Semisynthesis of Ht31(493 – 515): Involvement of PKA-Anchoring Proteins in the Regulation of the cAMP-Dependent Chloride Current in Heart Cells

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The synthesis of larger peptides or proteins by a protease-catalyzed segment condensation strategy, which does not lead to racemization and is independent of the side chain protection of trifunctional amino acids, can be a valuable alternative to the usual methods of peptide synthesis.[1] With enzymes, the segments can be ligated either at a specific amino acid residue or at a nonspecific amino acid moiety. The latter concept was invented in our laboratories and is based on the principle of enzymatic peptide synthesis by a substrate mimetics strategy.[2] In substrate mimetics, the site-specific amino acid moiety is transferred onto the leaving group of the acyl component enabling the acylation of the enzyme by a nonspecific acyl residue. Deacylation of the acyl enzyme by the amino component results in peptide synthesis regardless of the primary specificity of the proteases. Moreover, the newly formed peptide bond is not subject to secondary enzymatic hydrolysis due to the absence of a specific amino acid residue. In this respect, various ester moieties behave as artificial recognition sites for proteases. Obviously, it would be useful to combine the enzymatic ligation of segments with suitable methods of solid-phase peptide synthesis, especially for the preparation of acyl component peptide segments in the form of appropriate esters. We have previously demonstrated the general usefulness of Kaiser's oxime resin[3] for the syntheses of protected peptide esters in the form of various substrate mimetics that were used as acyl donor components in protease-catalyzed segment condensation of model peptides.[4]

Here we describe the application of this methodology to the semisynthesis of the biologically active 493 – 515 sequence of human thyroid PKA-anchoring** protein Ht31 [Ht31(493 – 515)][5] by α-chymotrypsin-catalyzed (8 + 16) segment condensation. The resulting peptide H-Asp-Leu-Ile-Glu-Glu-Ala-Ala-Ser-Arg-Ile-Val-Asp-Ala-Val-Ile-Glu-Gln-Val-Lys-Ala-Ala-Gly-Ala-Tyr-OH represents a minimum region of Ht31 required to bind to the regulatory subunit dimer of PKA.[5, 6] The Ht31(493 – 515) peptide synthesized was further used in biological studies that provided new insights into the PKA-mediated regulation of a chloride current in the heart.

In enzymatic peptide synthesis, the nucleophile specificity of proteases for the amino component represents one of the key parameters for obtaining high peptide product yields.[7] Generally, the presence of the Arg residue at the N terminus of the peptide amino component (P_1' position, binding-site notation according to Schechter and Berger[8]) significantly increases its nucleophilic efficiency in the acyl transfer reaction catalyzed by α-chymotrypsin.[9] In addition, the effect could be further enhanced by the presence of a large hydrophobic residue in the P_2' position.[9] The -Arg-Ile- motif in the sequence of peptide Ht31 evidently offers the choice of the Ser – Arg peptide bond as a coupling point for α-chymotrypsin-catalyzed segment ligation. The restriction of substrate specificity of the enzyme for aromatic amino acid residues in the P_1 position could be easily overcome in this case (Ser residue in P_1) by using an acylating peptide component as a substrate mimetic.[10]

The route to the final Ht31(493 – 515) peptide is illustrated in Scheme 1. The acylating component Boc-Asp-Leu-Ile-Glu-Glu-Ala-Ala-Ser-OPh (-OGp) [**2a** (**2b**)] used as a substrate mimetic was synthesized by using the oxime resin according to the same protocol as previously described by us.[4] The protected octapeptide esters **1a** and **1b** were released from the resin by aminolysis of the oxime ester linkage with two equivalents of TFA · Ser(Bzl)-OPh (-OGp)[11] in the presence of DIEA, catalyzed by acetic acid in THF. The crude fully protected octapeptide phenyl ester **1a** was somewhat purer than **1b** as determined by HPLC. The side chain deprotection was achieved by catalytic hydrogenation in a mixture of ethanol/THF at elevated temperature yielding **2a** and **2b**, respectively. Hexadecapeptide **3**, which was synthesized according to an Fmoc protocol on *p*-alkoxybenzyl alcohol resin,[12] was used for the enzymatic coupling as a crude product without further purification. The reaction conditions for α-chymotrypsin-catalyzed condensation of **2** with **3** were chosen empirically by using a mixture of sodium veronal buffer (pH 8.5) and DMSO (3:2). The best yields were achieved when the molar ratio of acyl component **2** to amino component **3** was 2:1. Under these conditions, regardless of the structure of the leaving group of the acylating component **2**, the complete consumption of both peptide segments accompanied by the formation of **4** was observed by HPLC analyses. The condensation of **2a** with **3** was monitored by HPLC analysis at 280 nm

