Synthesis of 5-{[(*R*,*S*)-5-[(9-Fluorenylmethoxycarbonyl)amino]-10,11-dihydrodibenzo[*a*,*d*]cyclohepten-2-yl]oxy}valeric Acid (CHA) and 5-{[(*R*,*S*)-5-[(9-Fluorenylmethoxycarbonyl)amino]dibenzo[*a*,*d*]-cyclohepten-2-yl]oxy}valeric Acid (CHE) Handles for the Solid-Phase Synthesis of C-Terminal Peptide Amides under Mild Conditions[1,2]

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Two novel handles for peptide amide preparation under mild conditions were developed for use in highly efficient solid-phase peptide synthesis. These handles, 5-{[(*R*,*S*)-5-[(9-fluorenylmethoxycarbonyl)amino]-10,11-dihydrodibenzo[*a*,*d*]cyclohepten-2-yl]oxy}valeric acid (CHA) and 5-{[(*R*,*S*)-5-[(9-fluorenylmethoxycarbonyl)amino]dibenzo[*a*,*d*]cyclohepten-2-yl]oxy}valeric acid (CHE), were attached to the solid support and were used for syntheses of peptides having a C-terminal amide by the fluorenylmethoxycarbonyl strategy. The cleavability of CHA and CHE was determined and compared with the that commercially available amide handles. CHA and CHE handles can be rapidly cleaved from the polymer support without significant side reactions using lower acid concentrations than those required for conventional handles. As CHA can be easily synthesized in large amounts, it is suitable for peptide amide preparation for pharmaceuticals. As CHE can be cleaved at very low concentrations of acid, it is especially suitable for preparing side chain-protected peptide amides. Several brain–gut peptides having a C-terminal amide were synthesized in high yield and high purity with these novel handles.

It is known that many naturally occurring biologically active peptides, such as neuropeptides and hormones, have an amide group at their C-terminus, which is generated from the propeptides through endogenous enzymatic cleavage during biological processing.[4] As these peptide amides can not be obtained easily by genetic engineering, chemical synthesis is necessary when such compounds are needed for biochemical research and pharmaceutical production. The peptide amides have been prepared by solid-phase peptide synthesis with benzyl and other ester anchoring linkages and ammonolysis,[5] or with benzhydrylamine resin and HF or trifluoromethanesulfonic acid treatment for the final cleavage.[6] However, there are several disadvantages to these methods, such as difficult ammonolysis of sterically hindered amino acids, retarded ammonolysis caused by the increased length of the peptide chain, partial racemization caused by prolonged treatment with ammonia, and also a tendency for some amino acid residues, such as tryptophan, to be alkylated under the strongly acidic conditions.[7] Since the introduction of the fluorenylmethoxycarbonyl (Fmoc) group for N^{α}-protection in peptide synthesis, the assembled peptide resin can be cleaved by TFA with scavengers,[8] conditions that can remove side chain protecting groups simultaneously. Handles for peptide amide formation in the Fmoc-*t*-Bu strategy have been prepared based on various (alkyloxy)benzylamines[9] and (alkyloxy)benzhydrylamines,[10] as well as a xanthenylamide derivative.[11] However, these handles require still a high concentration of TFA or a long reaction time

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(1) Abbreviations used are as follows: AcOEt, ethyl acetate; AcOH, acetic acid; CHA, 5-{[(*R*,*S*)-5-[(9-fluorenylmethoxycarbonyl)amino]-10,-11-dihydrodibenzo[*a*,*d*]cyclohepten-2-yl]oxy}valeric acid; CHE, 5-(((*R*,*S*)-5-[(9-fluorenylmethoxycarbonyl)amino]dibenzo[*a*,*d*]cyclohepten-2-yl)-oxy]valeric acid; DCM, dichloromethane; DIEA, diisopropylethylamine; EDT, ethanedithiol; EMS, ethyl methyl sulfide; EtOH, ethanol; Fmoc, 9-fluorenylmethoxycarbonyl; GRF, growth hormone releasing factor; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; LSIMS, liquid secondary ion mass spectrometry; MeOH, methanol; NMM, *N*-methylmorpholine; NMP, *N*-methyl-2-pyrrolidinone; PAL, trade name of Millipore Corp.; 5-{4-[[(9-fluorenylmethoxycarbonyl)amino]methyl]-3,5-dimethoxyphenoxy}valeric acid; PACAP38, pituitary adenylate activating polypeptide; PHI, peptide histidine isoleucine; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; PyBOP, (benzotriazol-1-yloxy)tris(pyrrolidino)-phosphonium hexafluorophosphate; RAM, {4-[(9-fluorenylmethoxycarbonyl)amino]-2,4-dimethoxybenzyl}phenoxyacetic acid; TEA, triethylamine; Trt, trityl; TsOH, *p*-toluenesulfonic acid; VIP, vasoactive intestinal peptide.

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for complete cleavage and these conditions generate several side reactions, especially for acid-labile side-chain functions. Recently xanthenyl handles have been reported to be useful for the synthesis of acid-sensitive peptide amides.[12] This paper describes the synthesis and application of handles derived from 10,11-dihydrodibenzo[*a*,*d*]cycloheptene and dibenzo[*a*,*d*]cycloheptene, which form the stable dibenzotropylium ion during acidolytic cleavage.[13] As the 5-dibenzosuberyl group has been reported as being useful for amino protection,[14] it is expected that the 5-dibenzosuberenyl group will be a potent function through stabilization of its carbonium ion. With the aim of performing further highly efficient peptide amide synthesis, we have developed two novel handles: 5-{[(*R*,*S*)-5-[(9-fluorenylmethoxycarbonyl)amino]-10,11-dihydrodibenzo[*a*,*d*]cyclohepten-2-yl]oxy}valeric acid (CHA) and 5-{[(*R*,*S*)-5-[(9-fluorenylmethoxycarbonyl)amino]dibenzo[*a*,*d*]cyclohepten-2-yl]oxy}valeric acid (CHE). The present handles were introduced onto a polymer support and were evaluated using a newly developed simultaneous multiple peptide synthesizer equipped with eight independent channels.[15] The cleavability of the CHA and CHE handles is compared with that of 5-{4-[[(9-fluorenylmethoxycarbonyl)amino]methyl]-3,5-dimethoxyphenoxy}valeric acid (PAL)[9c] and {4-[(9-fluorenylmethoxycarbonyl)amino]-2,4-dimethoxybenzyl}-phenoxyacetic acid (RAM).[10c] RAM and PAL are commercially available and require relatively high concentrations of TFA for complete cleavage. We have successfully synthesized several biologically active peptides having a C-terminal amide, such as neurokinin A[16] related peptides and the secretin-vasoactive intestinal polypeptide family,[4] by the use of simultaneous multiple peptide synthesis.

