A new solid-phase synthesis of porcine vasoactive intestinal peptide using N^{α}-9-fluorenylmethyloxycarbonyl amino acids

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Summary. The solid-phase synthesis of an octacosapeptide amide corresponding to the amino acid sequence of porcine vasoactive intestinal peptide (VIP) is described. No final treatment with strong, anhydrous acid was employed, since the use of base-labile 9-fluorenylmethyloxycarbonyl amino acids bearing tert-butyl based side chain protection enabled the peptide chain assembly to be performed on p-benzyloxybenzyl amine resin, which was cleaved from the whole peptide amide at the end of the synthesis by diluted trifluoroacetic acid.

A few years after the isolation of porcine vasoactive intestinal peptide (VIP) from pig small intestine[1], Mutt and Said[2] determined the amino acid sequence of this powerful hypotensive and vasodilatator peptide. It is an octacosapeptide amide

1 5 10
H-His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-

15 20
Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-

25 28
Ser-Ile-Leu-Asn-NH_2

characterized by a sequence homology with the hormones of the glucagon family; it differs from the corresponding chicken VIP[3] by replacement of Ser_{11}, Phe_{13}, Val_{26}, and Thr_{28} with Thr_{11}, Leu_{13}, Ile_{26}, and Asn_{28} respectively. This natural peptide exhibits a wide range of biological actions on the gastrointestinal tract[4] and has been localized in the gut[5], in the CNS (especially the hypothalamus)[6], and in hypophyseal portal blood[7].

Synthesis of porcine VIP by a classical solid-phase procedure[8] on a benzhydrylamine resin has been reported[9]. In the approach mentioned, which employed tosyl and benzyl-type side chain protecting groups, cleavage of peptide-amide to resin bond and protecting groups was performed at the end of the synthesis by treatment with anhydrous HF, a strongly acidic condition which may give rise to several side reactions[10]. In view of the encouraging results obtained by an alternative strategy in the synthesis of chicked VIP[11], I decided to undertake the solid-phase preparation of porcine VIP by a procedure in which treatment with HF is replaced by final acidolysis with diluted trifluoroacetic acid (TFA).

The 9-fluorenylmethyloxycarbonyl (Fmoc) group[12], a base-labile amino protecting group successfully employed in several solid-phase syntheses[13,14] of naturally occurring peptides, was employed as N^{α}-temporary protection in conjunction with tert-butyl based side-chain protecting groups and p-benzyloxybenzyl amide peptide to resin anchorage[15] which are both cleavable by mild acidolysis. This approach proved to be useful in order to reduce side reactions connected with repeated acidolytic removal of N^{α} protection and final HF treatment.

Materials and methods. The solid support, p-benzyloxybenzyl amine resin, was obtained from chloromethylpolystyrene-1%-divinylbenzene (0.7 mequiv./g, 200-400 mesh; Fluka AG, Switzerland) as described by Pietta and Brenna[15]. The α-amino protecting group was Fmoc in all but the last step (His_1), where tert-butyloxycarbonyl (Boc) was conveniently employed. Fmoc amino acids were prepared by the procedures of Carpino and Han[11,16] and of Chang et al.[17]. N^{α}-Boc-His (N^{im}-Boc)-OH was synthesized according to the literature[18]. The following groups were employed for side-chain protection: Arg, His, and Lys, Boc; Asp, tert-butyl ester; Ser, Thr, and Tyr, tert-butyl ether. Assembly of the porcine VIP sequence was initiated by coupling of Fmoc-Asn-OH to the amino resin with N,N'-dicyclohexylcarbodiimide in the presence of 2 equivalents of 1-hydroxybenzotriazole. After washings, the Fmoc-Asn-NH-resin was dried to constant weight in vacuo at 30 °C, and the Fmoc-Asn content per g of resin was determined to be 0.248 mmoles. The substituted resin (2.02 g, 0.5 mmoles) was transferred to the glass reaction vessel of a manual apparatus, acetylated with acetic anhydride (1 mmole in CH_2Cl_2 containing 0.5 mmoles of N,N-diisopropylethyl amine) for 20 min, washed with CH_2Cl_2, DMF, $(CH_3)_2$-CHOH and CH_2Cl_2, and submitted to the program of table 1 for stepwise synthesis from the carboxamide terminus. Fmoc-Gln-OH and Fmoc-Asn-OH were coupled to the resin, during the synthesis, as p-nitrophenyl esters (5 mmoles, 10 equiv.) in DMF in step 13 and 20. Immediately preformed symmetrical anhydrides of Fmoc-amino acids were used in all other couplings as previously described[11]. Completeness of coupling reactions was monitored both by the ninhydrin color test of Kaiser[19] and the fluorometric method[20]. Thrice-coupling was necessary for the introduction of Ile_{26}, Asn_{24}, Lys_{20}, Gln_{16}, Arg_{14}, Asn_9,