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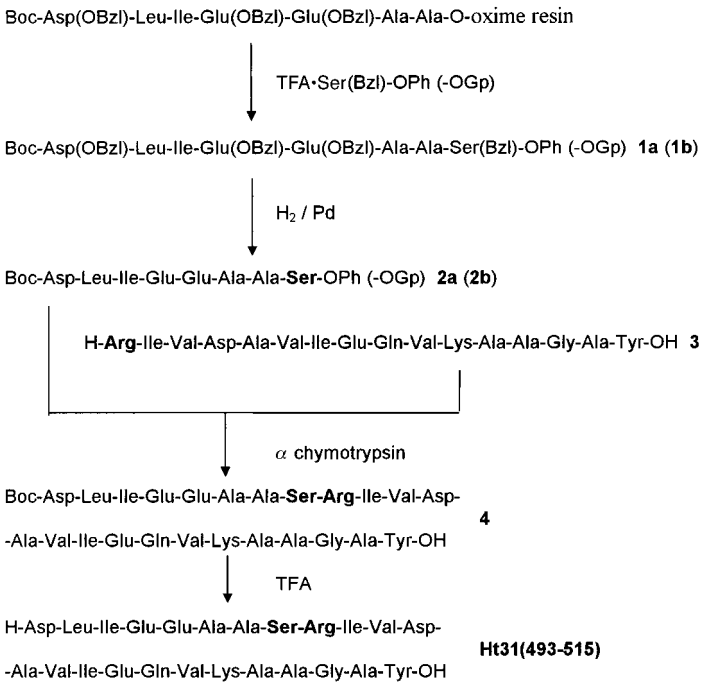
[**] For abbreviations see ref. [14].

(Figure 1). Chromatograms a) and b) show the composition of the mixture immediately after the addition of the enzyme and at the end of the reaction, respectively. Peak A represents the leaving group phenol, peak B represents **3**, and peak C was characterized as **4**, the product of the condensation. The



Scheme 1. *Schematic representation of the synthesis of peptide Ht31(493 – 515), which is based on an enzymatic segment condensation by using a substrate mimetics strategy.*

composition of the reaction mixture as detected by HPLC at 222 nm (not shown) also indicates complete conversion of **2a**. In a blank experiment, segment **3** was resistant to the enzymatic hydrolysis under the conditions used. For the enzymatic condensation we preferred to work with **2a** rather than with **2b** because of the higher stability of phenyl esters as compared to 4-guanidinophenyl esters at alkaline pH. The isolation of the product by preparative HPLC afforded the homogeneous Boc-protected 24-meric peptide **4** in 30% yield.

Biological studies with the deprotected synthetic peptide Ht31(493 – 515) revealed new findings about the PKA-dependent regulation of ion channels in mammalian heart cells. Figure 2 shows the time course of the membrane current of two whole-cell voltage-clamped single guinea pig ventricular myocytes in response to forskolin. Forskolin stimulates adenylyl cyclases leading to the activation of PKA through increased intracellular cAMP levels. Cells were dialyzed with pipette solutions containing the specific PKA inhibitor peptide PKI (20 μM) under experimental conditions designed to isolate the cAMP-dependent chloride current. In the absence of Ht31(493 – 515), application of 4 μM forskolin resulted in a marked transient activation of the chloride current (Figure 2). Since this activation occurred in the presence of PKI, it suggested a tight functional