Results and Discussion

The 10,11-dihydrodibenzo[*a*,*d*]cycloheptene moiety has been reported to be formed by the reduction of 3-benzalphthalide[17] with phosphorus and hydriodic acid and subsequent ring cyclization with $AlCl_3$ and PPA.[18] Although 3-(*m*-methoxybenzylidene)phthalide (1) could be prepared, the reduction of 1 with phosphorus and hydriodic acid was unsuccessful. Catalytic hydrogenation of 1 with Raney Ni gave the ring-opened product, 2-(*m*-methoxyphenethyl)benzoic acid (**2**), in 81% yield. Cyclization of **2** with PPA was cleanly performed to give 2-methoxy-10,11-dihydrodibenzo[*a*,*d*]cyclohepten-5-one (**3**) without regioisomers. Demethylation of **3** with $AlCl_3$ followed by alkylation of the resulting OH-group with ethyl 5-bromovalerate using *t*-BuOK afforded ester **5**, which was converted to corresponding acid **6** by hydrolysis with aqueous NaOH. The reduction of **6** with $NaBH_4$ gave an unstable alcohol. This alcohol was allowed to react with 9-fluorenylmethyl carbamate (Fmoc-NH_2),[19] which traps the cation resulting from treatment of the alcohol with AcOH and a catalytic amount of *p*-toluenesulfonic acid (TsOH), to give CHA (**7**) in an overall yield of 39% (Scheme 1). The bromination of **3** with NBS and subsequent dehydrobromination with triethylamine (TEA) formed 2-methoxydibenzo[*a*,*d*] cyclohepten-5-one (**8**). Compound **8** was converted to acid **11** in three steps: demethylation of **8** with $AlCl_3$, alkylation of **9** with ethyl 5-bromovalerate, and hydrolysis of **10** with aqueous NaOH. Compound 11 was reduced with $NaBH_4$ to an unstable alcohol, which was isolated as the TEA salt. The salt was converted to CHE (**12**) by trapping the cation derived from the alcohol with Fmoc-NH_2 in DMF in the same way described for **7**. Compound **12** was efficiently prepared in an eight-step synthesis in 21% overall yield (Scheme 2). CHA and CHE were coupled to polystyrene–polyethyleneglycol graft copolymer functionalized with the amino group (TentaGel S NH_2[20]) using (benzotriazol-1-yloxy)tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP), 1-hydroxybenzotriazole (HOBt), and diisopropylethylamine (DIEA) in DMF[21] to give 69–75% incorporation. Incorporation was improved to 78–80% when *N*-methyl-2-pyrrolidinone (NMP) was used as the solvent.

In order to assess the relative rates of cleavage of **7** and **12**, Fmoc-Val-CHA-, Fmoc-Val-CHE-, and Fmoc-Val-PAL-TentaGel S[22] were treated with TFA in dichloromethane (DCM). The remaining Fmoc-Val on the resin was quantified by a spectrophotometric method.[23] Half-lives of Fmoc-Val were ca. 5.5 min for PAL and ca. 1.5 min for CHA with 50% TFA/5% phenol in DCM as the cleavage cocktail, and ca. 11.5 min for CHA and 3.5 min for CHE with 10% TFA/5% phenol in DCM (Figure 1). These results indicated that CHA and CHE handles incorporated into a polymer support can be rapidly cleaved at lower concentrations of TFA than those required for the PAL handle. During cleavage, the CHE-resin was red but the CHA-resin was not. For further characterization of these two novel handles, a pentapep-

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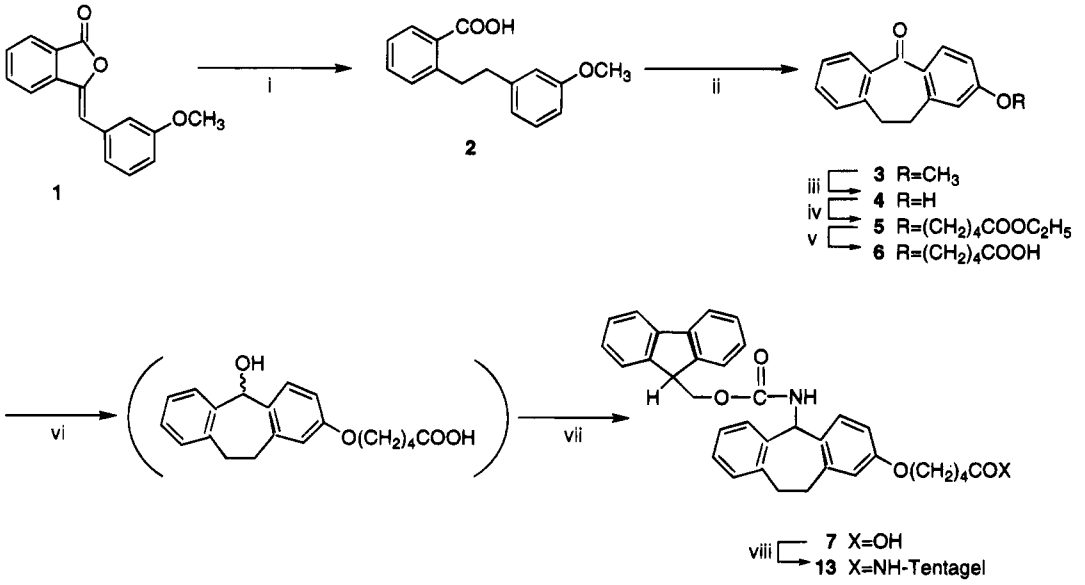
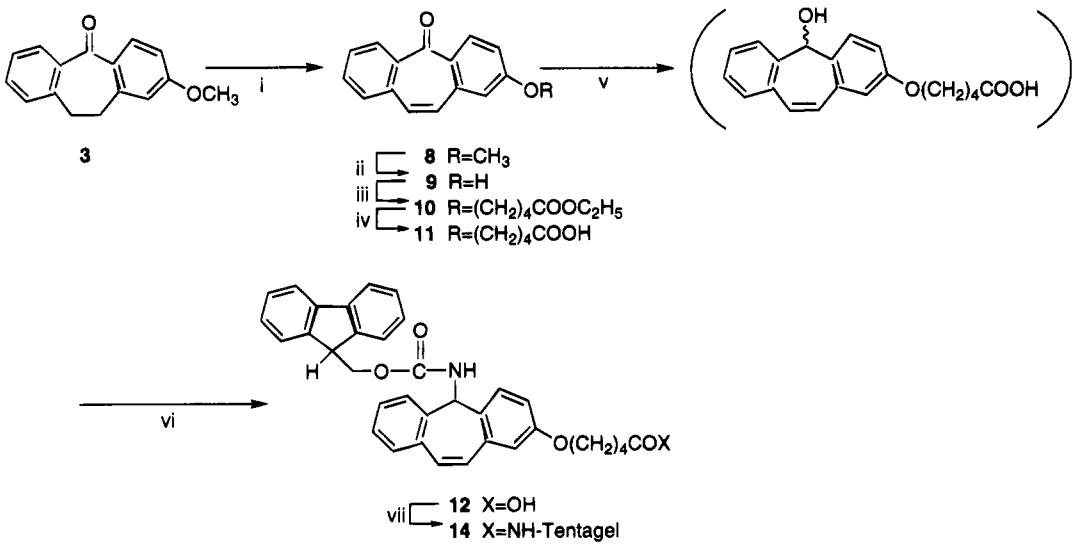
Scheme 1[a]



[a] (i) Raney Ni, H_2/THF; (ii) PPA; (iii) $AlCl_3$/benzene; (iv) *tert*-BuOK, $Br(CH_2)_4COOC_2H_5$/DMF; (v) aqueous NaOH/dioxane; (vi) $NaBH_4$/2-propanol; (vii) Fmoc-NH_2, PTS(cat.)/AcOH; (viii) PyBOP, HOBt, DIPEA/DMF or NMP.

