of asymmetrical and symmetrical disulfide fragments of AVP and OT (see also Fig. 1)[a].

Peptide	Method	Yield[b] (%)	TLC, R_f value (system)	$[\alpha]_D^{20}$ (*c* 1, DMF)	M.p. (°C) (dec.)
Z-Pro-Leu-O*t*Bu[c]	–ONp ester	78	0.66 (a)	–46.8°, –80.4° (MeOH)	89–90
Trt-Cys(Trt)-Pro-Leu-O*t*Bu	DCC/HOBt	82	0.90 (a)	+88.4°	~121
H-Cys(Trt)-Pro-Leu-O*t*Bu	80% HOAc	87	0.40 (a)	–25.1°	–
Z-Pro-Leu-Gly-NH_2[d]	mixed anh.	80	0.75 (e)	–73.2° (EtOH)	160–164
Trt-Cys(Trt)-Pro-Leu-Gly-NH_2[e]	DCC/HOBt	72	0.60 (a), 0.90 (b)	+81.1°	–
H-Cys(Trt)-Pro-Leu-Gly-NH_2	80% HOAc	95	0.32 (a), 0.50 (b), 0.60 (e)	[f]	–
Trt-Cys(Trt)-Pro-O*t*Bu	DCC/HOBt	62	0.70 (a)	[f]	–
H-Cys(Trt)-Pro-O*t*Bu	80% HOAc	97	0.41 (a)	[f]	–
Z-Phe-Gln-Asn-O*t*Bu[g]	DCC/HOBt	63	0.50 (b), 0.71 (d)	–20.8°	228–229
Z-Phe-Gln-Asn-OH	90% TFA	95	0.22 (d)	– 4.9°	220–222
Z-Ile-Gln-Asn-O*t*Bu[h]	DCC/HOBt	63	0.70 (c)	–14.3°	236–237
Z-Ile-Gln-Asn-OH[i]	90% TFA	90	0.30 (j)	[j]	194–197
Z-Phe-Gln-Asn-Cys(Trt)-Pro-Arg(Mbs)-Gly-NH_2	DCC/HOBt	75	0.68 (c)	– 4.5°	150–160
Z-Gln-Asn-Cys(Trt)-Pro-Arg(Mbs)-Gly-NH_2	DCC/HOBt	74	0.75 (d)	– 4.7°	–
Z-Asn-Cys(Trt)-Pro-Arg(Mbs)-Gly-NH_2	DCC/HOBt	78	0.60 (b), 0.90 (e)	[f]	–
Z-Ile-Gln-Asn-Cys(Trt)-Pro-Leu-Gly-NH_2	DCC/HOBt	63	0.74 (c)	[f]	–
Z-Gln-Asn-Cys(Trt)-Pro-Leu-Gly-NH_2	DCC/HOBt	89	0.63 (b)	–16.7°	–
Z-Gln-Asn-Cys(Trt)-Pro-Leu-O*t*Bu	DCC/HOBt	58	0.29 (a), 0.78 (b)	–13.7°	~115°
Boc-Asn-Cys(Trt)-Pro-Leu-Gly-NH_2	DCC/HOBt	95	0.84 (b)	[f]	–
Z-Gln-Asn-Cys(Trt)-Pro-O*t*Bu	DCC/HOBt	50	0.77 (b)	– 2.1°	~175°
(Z-Gln-Asn-Cys-Pro-Arg(Mbs)-Gly-NH_2)$_2$	I_2 in HOAc	82	0.58 (d)	–54.7°	–
(Z-Gln-Asn-Cys-Pro-Leu-Gly-NH_2)$_2$	I_2 in HOAc	90	0.73 (d)	[f]	–
(Z-Gln-Asn-Cys-Pro-Leu-O*t*Bu)$_2$	I_2 in HOAc	89	0.57 (a), 0.89 (d)	[f]	–