Table 1. Synthetic program for porcine VIP

Operation	Reagent or solvent[a]	Mixing time (min)	Applications
1	55% piperidine in DMF[b]	1	1
2	55% piperidine in DMF	10	1
3	DMF	1	3
4	CH_2Cl_2	1	3
5	$(CH_3)_2CHOH$	1	3
6	CH_2Cl_2	1	3
7-9	Repeat operation 1-3		
10	20% water in dioxane[b]	5	3
11	DMF	1	3
12	CH_2Cl_2		
13	Fmoc-amino acid anhydride[c] (4-fold excess)	180	1
14	CH_2Cl_2	1	3
15	DMF	1	3
16	$(CH_3)_2CHOH$	1	3
17-20	Repeat operation 12-15		
21	CH_2Cl_2	1	6
22	Coupling monitoring (if necessary, repeat operation 13-15)		
23	Acetic anhydride (2 equiv. in CH_2Cl_2 containing 0.5 equiv. of N,N-diiso propylethylamine)	15	1
24	CH_2Cl_2	1	6
25	DMF	1	3

[a] Wash volumes were 16 ml; [b] percentages express v/v ratios; [c] Fmoc-asparagine and Fmoc-glutamine were coupled to the peptide-resin as their p-nitrophenyl esters (5 mmoles, 10 equiv.). Mixing time: 8 h.

Table 2. Check of synthesis progress by amino acid analysis

Amino acid	Sequence spanned by peptide-resin[a]						
	25-28	21-28	17-28	13-28	9-28	5-28	1-28
Lys	(0)	1.00 (1)	1.94 (2)	2.88 (3)	2.97 (3)	3.02 (3)	2.94 (3)
His	(0)	(0)	(0)	(0)	(0)	(0)	1.00 (1)
Arg	(0)	(0)	(0)	0.98 (1)	1.04 (2)	2.00 (2)	1.94 (2)
Asx	1.05 (1)	2.14 (2)	2.04 (2)	1.99 (2)	2.97 (3)	3.89 (4)	5.12 (5)
Thr[b]	(0)	(0)	(0)	(0)	0.77 (1)	1.74 (2)	1.81 (2)
Ser[b]	0.87 (1)	0.84 (1)	0.81 (1)	0.89 (1)	0.85 (1)	0.79 (1)	1.75 (2)
Glu	(0)	(0)	(0)	0.97 (1)	0.99 (1)	1.06 (1)	0.98 (1)
Ala	(0)	(0)	1.09 (1)	1.00 (1)	1.14 (1)	1.03 (1)	2.07 (2)
Val	(0)	(0)	1.00 (1)	1.07 (1)	1.11 (1)	1.98 (2)	2.13 (2)
Met	(0)	(0)	0.98 (1)	0.95 (1)	0.97 (1)	0.91 (1)	0.92 (1)
Ile	1.07 (1)	1.12 (1)	1.05 (1)	1.01 (1)	0.98 (1)	1.10 (1)	1.01 (1)
Leu	1.00 (1)	1.97 (2)	2.03 (2)	2.97 (3)	3.00 (3)	2.98 (3)	3.02 (3)
Tyr	(0)	0.97 (1)	0.96 (1)	0.94 (1)	1.99 (2)	1.93 (2)	1.97 (2)
Phe	(0)	(0)	(0)	(0)	(0)	1.10 (1)	1.07 (1)

[a] The theoretical number of residues is indicated between brackets; [b] uncorrected for loss in hydrolysis.

Ala_4, Ser_2, and His_1. The progress of the synthesis was checked 7 times (table 2) by the procedure described for the synthesis of chicken VIP[11].