Scheme 2[a]



[a] (i) NBS/CCl_4, TEA; (ii) $AlCl_3/CH_2Cl_2$; (iii) *tert*-BuOK, $Br(CH_2)_4COOC_2H_5$/DMF; (iv) aqueous NaOH/dioxane; (v) $NaBH_4$/2-propanol; (vi) Fmoc-NH_2, PTS/DMF; (vii) PyBOP, HOBt, DIPEA/DMF or NMP.

tide amide, neurokinin A positions 6–10, which shows biological activity,[24] was simultaneously prepared by the same protocol using PAL, RAM, CHA, and CHE as handles. After completion of assembly, the N^{α}-Fmoc group remained on the peptide resin, and aliquots of the peptidyl resin were treated with a TFA-scavenger cocktail described in the Experimental Section. The amount remaining Fmoc group on the resin was determined and compared with the amounts before cleavage. The content of desired N^{α}-Fmoc-pentapeptide shown by a reverse-phase HPLC of cleaved peptides was more than 95%. Figure 2 shows the cleavability of the four handles. The half-lives for cleavage with 25% TFA were <2 min for CHE, <4 min for CHA, 35 min for PAL, and 38 min for RAM. The results show that CHA- and CHE-peptides can be easily and rapidly cleaved to liberate peptides from polymer support using lower TFA concentrations. The PAL handle has some disadvantages. In the preparation of the PAL handle, an isomer is obtained as a byproduct, which is more stable in TFA. Since side reactions during cleavage are possible, the yield of the liberated desired peptide amide is low, and scavengers must be chosen carefully.

Four neuropeptides and hormones, secretin, peptide histidine isoleucine (PHI), pituitary adenylate activating polypeptide (PACAP38), and growth hormone releasing factor (GRF) (of which the primary structures are indicated in Figure 3.) were successfully synthesized in high yield using these novel handles. It had been suggested that one of the acid labile handles itself was unstable toward HOBt, and losses of the peptide chain were observed.[10c] The CHA and CHE handles were stable

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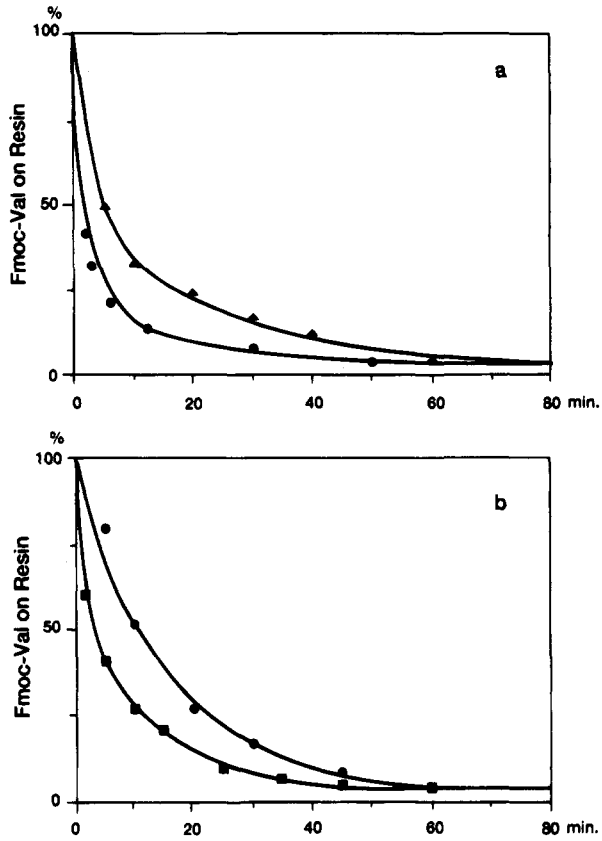

Figure 1. Efficiency of cleavage from resins. a: 50% TFA/5% phenol in DCM. b: 10% TFA/5% phenol in DCM. PAL-resin (▲), CHA-resin (●), CHE-resin (■). Mean value is shown.

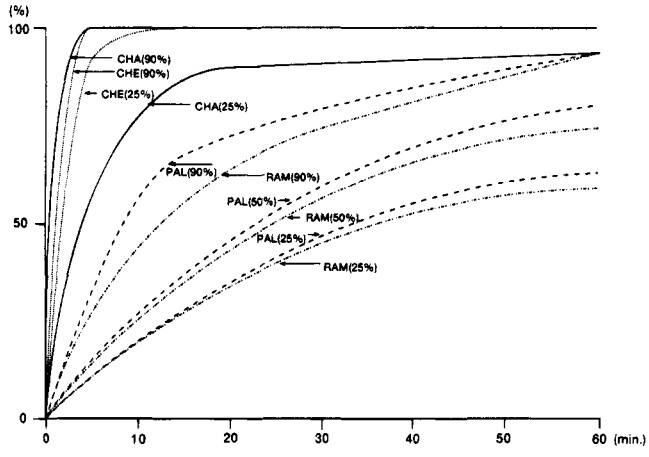

Figure 2. 2. Cleavability of a pentapeptide from different handles. Background was corrected. Mean value is given.

during peptide chain assembly in the conventional protocol. Their stability was judged from the yield of peptide amides after cleavage. Since the four desired peptides contain Arg(Pmc) residues, cleavage was carried out with 82.5% TFA for complete removal of Pmc-group. Cleaved peptides were judged to be high quality on HPLC and were easily purified by a single HPLC. The purified peptides were analyzed by HPLC, liquid secondary ion mass spectrometry (LSIMS), and sequencing and amino acid analysis and were found to be highly homogeneous. HPLC profiles of cleaved crude secretin, PHI, PACAP38, and GRF prepared using CHA and CHE are shown in Figure 4a–h. The peptides prepared in the present study were used for physiological studies.[25] As the overall yield for CHE was lower than that of CHA, it was concluded that CHA is a superior handle for routine preparation of peptide amides, especially peptides containing acid labile residues such as Trp and/or Tyr(SO_3H). For the maximum advantages of these handles, a novel protecting group for the side chain of Arg, which is much more acid labile than Pmc, should be considered.

Experimental Section

^1H NMR spectra were recorded on a General Electric Model 9E-300 spectrometer (San Francisco, CA), and chemical shifts are expressed in ppm (δ) related to TMS as an internal standard. Melting points were determined on a Shibayama Micro-melting Point Apparatus and are uncorrected. Open column chromatography was carried out on Wakogel C-200 (100–200 mesh). Elemental analyses were performed in the Microanalytical Service Center, Faculty of Pharmaceutical Sciences, Kyoto University. Chemicals for peptide assembly were of the SynProPep products of Shimadzu Corp. (Kyoto, Japan) unless otherwise cited. All other solvents and reagents were of analytical grade and used as received. Peptides were assembled by the stepwise procedure using a simultaneous multiple peptide synthesizer, Shimadzu Model PSSM-8, by the Fmoc-method. Side-chain protecting groups of Fmoc-amino acids were 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for arginine; *t*-Bu for aspartic acid, glutamic acid, serine, threonine, and tyrosine; trityl (Trt) for asparagine, glutamine, and histidine; and Boc for lysine. The syntheses were carried out by the standard protocol on the PSSM-8. Cleavage was carried out simultaneously using the PSSM-C8. All peptides were analyzed by a reverse phase HPLC employing a SynProPep RPC18 column (4.6 × 150 mm) (Shimadzu Corp.) using a linear gradient with 0.01 N HCl and acetonitrile at a flow rate of 1.0 mL/min and monitored at 210 nm. High resolution LSIMS was carried out on a Shimadzu-Kratos Concept II H (Manchester, UK). Acid hydrolysis was carried out at 110 °C for 24 h with 6 N HCl using a Shimadzu Model AHST-1, and the hydrolyzate was analyzed on a Shimadzu-CAT Model DLAA-1. Sequence analysis was performed by a gas-phase protein sequencer, Shimadzu Model PSQ-1, according to the manufacturer's protocol.