[a] The Cys(Scm)-containing intermediates are not included in the Table since in general only R_f values are available. [b] The yield of the final step is mentioned (see also Fig. 1). Yields given are after purification and concern only the first crops. [c] Lit.[40] $[\alpha]_D^{25}$ –76.5° (*c* 5, MeOH); m.p. 89–90°C. [d] Coupling of Z-Pro-OH and H-Leu-Gly-NH_2. Lit.[41] $[\alpha]_D^{18.5}$ –73.3° (*c* 2, 95% EtOH); m.p. 163–163.5°C. [e] Lit.[42] $[\alpha]_D$ +85.9° (*c* 2, DMF); m.p. 206–209°C. [f] Not completely homogeneous (traces of DCU and/or a few by-products). [g] Reaction of Z-Phe-OH with H-Gln-Asn-O*t*Bu. [h] Reaction of Z-Ile-OH with H-Gln-Asn-O*t*Bu. [i] Lit.[43] $[\alpha]_D^{21}$ –35.5° (*c* 1, 0.5 N $KHCO_3$); m.p. 203–204°C; Lit.[44] $[\alpha]_D$ –43.1° (*c* 1, 0.5 N $KHCO_3$); m.p. 218–220°C. [j] No accurate value because of turbid solution.

ether. The precipitate was filtered, washed with pet. ether and dried. Yield 3.54 g (88.5%); m.p. 151–152°C; $[\alpha]_D^{20}$ +71.5° (*c* 0.5, DMF). TLC: R_f 0.50 (a).

Synthesis of Trt-Cys(Trt)-Pro-Arg(Mbs)-Gly-NH_2

Z-Pro-Arg(Mbs)-OH. 40.4 g (64.0 mmol) of Z-Pro-Arg(Mbs)-O*t*Bu were dissolved in 200 ml of TFA/H_2O (9/1, v/v) and 1% of anisole was added. After 1 h at room temperature, the solution was added to ether and the resulting oily product stirred with ether. Precipitation of the product from a CH_2Cl_2 solution with ether gave 31.9 g (86.5%) of free acid. $[\alpha]_D^{20}$ –17.6° (*c* 1, DMF), m.p. 80–84°C (dec.). Lit.[37]: $[\alpha]_D^{24}$ –16.3° (*c* 0.58), DMF); m.p. 80–85°C (dec.). TLC: R_f 0.54 (d).

Z-Pro-Arg(Mbs)-Gly-NH_2. *a.* Coupling with H-Gly-NH_2. To a cold solution (–10°C) of 12.0 g (20.8 mmol) of Z-Pro-Arg(Mbs)-OH and 5.64 g (41.7 mmol) of HOBt in 180 ml of DMF was added a cold solution of 2.54 g (22.9 mmol) of H-Gly-NH_2·HCl and 2.89 ml (one equiv.) of NEM in 25 ml DMF followed by 4.75 g (22.9 mmol) of DCC. After stirring at –10°C for 1 h and at 0–4°C overnight, the formed precipitate (DCU) was filtered off and the filtrate evaporated to dryness. The resulting oil was dissolved in 200 ml of a 2/3 mixture of 2-BuOH/CH_2Cl_2 and the solution extracted with 3 portions of 5% $KHSO_4$, 5% $NaHCO_3$, H_2O and 30% NaCl solution. After drying on anh. Na_2SO_4, pet. ether was added to the filtered and concentrated solution. The precipitate was removed by filtration and washed with pet. ether. TLC: R_f 0.77 (d). The yield was about 90% but the compound was not completely homogeneous (TLC). Large variations in yield and quality were observed.

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Table II Data on thirteen fragments and analogues of AVP and OT (see also Fig. 1).