At the end of the chain assembly, after the last operation (25), the protected octacosapeptide amide resin was thoroughly washed with dioxane and CH_2Cl_2, and dried to constant weight (4.25 g) in vacuo over P_2O_5 (30 °C). To a suspension of the peptide resin in dry CH_2Cl_2 (10 ml) containing anisole (3 ml) and thiophenol (1 ml), TFA (50 ml) was added at 25 °C with vigorous stirring under N_2 to cleave all the protecting groups and the peptide amide to resin bond. After 2.5 h at room temperature and with stirring the resin particles were removed by filtration, and the solvent evaporated under reduced pressure (bath temperature: 30 °C). The obtained residue was triturated with anhydrous diethyl ether to give 1.115 g of crude product (62% yield based on starting Fmoc-Asn-NH-resin).

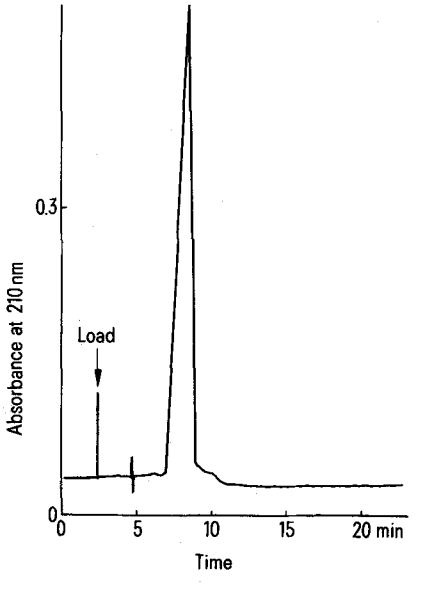
Results and discussion. The synthetic peptide was purified by gel filtration on Sephadex G-50 using the biphasic solvent system n-butanol-1 M acetic acid-pyridine (11:5:3), followed by desalting on Sephadex G-25 and ion-exchange chromatography on CM-cellulose by gradient elution with ammonium acetate buffers (0.1 M, pH 6–0.6 M, pH 7)[9]. The elution profile was determined by absorbance at 275 nm. After desalting on Sephadex G-25 in 2 M acetic acid, lyophilization gave a colorless fluffy powder, 352 mg (20% overall yield). TLC (Silica Gel, Merck 60 F-254) in pyridine-ethyl acetate-acetic acid-water (5:5:1:3), R_f 0.16; n-butanol-ethyl acetate-acetic acid-water (1:1:1:1), R_f 0.19; and isopropanol-1 M acetic acid (2:1), R_f 0.26, gave a single chlorine-positive, ninhydrin-positive, and Sakaguchi-positive spot. HPLC analysis of synthetic porcine VIP (fig.) was performed as described by Coy and Gardner[9]. $[\alpha]_D^{26}$ was found to be −67.5° (c = 1, AcOH 2 M) (lit.[9] $[\alpha]_D^{28} = -58°$). Histidine was found to be the only amino terminal amino acid by the dansyl technique[21]. The results of amino acid analysis of acid and enzyme hydrolysates of a sample of purified peptide are reported in table 3. Treatment of synthetic porcine VIP with CNBr in 70% formic acid gave 2 peaks on HPLC analysis, and the material collected from each peak was found to have the amino acid composition corresponding to porcine VIP 1-16 (+homoserine) and 18-28 fragments.

The above reported physico-chemical data on synthesized porcine VIP show that it is endowed with an acceptable degree of homogeneity; furthermore, the synthetic peptide is able to inhibit the release of gastric acid induced by pentagastrin infusion in the dog[22] (maximal inhibition at an infusion rate of 7 µg/kg/h) and is active in the effect on the peak rate of pancreatic blood flow. As previously reported[9],

Table 3. Amino acid analyses of synthetic porcine VIP

Amino acid	Theoretical	Acid hydrolysate[a,c]	Enzyme digest[b,c]
Lys	3	3.09	3.17
His	1	1.04	0.98
Arg	2	1.93	1.97
Asp	2	5.12	2.04
Asn	3		
Thr	2	1.78[d]	8.15
Ser	2	1.73[d]	
Gln	1	1.06[e]	
Ala	2	2.08	2.05
Val	2	1.99	2.10
Met	1	1.00	0.98
Ile	1	0.97	1.00
Leu	3	3.11	3.04
Tyr	2	1.98	2.14
Phe	1	0.99	0.98

[a] Hydrolysis was carried out with 6N HCl containing 0.05% v/v β-mercaptoethanol for 24 h at 110 °C in sealed evacuated tubes; [b] digestion with acid protease for 24 h at 37 °C, followed by 24 h digestion with trypsin and chymotrypsin, followed by 48 h digestion with leucine amino-peptidase; [c] average of 3 determinations; [d] uncorrected for loss in hydrolysis; [e] determined as Glu.