3-(*m*-Methoxybenzylidene)phthalide (1). Phthalic anhydride (44.44 g, 0.3 mol), *m*-methoxyphenylacetic acid[26] (50 g, 0.3 mol), and anhyd sodium acetate (0.82 g, 0.012 mol) were heated with stirring at 230–245 °C for 6 h. The water formed was distilled off. The residual brown mass was recrystallized from EtOH to give **1** as pale yellow prisms (58.324 g, 77%): mp 222–223 °C; ^1H NMR ($CDCl_3$) δ 3.88 (3H, s, OCH_3), 6.41 (1H, s, CH=C), 6.89 (1H, ddd, J = 8.1, 2.4, 0.9 Hz, 4′-H), 7.33 (1H, t, J = 8.1 Hz, 5′-H), 7.42–7.45 (2H, m, 2′-H, 6′-H), 7.56 (1H, dt, J = 7.8(t), 0.9(d) Hz, 6-H), 7.73 (1H, dt, J = 7.8(t), 0.9(d) Hz, 5-H), 7.78 (1H, dt, J = 7.8(t), 0.9(d) Hz, 4-H), 7.95 (1H, dt, J = 7.8(t), 0.9(d) Hz, 7-H). Anal. Calcd for $C_{16}H_{12}O_3$: C, 76.18; H, 4.80. Found: C, 76.10; H, 4.57.

2-(*m*-Methoxyphenethyl)benzoic Acid (2). To a solution of W-2 Raney nickel (prepared from 80 g of 50% Raney nickel alloy) in THF (150 mL) in an autoclave (500 mL) were added 2-(*m*-methoxybenzylidene)phthalide (**1**) (20 g, 0.079 mol) and TEA (16.07 g, 0.158 mol). The mixture was hydrogenated with H_2 under a pressure of 4 kg/cm^2 at 50 °C for 30 h. The catalyst was filtrated off and washed with EtOH, and the combined filtrates were evaporated. The residual oil was extracted with AcOEt, washed with 3% HCl, water, and brine, and dried over anhyd $MgSO_4$. After evaporation, the residue was recrystallized from AcOEt–hexane to give **2** as colorless needles (16.492 g, 81%): mp 118–119 °C; ^1H NMR ($CDCl_3$) δ 2.92 (2H, t, J =

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(26) Purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI).

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                1                                   10                                  20
h Secretin:     His-Ser-Asp-Gly-Thr-Phe-Thr-Ser-Glu-Leu-Ser-Arg-Leu-Arg-Glu-Gly-Ala-Arg-Leu-Gln-
PHI       :     His-Ala-Asp-Gly-Val-Phe-Thr-Ser-Asp-Phe-Ser-Arg-Leu-Leu-Gly-Gln-Leu-Ser-Ala-Lys-
PACAP38   :     His-Ser-Asp-Gly-Ile-Phe-Thr-Asp-Ser-Tyr-Ser-Arg-Tyr-Arg-Lys-Gln-Met-Ala-Val-Lys-
h GRF     :     Tyr-Ala-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-

21                        27          30                                  40
Arg-Leu-Leu-Gln-Gly-Leu-Val-NH2
Lys-Tyr-Leu-Glu-Ser-Leu-Ile-NH2                                  38
Lys-Tyr-Leu-Ala-Ala-Val-Leu-Gly-Lys-Arg-Tyr-Lys-Gln-Arg-Val-Lys-Asn-Lys-NH2                  44
Lys-Leu-Leu-Gln-Asp-Ile-Met-Ser-Arg-Gln-Gln-Gly-Glu-Ser-Asn-Gln-Glu-Arg-Gly-Ala-Arg-Ala-Arg-Leu-NH2
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Figure 3. 3. Primary structures of peptides synthesized in this study.

7.8 Hz, CH_2), 2.34 (2H, t, J = 7.8 Hz, CH_2), 3.78 (3H, s, OCH_3), 6.74 (1H, dd, J = 7.8, 1.2 Hz, 4'-H), 6.78 (1H, t, J = 1.2 Hz, 2'-H), 6.83 (1H, d, J = 7.8 Hz, 6'-H), 7.19 (1H, t, J = 7.8 Hz, 5'-H), 7.25 (1H, dd, J = 7.5, 1.2 Hz, 3-H), 7.32 (1H, dt, J = 7.5(t), 1.2(d) Hz, 5-H), 7.47 (1H, dt, J = 7.5(t), 1.5(d) Hz, 4-H), 8.08 (1H, dd, J = 7.5, 1.2 Hz, 6-H). Anal. Calcd for $C_{16}H_{16}O_3$: C, 76.10; H, 4.80. Found: C, 76.10; H, 4.57.

2-Methoxy-10,11-dihydrodibenzo[*a*,*d*]cyclohepten-5-one (3). 2-(*m*-Methoxyphenethyl)benzoic acid **(2)** (30 g, 0.117 mol) was added to PPA, which was prepared from P_2O_5 (144 g) and 85% H_3PO_4 with heating at 95 °C for 1 h. The mixture was heated at 145–150 °C with stirring for 50 min under a N_2 atmosphere. The mixture was poured into a large volume of water with ice and then extracted with AcOEt. The organic layer was washed with 10% $NaHCO_3$ and brine and dried over anhyd $MgSO_4$. After evaporation of the solvent, the residue was recrystallized from AcOEt-hexane to give **3** as colorless prisms (23.95 g, 86%): mp 72–73 °C; ^{1}H NMR ($CDCl_3$) δ 3.18 (4H, s, CH_2CH_2), 3.86 (3H, s, OCH_3), 6.70 (1H, d, J = 2.7 Hz, 1-H) 6.86 (1H, dd, J = 8.7, 2.7 Hz, 3-H), 7.21 (1H, dd, J = 7.5, 0.9 Hz, 9-H) 7.32 (1H, dt, J = 7.5(t), 1.5(d) Hz, 7-H), 7.42 (1H, dt, J = 7.5(t), 1.5(d) Hz, 8-H), 8.01 (1H, dd, J = 7.8, 1.5 Hz, 6-H), 8.17 (1H, d, J = 8.7 Hz, 4-H). Anal. Calcd for $C_{16}H_{14}O_2$: C, 80.65; H, 5.92. Found: C, 80.93; H, 5.88.