Peptide	Amino acid analysis								Peptide content (%)	HPLC main component (%)	Isotacho-phoresis (% HOAc)	TLC R_f value (system)	$[\alpha]_D^{20}$ (*c* 0.25, 10% HOAc)
	Phe or Ile	Glu	Asp	Cyt	Pro	Arg or Leu	Gly	NH_3					
1 [Cyt^6]AVP-(3–9)-NH_2	0.98	1.02	1.01	0.98	1.00	1.00	1.03	2.47	79.4	98.4[a]	10.1	0.14 (f; W); 0.08 (k); 0.23 (l)	−132.6°
2 [pGlu^4, Cyt^6]AVP-(4–9)-NH_2	–	1.01	1.02	0.92	0.99	1.03	1.04	1.94	76.0	95.5[b]	11.1	0.14 (f; W); 0.07 (k); 0.11 (l)	−150.9°
3 [pGlu^4, Cyt^6]AVP-(4–8)-OH	–	1.05	1.01	0.95	1.02	0.99	–	0.90	84.6	98.3[c]	2.9	0.21 (f; W); 0.05 (k); 0.21 (l)	−165.1°
4 [Cyt^6]AVP-(5–9)-NH_2	–	–	1.03	0.96	1.01	1.03	0.99	3.71	72.5	87.6[d]	6.8	0.12 (f; W); 0.09 (k; W); 0.12 (l)	−115.8°
5 [pGlu^4]AVP-(4–9)-NH_2 dimer	–	2.07	2.05	0.93	2.06	2.04	1.88	3.41	82.2	88.5[e]	9.8	0.27 (h; W); 0.07 (k;W); –[f]	−134.4°
6 [pGlu^4]AVP-(4–8)-OH dimer	–	1.93	2.01	0.93	2.12	2.02	–	1.75	88.8	94.1[g]	1.1	0.09 (f; W); 0.42 (h; W); –[f]	−137.1°
7 [Cyt^6]OT-(3–9)-NH_2	1.05	1.03	1.01	0.91	0.94	1.05	1.03	2.86	79.0	96.9[h]	6.3	0.21 (f); 0.26 (k); 0.45 (l)	−160.6°
8 [pGlu^4, Cyt^6]OT-(4–9)-NH_2	–	1.02	0.97	0.95	1.11	0.99	0.98	1.91	87.0	98.3[g]	0.9	0.27 (f); 0.26 (k); 0.42 (l)	−190.7°
9 [pGlu^4, Cyt^6]OT-(4–8)-OH	–	1.00	1.01	0.96	1.06	0.98	–	1.00	94.0	98.5[g]	3.5	0.39 (f); 0.21 (k); 0.34 (l)	−192.3°
10 [Cyt^6]OT-(5–9)-NH_2	–	–	1.02	0.97	0.99	0.98	1.05	1.60	78.6	96.0[g]	7.3	0.23 (f); 0.21 (k); 0.41 (l)	−151.4°
11 [pGlu^4]OT-(4–9)-NH_2 dimer	–	2.05	2.03	0.94	2.09	1.97	1.92	2.86	83.9	96.6[i]	4.2[k]	0.30 (f); 0.37 (k); 0.50 (l)	−147.0°
12 [pGlu^4]OT-(4–8)-OH dimer	–	1.98	2.01	0.97	2.12	1.94	–	1.73	86.6	96.1[j]	1.3[l]	0.51 (f); 0.27 (k); 0.39 (l)	−158.8°
13 pGlu-Asn-Cyt-Pro-OH	–	1.04	0.99	0.93	1.05	–	–	0.96	87.8	99.3[b]	3.7	0.23 (f); 0.13 (k; W); 0.31 (l)	−192.5°

[a] Mobile phase: A = 0.1 Mol/l NaH_2PO_4, pH 4.5, with 0.01 Mol/l hexanesulfonic acid, B = MeOH; gradient: time–%A–%B = 0–100–0; 40–50–50. [b] Mobile phase: A = 0.1% H_3PO_4 (pH 2.1) and B = MeOH/H_2O = 50/50 (v/v) with 0.05 Mol/l TMAH and H_3PO_4 till pH 2.8. Gradient: time–%A–%B = 0–100–0; 4–100–0; 25–0–100. [c] Mobile phase: A = MeOH/H_2O = 25/75 (v/v) and B = MeOH/H_2O = 80/20 (v/v) both with 0.05 Mol/l TMAH and H_3PO_4 till pH 2.8. Gradient: time–%A–%B = 0–100–0; 4–100–0; 25–0–100. [d] Mobile phase: A = 0.5 Mol/l NaH_2PO_4, pH 4.5, with 0.05 Mol/l hexanesulfonic acid, B = H_2O, C = MeOH. Gradient: time–%A–%B–%C = 0–20–80–0; 40–20–30–50; 45–20–30–50. [e] Mobile phase: A = 0.5 Mol/l NaH_2PO_4 and 0.25 Mol/l TMAH with H_3PO_4 till pH 2.5; B = H_2O, C = CH_3CN/H_2O = 60/40 (v/v). Gradient: time–%A–%B–%C = 0–20–80–0; 40–20–30–50; 45–20–30–50. Temp. 35°C. [f] In the basic system (l) (both on Merck and Woelm plates), R_f values of <0.02 were obtained. [g] See b. Gradient: 0–80–20; 35–0–100; 40–0–100. [h] See c. Gradient: 0–100–0; 25–30–70; 30–30–70. [i] See e. Gradient: 0–20–64–16; 40–20–22–58; 45–20–22–58. Temp. 35°C. [j] Mobile phase: A = 0.5 Mol/l Na_2HPO_4, pH 2.5 by addition of H_3PO_4; B = H_2O; C = CH_3CN. Gradient: 0–20–70–10; 40–20–45–35; 45–20–45–35. Temp. 35°C. [k] Residual MSA (2.0%) but no TFA was found. [l] Residual MSA (1.6%) but no TFA was found.