HPLC of synthetic porcine VIP on LiChrosorb RP-18 reverse phase column (10 µm particle size, 0.4 g/ml density) with 30% CH_3CN-0.1% Et_3N-CH_3COONH_4 (pH 4, 0.01 M) as mobile phase. Flow rate: 1.4 ml/min. Load: 250 µg.

storage of the lyophilized peptide in vacuo at +4 °C for several months produces faint traces of a decomposition product which is detectable by HPLC.

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Stereospecific reduction of geraniol to (*R*)-(+)-citronellol by *Saccharomyces cerevisiae*

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Summary. (*R*)-(+)-citronellol, a useful C_{10} chiral synthon for natural terpenoid products, can be obtained in enantiomerically pure form and satisfactory yield by yeast reduction of geraniol.

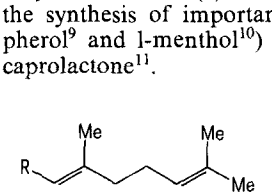
Although it is well recognized that yeasts are capable of reducing stereospecifically cinnamyl alcohols to the corresponding dihydroderivative[1], to our knowledge no analogous transformation has been reported for aliphatic allylic alcohols. We report here a simple route which allows (*R*)-(+)-citronellol (**2**) to be prepared in enantiomerically pure form[2] and satisfactory yield from geraniol (**1**), an achiral readily-available starting material, via microbiological *(Saccharomyces cerevisiae)* reduction.

A highly dense suspension of resting cells of *S. cerevisiae*[3] was incubated with geraniol (**1a**, 100 mg) and shaken at 27 °C for 24 h. The ether extract of the fermentation medium and the acetone extract of centrifuged cells were evaporated at room temperature under vacuum and chromatographed on preparative silica gel (TLC; benzene: ethyl acetate 8:2) to give (*R*)-(+)-citronellol (**2**) (yield 25%)[4] ($[\alpha]_D^{20} = +4.87°$ (MeOH); lit. +4.97° (MeOH)[5a], +2.32° to +2.7° (from Java citronella oil)[5b], +4.17°[5c], +5.2[6]) pure by NMR and GLC-MS (FFAP 10%, T = 160 °C).

Taking into account that, in the case of citronellol, optical rotation cannot be used as a criteria for enantiomeric purity (see above $[\alpha]_D$ data and Valentine et al[7]), citronellol from yeast reduction was examined by ^{1}H-NMR-spectrum in the presence of a chiral lanthanide shift reagent. In the NMR-spectrum ($CDCl_3$), registered in the presence of Eu(tfe)$_3$ (molar ratio = 1:1), only a doublet due to 3-methyl group was observed, thus indicating that this material was essentially enantiomerically homogeneous[7]. As a further confirmation, mixtures of commercial (*S*)-(−)-citronellol and (*R*)-(+)-citronellol, derived from fermentation, showed 2 well resolved (1 Hz) doublets, that at higher fields being assignable to the (*R*)-(+)-isomer.

Geranial (**1b**) was found to give (*R*)-(+)-citronellol, as expected[1], whilst nerol (**4a**) and neral (**4b**), reduced in analogous way, afforded a mixture of the 2 enantiomers of (**2**). The enantiomeric ratio, determined by NMR as described, was (*R*):(*S*) = 6:4. This result is consistent with a partial cis-trans isomerisation of neral (**4b**) which appear to be an obligatory intermediate in the microbiological conversion of nerol into citronellol[1].

These findings, together with the ready availability of the diastereomerically pure geraniol (**1a**) appear to be of relevant synthetic value. (*R*)-(+)-citronellol (**2**) and its 6,7-dihydroderivative (**3**)[8] are known as key intermediates in the synthesis of important natural products (e.g. α-tocopherol[9] and l-menthol[10]) and optically active γ-methyl-ε-caprolactone[11].



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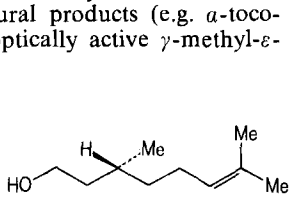
1b R = CHO



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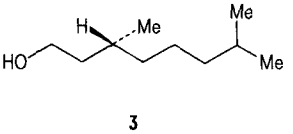

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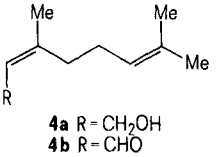

4a R = CH_2OH
4b R = CHO