2-Hydroxy-10,11-dihydrodibenzo[*a*,*d*]cyclohepten-5-one (4). To a cold solution of 2-methoxy-10,11-dihydrodibenzo-[*a*,*d*]cyclohepten-5-one **(3)** (30 g, 0.126 mol) in benzene (450 mL) was added $AlCl_3$ (35.3 g, 0.265 mol). The mixture was refluxed for 1.5 h under a N_2 atmosphere, poured into water with ice, and extracted with AcOEt. The organic layer was washed with brine, dried over anhyd $MgSO_4$, and evaporated to give a solid, which was recrystallized from AcOEt–hexane to give **4** as colorless plates (27.264 g, 97%): mp 139–140 °C; ^{1}H NMR ($CDCl_3$) δ 3.15 (4H, s, CH_2CH_2), 6.30 (1H, broad s, OH), 6.18 (1H, d, J = 2.4 Hz, 1-H), 6.80 (1H, dd, J = 8.7, 2.4 Hz, 3-H), 7.24 (1H, dd, J = 7.5, 0.9 Hz, 9-H), 7.32 (1H, dt, J = 7.5(t), 1.5(d) Hz, 7-H), 7.42 (1H, dt, J = 7.5(t), 1.5(d) Hz, 8-H), 8.0 (1H, dd, J = 7.8, 1.5 Hz, 6-H), 8.13 (1H, d, J = 8.7 Hz, 4-H). Anal. Calcd for $C_{15}H_{12}O_2$: C, 80.33; H, 5.40. Found: C, 80.17; H, 5.56.

Ethyl 5-[(5-Oxo-10,11-dihydrodibenzo[*a*,*d*]cyclohepten-2-yl)oxy]valerate (5). To a solution of 2-hydroxy-10,11-dihydrodibenzo[*a*,*d*]cyclohepten-5-one **(4)** (12 g, 0.054 mol) in DMF (60 mL) at 0 °C was added t-BuOK (6.61 g, 0.059 mol) under a N_2 atmosphere. The mixture was stirred at rt for 5 min. To the mixture was added dropwise ethyl 5-bromovalerate[26] (12.33 g, 0.059 mol), and the resulting solution was heated at 110 °C with stirring for 5 h. After removal of the solvent at 80 °C under reduced pressure, the residue was dissolved in AcOEt, washed with water and brine, dried over anhyd $MgSO_4$, and then evaporated to give the crude material. This material was recrystallized from AcOEt–hexane to give **5** as colorless needles (17.26 g, 91%): mp 55–56 °C; ^{1}H NMR ($CDCl_3$) δ 1.26 (3H, t, J = 7.2 Hz, CH_3), 1.80–1.90 (4H, m, CH_2CH_2), 2.39 (2H, t, 6.9 Hz, OCH_2), 3.17 (4H, s, CH_2CH_2), 4.04 (2H, t, J = 6.0 Hz, CH_2CO_2), 4.14 (2H, q, J = 7.2 Hz, CH_2), 6.69 (1H, d, J = 2.7 Hz, 1-H), 6.84 (1H, dd, J = 8.7, 2.7 Hz, 3-H), 7.21 (1H, dd, J = 7.5, 1.2 Hz, 9-H), 7.32 (1H, dt, J = 7.5(t), 1.5(d) Hz, 7-H), 7.42 (1H, dt, J = 7.5(t), 1.5(d) Hz, 8-H), 8.02 (1H, dd, J = 7.8, 1.5 Hz, 6-H), 8.16 (1H, d, J = 8.7 Hz, 4-H). Anal. Calcd for $C_{22}H_{24}O_4$: C, 74.98; H, 6.86. Found; C, 74.89; H, 6.77.

5-[(5-Oxo-10,11-dihydrodibenzo[*a*,*d*]cyclohepten-2-yl)oxy]valeric Acid (6). Ethyl 5-[(5-oxo-10,11-dihydrodibenzo-[*a*,*d*]cyclohepten-2-yl)oxy]valerate **(5)** (30 g, 0.085 mol) was dissolved in 2 N NaOH in dioxane (100 mL). The mixture was stirred at rt for 16 h and then acidified with 3% HCl to give crystals. Recrystallization from AcOEt–hexane gave **6** as colorless plates (26.445 g, 96%): mp 121–122 °C, ^{1}H NMR ($CDCl_3$) δ 1.58–1.59 (1H, m, CH_2CH_2), 2.46 (2H, t, J = 6.8 Hz, OCH_2), 3.17 (4H, s, CH_2CH_2), 4.05 (2H, t, J = 5.6 Hz, CH_2-CO_2), 6.70 (1H, d, J = 2.7 Hz, 1-H), 6.84 (1H, dd, J = 9.0, 2.7 Hz, 3-H), 7.21 (1H, dd, J = 7.5, 1.2 Hz, 9-H), 7.32 (1H, dt, J = 7.5(t), 1.2(d) Hz, 7-H), 7.42 (1H, dt, J = 7.5(t), 1.5(d) Hz, 8-H), 8.01 (1H, dd, J = 7.8, 1.2 Hz, 6-H), 8.15 (1H, d, J = 9.0 Hz, 4-H). Anal. Calcd for $C_{20}H_{20}O_4$: C, 74.05, H, 6.22. Found: C, 73.93; H, 6.12.

5-{[(*R*,*S*)-5-(Fmoc-amino)-10,11-dihydrodibenzo[*a*,*d*]-cyclohepten-2-yl]oxy}valeric Acid (7). To a solution of 5-[(10,11-dihydrodibenzo[*a*,*d*]cyclohepten-2-yl)oxy]valeric acid **(6)** (1.0 g, 3.09 mmol) in *i*-PrOH (20 mL) were added TEA (0.313 g, 3.09 mol) and $NaBH_4$ (0.584 g, 15.43 mmol). The mixture was refluxed for 2 h. After evaporation of the solvent, water was added to the residue. The resulting mixture was acidified with 3% HCl (pH 4.0) and then extracted with AcOEt. The organic layer was washed with brine, dried over anhyd $MgSO_4$, and evaporated to give the crude alcohol. The alcohol and Fmoc-NH_2[27] (0.738 g, 3.09 mmol) were dissolved in glacial AcOH (35 mL). A catalytic amount of TsOH was added, and the resulting pale yellow solution was stirred at rt for 1 h. This mixture was diluted with water to provide crystals, which were collected and recrystallized from MeOH–acetone to give **7** as a colorless powder (1.449 g, 86%): mp 187–189 °C. ^{1}H NMR (DMSO-d_6) δ 1.60–1.80 (4H, m, CH_2CH_2), 2.27 (2H, t, J = 7.1 Hz, OCH_2), 3.00–3.30 (4H, m, CH_2CH_2, 10-H, 11-H), 3.92 (2H, t, J = 6.0 Hz, CH_2CO_2), 4.25 (3H, broad s, CO_2CH_2CH), 6.03 (1H, d, J = 9.1 Hz, 5-H), 6.72 (2H, broad s, 1-H, 3H), 7.15–7.90 (13H, m, aromatic), 8.54 (1H, broad d, J = 9.1 Hz, NH), 12.03 (1H, broad s, COOH). Anal. Calcd for $C_{35}H_{33}O_5N$; C, 76.76; H, 6.07; N, 2.56. Found: C, 76.57; H, 6.08; N, 2.74.