b. Via H-Gly-O*t*Bu. Z-Pro-Arg(Mbs)-OH (25.0 g, 43.4 mmol) was coupled with 5.75 g (43.8 mmol) of H-Gly-O*t*Bu using the DCC/HOBt method. After extraction, the ester was precipitated with ether. Yield 88.6% (26.5 g) of a TLC-homogeneous product, R_f 0.68 (a), 0.75 (d); $[\alpha]_D^{20}$ −28.2° (*c* 1, DMF). Treatment of 25.0 g of this product with 90% TFA (1½ h at room temperature) and precipitation of the tripeptide acid with ether gave Z-Pro-Arg(Mbs)-Gly-OH in nearly 100% yield. M.p. 75–83°C (dec.); $[\alpha]_D^{20}$ −26.8° (*c* 1, DMF). TLC: R_f 0.30 (a), 0.37 (d). Via a mixed-anhydride procedure using one equiv. of isobutyl chloroformate (−10°C, 30 min) the peptide acid (23.0 g, 36.3 mmol) was converted into the corresponding amide by bubbling NH_3 through the solution at −10°C for 30 min. Isolation of the product from DMF/H_2O was followed by recrystallization from EtOH/ether. Yield 85% (19.5 g); m.p. ~80°C (dec.); $[\alpha]_D^{20}$ −25.5° (*c* 1, DMF). TLC: R_f 0.71 (d).

Trt-Cys(Trt)-Pro-Arg(Mbs)-Gly-NH₂. The same procedure as described for the synthesis of Trt-Cys(Trt)-Pro-Arg(Mbs)-O*t*Bu was used to obtain this protected tetrapeptide. After the extraction procedure (the oily reaction product was dissolved in 2-BuOH/CH_2Cl_2 = 2/3, v/v), the yield was about 90%. $[\alpha]_D^{20}$ +69.8° (*c* 1, DMF), m.p. ≥145°C. TLC: R_f 0.82 (c).

Synthesis of pGlu-Asn-Cyt-Pro-Arg-OH (**3**)

Z-Gln-Asn-Cys(Trt)-Pro-Arg(Mbs)-OtBu. 2.92 g (2.69 mmol) of Trt-Cys(Trt)-Pro-Arg(Mbs)-O*t*Bu were dissolved in 10 ml of HOAc and 2 ml of water were slowly added. After one hour at room temperature, 100 ml of H_2O, 40 ml of 30% NaCl solution and 100 ml of EtOAc were added, and, after mixing, the layers separated. The organic phase was washed with 30% NaCl solution, 3× with 5% $NaHCO_3$ solution and again with the NaCl solution. Three volumes of ether were added to the concentrated EtOAc solution, the precipitate removed by filtration and washed with ether. Yield of the N^{α}-detritylated tripeptide 1.61 g (70.5%); m.p. 191.5° (dec.); $[\alpha]_D^{20}$ +4.7° (*c* 1, DMF); TLC: R_f 0.20 (a). In 50 ml of DMF were dissolved successively 4.26 g (10.8 mmol) of Z-Gln-Asn-OH [obtained from the corresponding O*t*Bu ester after treatment with 90% TFA and precipitation with ether; m.p. 192–195°C, lit.[39] 206°C; $[\alpha]_D^{20}$ +8.9° (*c* 1, DMF)], 9.10 g (10.8 mmol) of H-Cys(Trt)-Pro-Arg(Mbs)-O*t*Bu and 1.5 equiv. of HOBt (2.19 g). The solution was cooled to −20°C and 2.46 g (11.9 mmol) of DCC were added. After 1 h at −20°C, overnight at 0–4°C and finally 4 h at room temperature, the precipitated DCU was filtered and the filtrate evaporated to dryness. The residue was then dissolved in 2-BuOH/CH_2Cl_2 (2/3, v/v) and the solution extracted as usual. The addition of ether to the dried, concentrated, solution gave a precipitate that was washed with pet. ether. Yield 12.0 g (91.2%). TLC: R_f 0.22 (a); 0.75 (b), not completely homogeneous.