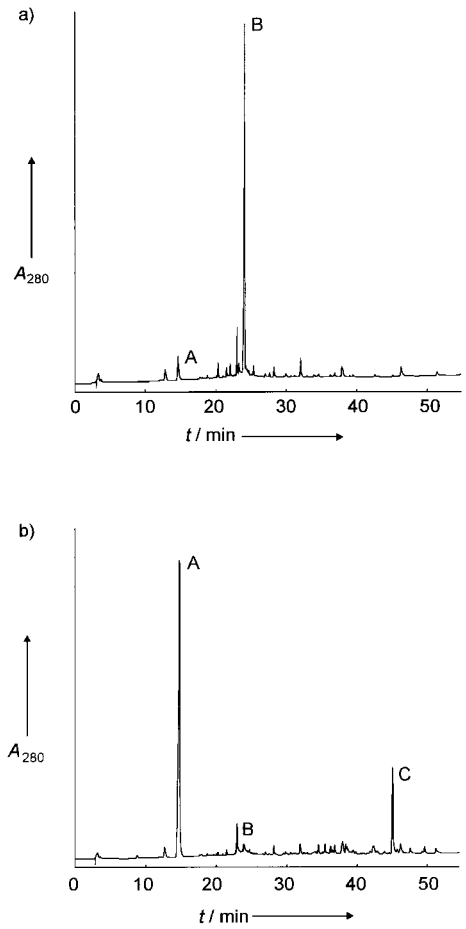


Figure 1. *Reversed-phase HPLC analyses of the reaction mixture from the α-chymotrypsin-catalyzed condensation of **2a** with **3** (detection at 280 nm, linear gradient from 5% acetonitrile in 0.1% TFA to 0.1% TFA in 95% acetonitrile over 60 min). Peaks A, B, and C represent phenol as the ester leaving group, hexadecapeptide **3**, and condensation product **4**, respectively. a) Composition of the mixture immediately after the addition of the enzyme, b) at the end of the reaction (30 min).*

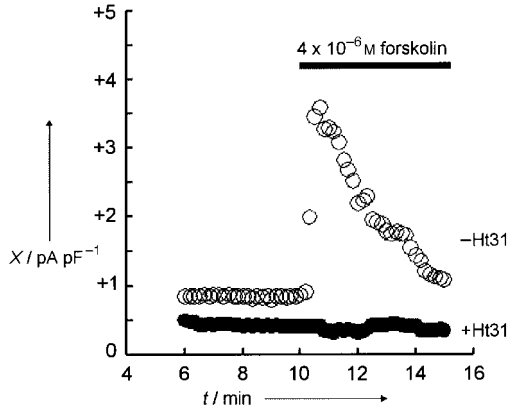


Figure 2. *Effect of peptide Ht31(493 – 515) on the forskolin-dependent activation of the cAMP-dependent chloride current X in guinea pig ventricular myocytes. In the absence of Ht31(493 – 515) (open circles, − Ht31), forskolin elicits a large, transient outward chloride current (the application of forskolin is indicated by the horizontal bar above the current trace). By contrast, inclusion of Ht31(493 – 515) in the pipette solution (filled circles, + Ht31) abolishes the forskolin-related effect.*

coupling between the PKA molecules and the chloride channels. This coupling may be due to specific anchoring proteins targeting the kinases to the plasma membrane in close proximity to the channels. To test this hypothesis, experiments were repeated in the presence of 60 μM Ht31(493 – 515) in the pipette solution to dislocate the PKA molecules from their respective anchoring proteins. Under these conditions, forskolin did not activate the chloride current (Figure 2) suggesting that PKA-anchoring proteins are involved in the regulation of the cAMP-dependent chloride current in heart cells and demonstrating that the synthetic peptide Ht31(493-515) is biologically active.

Experimental Section

Materials: TLCK-treated α chymotrypsin (EC 3.4.21.1) was purchased from Fluka (Switzerland), Fmoc-Tyr(*t*Bu)-*p*-alkoxybenzyl alcohol resin from Bachem (Germany). Substituted oxime resin (0.53 mmol g^{-1}), amino acid derivatives, and reagents for the synthesis were purchased either from NovaBiochem (Germany) or from Bachem. Forskolin was obtained from Sigma (Germany) and PKI from Alexis (Germany).