2-Methoxydibenzo[*a*,*d*]cyclohepten-5-one (8). To a solution of 2-methoxy-10,11-dihydrodibenzo[*a*,*d*]cyclohepten-5-one **(3)** (6.0 g, 0.025 mol) in CCl_4 (200 mL) was added NBS (4.487 g, 0.025 mol). The reaction mixture was refluxed for 16 h. The insoluble precipitates were filtered off, and the filtrate was evaporated. The residual oil was dissolved in TEA (100 mL), and the reaction mixture was refluxed for 16 h. The solvent was evaporated, and the residue was dissolved in AcOEt. This solution was washed with water, 3% HCl, and brine, dried over anhyd $MgSO_4$, and evaporated. The residue was recrystallized from AcOEt–hexane to give **8** as colorless needles (4.115 g, 69%): mp 75–76 °C; ^{1}H NMR ($CDCl_3$) δ 3.92 (3H, s, OCH_3), 6.96 (1H, d, J = 2.4 Hz, 1-H), 6.98 (1H, d, J = 12.3 Hz, 11-H), 7.05 (1H, d, J = 12.3 Hz, 10-H), 7.10 (1H, dd, J = 8.7, 2.4 Hz, 3-H), 7.54 (1H, s, 9-H), 7.54 (1H, dt, J = 6.9-(t), 2.1(d) Hz, 7-H), 7.62 (1H, dt, J = 6.9(t), 2.1(d) Hz, 8-H), 8.26 (1H, d, J = 8.7 Hz, 4-H), 8.29 (1H, dd, J = 6.9, 2.1 Hz, 6-H). Anal. Calcd for $C_{16}H_{12}O_2$: C, 81.33; H, 5.12. Found: C, 81.44; H, 4.98.

2-Hydroxydibenzo[*a*,*d*]cyclohepten-5-one (9). To a solution of 2-methoxydibenzo[*a*,*d*]cyclohepten-5-one **(8)** (1.0 g,

(27) Fmoc-amide was prepared from 9-fluorenemethanol (ref 26) by means of the procedure described in ref 19.

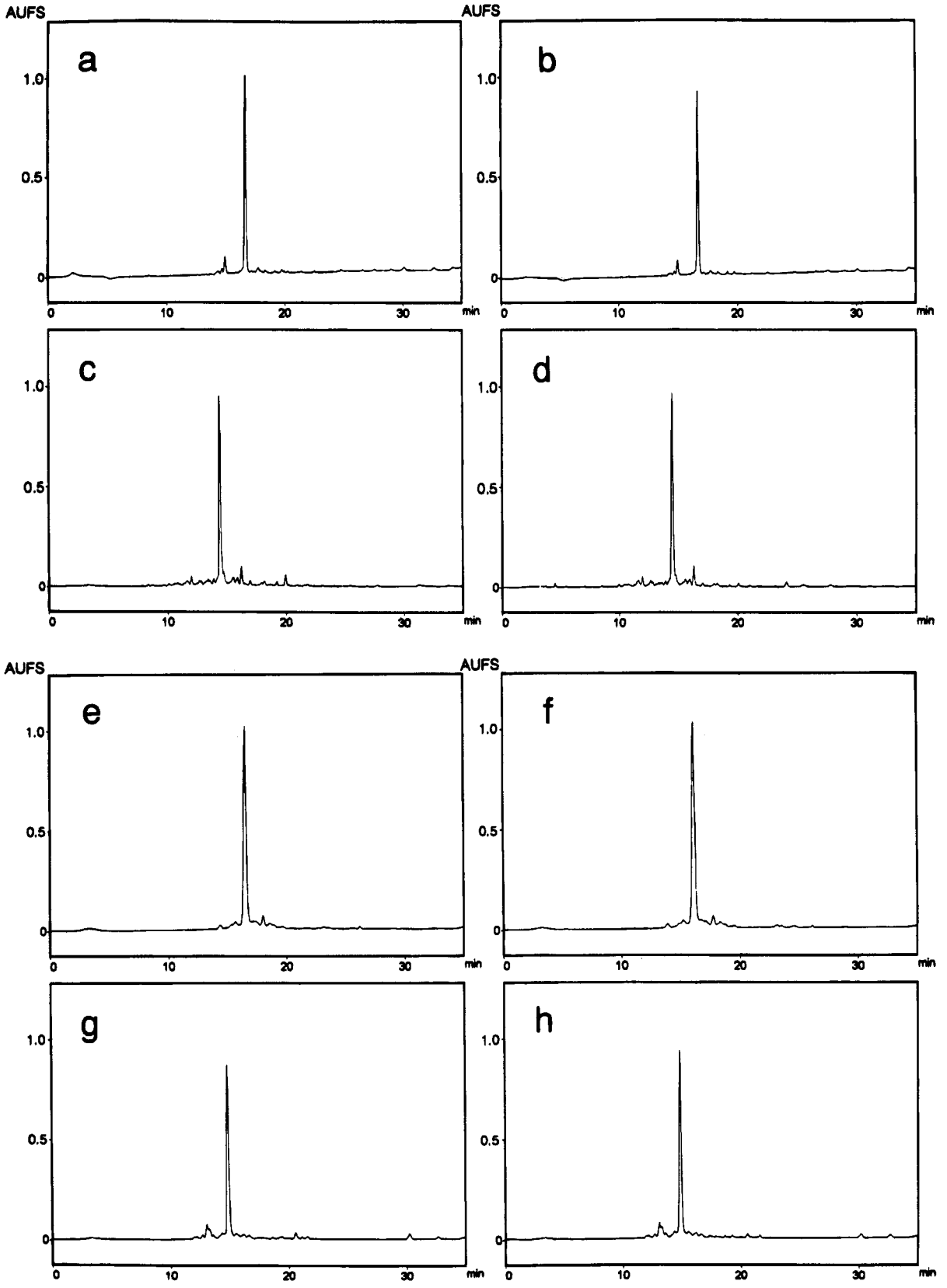

Figure 4. 4. RP-HPLC of cleaved crude peptides: a, secretin from CHA; b, secretin from CHE; c, PHI from CHA; d, PHI from CHE, e, PACAP38 from CHA; f, PACAP38 from CHE; g, GRF from CHA; h, GRF from CHE. Column: SynProPep RPC18 (4.6 × 150 mm); eluent: 0.01 N HCl/CH_3CN = 80/20–50/50 (a, b, g, h), 75/25–45/55 (c, d), 90/10–60/40 (e, f) in 30 min; flow rate of 1.0 mL/min; absorbance at 210 nm.

4.24 mmol) DCM (30 mL) was added $AlCl_3$ (1.70 g, 12.72 mmol), and the mixture was refluxed for 24 h. The reaction mixture was poured into water with ice. The DCM solution was separated, and the water layer was extracted with AcOEt. The combined extracts were washed with brine, dried over anhyd $MgSO_4$, and evaporated to give the crude product. The crude product was purified on a silica gel column chromatography, with AcOEt–hexane (1:4 and 1:2) as eluant, to give **9**

(0.82 g, 87%): colorless needles, mp 198–199 °C (from AcOEt-hexane), ^{1}H NMR ($CDCl_3$) δ 5.59 (1H, broad, s, OH), 6.94 (1H, d, J = 12.0 Hz, 11-H), 6.94 (1H, d, J = 2.4 Hz, 1-H), 7.02 (1H, dd, J = 8.4, 2.4 Hz, 3-H), 7.05 (1H, d, J = 12.0 Hz, 10-H), 7.54 (1H, s, 9-H), 7.54 (1H, dt, J = 6.9(t), 2.1(d) Hz, 7-H), 7.63 (1H, dt, J = 6.9(t), 2.1(d) Hz, 8-H), 8.23 (1H, d, J = 8.7 Hz, 4-H), 8.29 (1H, dd, J = 6.9, 2.1 Hz, 6-H). Anal. Calcd for $C_{15}H_{10}O_2$: C, 81.06; H, 4.54. Found: C, 80.81; H, 4.35.