Z-Gln-Asn-Cys(Scm)-Pro-Arg(Mbs)-OtBu. 3.00 g (2.48 mmol) of the previous preparation were dissolved in 40 ml of CH_2Cl_2 and 0.6 ml of Scm-Cl was added. The reaction mixture was stirred for 10 min at room temperature and the precipitate that was formed removed by filtration, washed with ether and dried. Yield 2.32 g (87.8%). TLC: R_f 0.59 (b); 0.76 (d), not completely homogeneous. $[\alpha]_D^{20}$ −60.6°(*c* 1, DMF).

Z-Gln-Asn-Cys(Cys)-Pro-Arg(Mbs)-OtBu. Since the solubility of the Scm-containing peptide in a small volume of MeOH was not sufficient, DMF was chosen as a solvent. 1.50 g (1.40 mmol) of the peptide were dissolved in approx. 50 ml of DMF and with stirring 345 mg (1.96 mmol) of H-Cys-OH·HCl·H_2O were added. After 1 h at room temperature about 4 volumes of ether were added and the formed precipitate filtered and washed with ether. Yield 1.19 g (77.5%). TLC: R_f 0.42 (d), not homogeneous. Chromatography on a SiO_2 column (Merck Fertigsäule) using solvent system d gave, after precipitation of the isolated first crop from MeOH/ether, 772 mg (65.0%) of the title peptide. TLC: R_f 0.40 (d), 0.55 (k). M.p. ca. 152°C (dec.); $[\alpha]_D^{20}$ −55.5° (*c* 1, DMF).

pGlu-Asn-Cyt-Pro-Arg-OH (**3**)

700 mg (0.62 mmol) of the previous preparation were dissolved in a mixture of 7 ml of TFA, 1.6 ml of MSA and 0.16 ml of thioanisole. After 4 h at room temperature, the solution was added dropwise to ether and the precipitate was filtered and washed. The peptide was dissolved in *t*-BuOH/H_2O (1/1, v/v) and treated with Dowex 2 X-8 in the acetate form. After lyophilization, the product was dissolved in 50% aqueous HOAc and kept at 50°C for about 5 h after which the peptide was isolated again by lyophilization. Purification was performed by chromatography on a SiO_2 column (Merck Fertigsäule) using solvent system i. Overall yield, starting with the protected peptide, was 260 mg (58% based on naked peptide). TLC: R_f 0.12 (f); 0.21 (l). Other analytical data are given in Table II.

Synthesis of (pGlu-Asn-Cys-Pro-Arg-OH)₂ (**6**)

(Z-Gln-Asn-Cys-Pro-Arg(Mbs)-OtBu)₂. 1.62 g (1.33 mmol) of Z-Gln-Asn-Cys(Trt)-Pro-Arg(Mbs)-O*t*Bu were dissolved in 292 ml of a 0.005 M solution of iodine in acetic acid and the solution kept at room temperature for 1 h. A solution of 1 N sodium thiosulphate was then added dropwise with stirring until the reddish colour of the solution became pale yellow. 1.33 ml of a 1 M NaOH solution were added followed by 250 ml of water. The formed precipitate was filtered, washed with H_2O and dried. Recrystallization from MeOH/ether gave 1.03 g of the protected 4–8 dimer (80%). TLC: R_f 0.22 (a), 0.76 (b), 0.87 (d), not completely homogeneous.

(pGlu-Asn-Cys-Pro-Arg-OH)₂ (**6**). 1.03 g of the previous preparation were dissolved in 10 ml TFA to which 4.1 ml MSA and 0.67 ml thioanisole had been added. The solution was kept at room temperature for 5 h and then added dropwise to 100 ml of ether. The precipitate was filtered, washed with ether and dissolved in *t*-BuOH/H_2O (1/1, v/v). After the exchange for acetate ions (using Dowex 2 X-8 in the acetate form), the solution was lyophilized. The resulting product was dissolved in 50% aqueous HOAc and the solution stirred at 50°C for about 5 h. The conversion of the fluorescamine-positive main component into a negative higher-running product was followed on TLC.
After isolation of the peptide by lyophilization the pGlu-containing dimer (630 mg) was purified by chromatography on a Merck SiO_2 column using solvent system f. Overall yield (deprotection, pGlu formation and purification) was 34%. TLC: R_f 0.14 (g); 0.42 (h; W). For analytical data, see Table II.
The same procedures have been used to obtain the dimers of the pGlu-containing fragments 4–9 of AVP and OT and the 4–8 fragment of OT. See Table II for details of the end-products.

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