Ethyl 5-[(5-Oxo-dibenzo[*a*,*d*]cyclohepten-2-yl)oxy]valerate (10). To a solution of 2-hydroxydibenzo[*a*,*d*]cyclohepten-5-one (**9**) (1.415 g, 6.37 mmol) in DMF (15 mL) at 0 °C was added *t*-BuOK (0.786 g, 7.0 mmol) under a N_2 atmosphere. The mixture was stirred for 5 min at rt, combined with ethyl 5-bromovalerate (1.465 g, 7.0 mmol), and heated at 110 °C with stirring for 5 h. After removal of the solvent at 80 °C under reduced pressure, the residue was dissolved in AcOEt, washed with brine, and dried over anhyd $MgSO_4$. After evaporation, the residue was purified by silica gel column chromatography, with AcOEt–hexane (1:7) as eluant, to give **10** (1.876 g, 84%): colorless needles, mp 56–57 °C (from AcOEt–hexane); ^{1}H NMR ($CDCl_3$) δ 1.26 (3H, t, 7.2 Hz, CH_3), 1.8–1.9 (4H, m, CH_2CH_2), 2.40 (2H, t, J = 6.9 Hz, OCH_2), 4.07 (2H, t, J = 6.0 Hz, CH_2CO_2), 4.14 (2H, q, J = 7.2 Hz, CH_2), 6.94 (1H, d, J = 2.4 Hz, 1-H), 6.95 (1H, d, J = 12.0 Hz, 11-H), 7.03 (1H, d, J = 12.0 Hz, 10-H), 7.07 (1H, dd, J = 8.7, 2.4 Hz, 3-H), 7.53 (1H, d, J = 7.2 Hz, 9-H), 7.53 (1H, dt, J = 7.2(t), 1.2(d) Hz, 7-H), 7.61 (1H, dt, J = 7.2(t), 1.2(d) Hz, 8-H), 8.25 (1H, d, J = 8.7 Hz, 4-H), 8.28 (1H, dd, J = 7.8, 1.5 Hz, 6-H). Anal. Calcd for $C_{22}H_{22}O_4$: C, 75.41; H, 6.33. Found: C, 75.37; H, 6.34.

5-[(5-Oxo-dibenzo[*a*,*d*]cyclohepten-2-yl)oxy]valeric Acid (11). To a solution of 5-[(5-oxo-dibenzo[*a*,*d*]cyclohepten-2-yl)-oxy]valerate (**10**) (1.7 g, 4.86 mmol) in dioxane (20 mL) was added 2 N NaOH (10 mL), and the reaction mixture was stirred at rt for 4 h. After evaporation of the solvent, the residue was suspended in water and acidified with 3% HCl to give a precipitate. The precipitate was collected, washed with water, and dried *in vacuo* to give **11** (1.502 g, 96%): colorless prisms, mp 123–124 °C (from AcOEt–hexane); ^{1}H NMR ($CDCl_3$) δ 1.80–1.95 (4H, m, CH_2CH_2), 2.46 (2H, t, J = 6.9 Hz, OCH_2), 4.09 (2H, t, J = 5.7 Hz, CH_2CO_2), 6.94 (1H, d, J = 2.4 Hz, 1-H), 6.96 (1H, d, J = 12.0 Hz, 11-H), 7.04 (1H, d, J = 12.0 Hz, 10-H), 7.07 (1H, dd, J = 8.7, 2.4 Hz, 3-H), 7.52 (1H, d, J = 7.2 Hz, 9-H), 7.54 (1H, dt, J = 7.2(t), 1.2(d) Hz, 7-H), 7.62 (1H, dt, J = 7.2(t), 1.2(d) Hz, 8-H), 8.25 (1H, d, J = 8.7 Hz, 4-H), 8.29 (1H, dd, J = 7.2, 1.2 Hz, 6-H). Anal. Calcd for $C_{20}H_{18}O_4$: C, 74.52; H, 5.63. Found: C, 74.31; H, 5.54.

5-{[(*R*,*S*)-5-(Fmoc-amino)dibenzo[*a*,*d*]cyclohepten-2-yl]oxy}valeric Acid (12). To a solution of 5-[(5-oxo-dibenzo-[*a*,*d*]cyclohepten-2-yl)oxy]valeric acid (**11**) (1.0 g, 3.1 mmol) in *i*-PrOH (20 mL) were added TEA (0.313 g, 3.1 mmol) and $NaBH_4$ (0.587 g, 15.5 mmol). The reaction mixture was refluxed with stirring for 2 h. After evaporation of the solvent, the residue was dissolved in water, acidified (pH 4.0) at 0 °C, and then extracted with AcOEt. The organic layer was washed with brine and dried over anhyd $MgSO_4$. TEA (2 mL) was added to the washed and dried organic layer, and it was then evaporated to dryness to provide the crude alcohol. This alcohol was dissolved in DMF (12 mL) and combined with Fmoc-NH_2 (0.815 g, 3.41 mmol) and TsOH (0.59 g, 3.1 mmol). The reaction mixture was stirred at rt for 1 h and then mixed with ice and water. The precipitated crystals were collected, washed with water, and dried *in vacuo* to give the crude material, which was recrystallized from MeOH–acetone to give **12** (1.352 g, 80%): colorless powder, mp 134–135 °C. ^{1}H NMR (DMSO-d_6) δ 1.59–1.80 (4H, m, CH_2CH_2), 2.28 (2H, t, J = 7.1 Hz, OCH_2), 3.97 (2H, t, J = 6.0 Hz, CH_2CO_2), 4.25 (3H, broad, s, CH_2CH=), 5.36 (1H, broad, s, 5-H), 6.98–7.90 (17H, m, aromatic), 8.4–8.8 (1H, broad, s, NH), 12.0 (1H, broad, s, COOH). Anal. Calcd for $C_{35}H_{31}O_5N$: C, 77.04; H, 5.73; N, 2.57. Found: C, 76.77; H, 5.84; N, 2.52.

General Procedure for Loading the CHA or CHE Handle onto the Amino Group of Polystyrene–Polyethylene Glycol Graft Copolymer (TentaGel S NH_2). To a solution of Fmoc-CHA (0.213 g, 0.39 mmol) or Fmoc-CHE (0.213 g, 0.39 mmol) in NMP (2 mL) were added PyBOP (0.203 g, 0.39 mmol), HOBt (0.06 g, 0.39 mmol), and TentaGel S NH_2 (0.5 g, 0.26 mequiv/g). Then DIEA (68 μL 0.39 mmol) was added and the whole mixture was shaken at rt for 5 h. The resin was filtered and washed with DCM. For capping, the resin was treated with acetic anhydride (24 μL, 0.26 m mol) and pyridine (21 μL, 0.26 mmol) in DCM (2 mL) for 1 h. The resin was filtered and washed with DMF, DCM, MeOH, and ether and dried *in vacuo*. An aliquot of the resin (*ca.* 10 mg) was quantified by a spectrophotometric method,[21] which indicated 78–80% incorporation.

Efficiency of Cleavage from Handles. Fmoc-Val was manually coupled to the above resin. Aliquots (ca. 10 mg) of the PAL, CHA, and CHE resins were treated with 50% TFA/5% phenol in DCM and 10% TFA/5% phenol in DCM, respectively. At various cleavage times the mixture was quickly diluted with DMF; washed with DMF, DCM, MeOH, and ether, and dried *in vacuo*. The remaining Fmoc-group on the resin was quantified by a spectrophotometric method.[23] This experiment was carried out three times.

Synthesis of Protected Pentapeptide Resin. A pentapeptide (Fmoc-Phe-Val-Gly-Leu-Met-NH_2) was synthesized by means of the standard protocol and by the N^{α}-Fmoc protocol. The assembly was performed with PyBOP, HOBt, and NMM (1:1:1.5 molar ratio) using the PAL-Resin[28] (400 mg, 0.32 mequiv/g), RAM-TentaGel S (400 mg, 0.20 mequiv/g), and the resins with the novel handles, CHA-TentaGel S (400 mg, 0.17 mequiv/g), and CHE-TentaGel S (400 mg, 0.14 mequiv/g). D/L amino acid analysis revealed that there was no racemization.

Determination of Cleavability for the Above Pentapeptide. The above N^{α}-Fmoc-pentapeptide resin (10 mg) was placed in the reaction vessel of the synthesizer and cleaved with 600 μL of a freshly prepared cocktail consisting of TFA–*thioanisole*–EDT–DCM (90:5:5:0 v/v, 50:5:5:40 v/v, or 25:5:5:65 v/v) at rt for 5, 10, 20, 30, and 60 min. The cocktail was then collected by filtration under a stream of nitrogen, and the remaining resin was washed with DCM (3 mL) and dried *in vacuo*. The amount of Fmoc groups of the remaining resin was determined as follows: The resin was treated with piperidine–DCM (1:1 v/v) (0.5 mL) for 30 min and filtered. The resin was washed with additional DCM (5 mL), and the filtrates were combined and concentrated to dryness. The resulting residue was dissolved in DCM (50 mL) and quantified by UV. The major peak on analytical HPLC of the cleaved crude N^{α}-Fmoc-peptide contained more than 95% of the desired material. This experiment was repeated three times. The same procedure was carried out without resin to determine the background.

Synthesis of Human Secretin. The synthesis was carried out with HBTU/HOBt/DIEA (1:1:2 molar ratio), and Fmoc-amino acids were coupled to the CHA-TentaGel S (30 mg, 0.17 mequiv/g) and CHE-TentaGel S (30 mg, 0.14 mequiv/g). After assembly of the 27 amino acid residues, the resulting peptide resin was simultaneously cleaved with a cocktail consisting of TFA–H_2O–thioanisole–EMS–EDT–thiophenol (82.5:5:5:3:2.5:2, v/v) at rt for 6 h and was precipitated with absolute ether. The precipitate was collected by centrifugation, dissolved in aqueous AcOH, and lyophilized to give crude peptides in quantitative amounts. This crude peptide was purified by semipreperative HPLC (same packings material as used for analysis, 20 × 150 mm). Yield 54%. MS *m/z*: calcd for $C_{130}H_{221}N_{44}O_{40}$ 3039.7 ([M + H]), found 3039.7 ([M + H]$^+$) for the CHA-resin; 3040.5 ([M + H]$^+$) by the use of CHE-resin. Sequence analysis of the peptide prepared from both resins confirmed the desired primary structure. Amino acid analysis: Ala 1.10 (1), Arg 4.22 (4), Asx 1.02 (1), Glx 3.65 (4), Gly 3.03 (3), His 0.83 (1), Leu 5.85 (6), Phe 0.92 (1), Ser 3.36 (3), Thr 1.91 (2), Val 1.11 (1). Peptide contents: 81.6%.

Syntheis of Porcine PHI. Synthesis was carried out as described for the synthesis of secretin. Starting from CHA-TentaGel S (80 mg, 0.17 mequiv/g) and CHE-TentaGel S (41 mg, 0.14 mequiv/g). Cleavage was performed as above. Ly-

(28) Purchased from Millipore Corp. (Bedford, MA).

ophilized material, which was obtained in quantitative yield, was further purified by HPLC. Yield 51%. MS m/z: calcd for $C_{136}H_{217}N_{36}O_{40}$ 2995.6 ([M + H]), found 2995.6 ([M + H]$^+$) for the CHA-resin, 2995.5 ([M + H]$^+$) for the CHE-resin. Sequencing of peptides prepared from both resins confirmed the desired primary structure. Amino acid analysis: Ala 2.15 (2), Arg 0.88 (1), Asx 1.87 (2), Glx 2.02 (2), Gly 2.21 (2), His 0.91 (1), Ile 1.12 (1), Leu 4.82 (5), Lys 2.19 (2), Phe 1.89 (2), Ser 3.95 (4), Thr 1.06 (1), Tyr 0.85 (1), Val 0.94 (1). Peptide contents: 81.3%.

Synthesis of PACAP38. By means of a coupling method with PyBOP/HOBt/NMM (1:1:1.5 molar ratio), PACAP38 was assembled on CHA-TentaGel S (60 mg, 0.17 mequiv/g) and CHE-TentaGel S (56 mg, 0.14 mequiv/g). After cleavage and lyophilization, as previously described, the crude peptides, obtained in quantitative amounts, were purified by HPLC. Yield 50%. MS m/z: PACAP38, calcd for $C_{203}H_{332}N_{64}O_{53}S_1$ 4534.5 ([M + H]), found 4534.5 ([M + H)$^+$) for the CHA-resin, 4534.3 ([M + H]$^+$) for the CHE-resin. Sequencing of peptides prepared from both resins confirmed the desired primary structure. Amino acid analysis: Ala 3.20 (3), Arg 3.90 (4), Asx 2.76 (3), Glx 1.80 (2), Gly 2.25 (2), His 0.93 (1), Ile 0.94 (1), Leu 1.87 (2), Lys 7.27 (7), Met 0.80 (1), Phe 1.00 (1), Ser 2.96 (3), Thr 1.03 (1), Tyr 3.94 (4), Val 2.69 (3). Peptide contents: 81.5%.

Synthesis of Human GRF. Human GRF was assembled, cleaved, and lyophilized as for PACAP38. The crude peptides, which were obtained in quantitative amounts, were purified by HPLC. Yield 45%. MS m/z: calcd for $C_{215}H_{359}N_{72}O_{66}S_1$ 5039.7 ([M + H]), found 5039.6 ([M + H]$^+$) for the CHA-resin, 5039.8 ([M + H]$^+$) for the CHE-resin. Sequencing of the peptides from both resins confirmed the desired primary structure. Amino acid analysis: Ala 5.00 (5), Arg 6.03 (6), Asx 3.79 (4), Glx 6.70 (7), Gly 3.31 (3), Ile 1.97 (2), Leu 4.69 (5), Lys 2.12 (2), Met 0.86 (1), Phe 0.91 (1), Ser 3.75 (4), Thr 1.05 (1), Tyr 1.93 (2), Val 1.01 (1). Peptide contents: 84.2%.

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