Chemical syntheses

1a: The synthesis of peptide **1a** started with the attachment of Boc-Ala-OH (1.2 mmol) to the oxime resin (1 g) by standard DCC coupling.[3] The substitution level determined by amino acid analysis was 0.21 mmol g^{-1}. After placing the resin in the peptide synthesizer, the rest of the synthesis was carried out semiautomatically by manual addition of N^{α}-Boc-protected amino acid (3 equiv) and HBTU (3 equiv) in the presence of DIEA (5 equiv) in each coupling step. The acetylation of free hydroxy groups, deprotection, and washing procedures were the same as previously described.[3] The Kaiser test was performed after each deprotection and coupling step. The protected heptapeptide linked to the resin (1 g) was treated with TFA · Ser(Bzl)-OPh (148 mg, 0.4 mmol), DIEA (68 μL, 0.4 mmol), and acetic acid (23 μL, 0.4 mmol) in THF (15 mL) for 2 d at room temperature. The resin was filtered, washed with THF, the combined filtrate was evaporated, the residue triturated with a mixture of ethyl acetate/*n*-hexane (1:2), suspended in methanol, and precipitated with water. The solid material was washed with water, 5% citric acid, water, and, after drying, crystallized from ethanol/diethyl ether (4:1) yielding 130 mg (47%) of **1a**. Elemental analysis (%): calcd for $C_{74}H_{94}N_8O_{18} \cdot 2H_2O$ (1419.6): C 62.61, H 6.96, N 7.89; found: C 62.76, H 6.66, N 8.05; MS (FAB): *m/z*: 1384.4 $[M+H]^+$.

1b: Peptide **1b** was released from the resin with TFA · Ser(Bzl)-OGp(Z)$_2$ by using the same procedure as for **1a**. The similar work-up including washing the crude peptide with aqueous acidic solvent, crystallization from the mixture of ethyl acetate/*n*-hexane, and finally recrystallization from hot methanol yielded 170 mg (50%) of **1b**. MS (FAB): *m/z*: 1709.6 $[M+H]^+$.

2a: Peptide **2a** resulted from deprotection of **1a** (100 mg) by catalytic hydrogenation over Pd black in a mixture (20 mL) of ethanol/THF (4:1) for 20 h at elevated temperature. The catalyst was filtered off, the filtrate was evaporated, and the residue was triturated with the mixture of diethyl ether/*n*-hexane to give 55 mg (74%) of **2a**. Amino acid analysis: Asp 0.92, Ser 0.95, Glu 2.12, Ala 2.12, Ile 0.91, Leu 0.98; MS (FAB): *m/z*: 1045.2 $[M+Na]^+$.

2b: Peptide **2b** resulted from deprotection of **1b** (80 mg) by hydrogenation in the presence of an equimolar amount of *p*-toluenesulfonic acid (10 mg) in the same way as described for **2a**. After trituration with diethyl ether, 50 mg (85%) of **2b** was obtained. Amino acid analysis: Asp 0.92, Ser 1.03, Glu 2.10, Ala 2.08, Ile 0.92, Leu 0.94; MS (FAB): *m/z*: 1081.4 $[M+H]^+$.

3: Peptide **3** was synthesized by standard Fmoc strategy starting from Fmoc-Tyr(*t*Bu)-*p*-alkoxybenzyl alcohol resin (substitution 0.48 mmol g^{-1}; 1 g) by using the DIC/HOBt coupling method. The last three amino acid residues were attached with the help of HBTU in the presence of HOBt and DIEA. The hexadecapeptide was cleaved from the resin with a mixture of TFA/H_2O/triethylsilane (95:2.5:2.5) yielding 600 mg of crude peptide **3**, which was recrystallized from ethyl acetate and used for the enzymatic condensation without further purification. Amino acid analysis: Asp 1.19, Glu 2.25, Gly 1.15, Ala 4.32, Val 2.45, Ile 1.51, Tyr 1.11, Lys 1.06, Arg 0.95; MS (FAB): *m/z*: 1703.8 $[M+H]^+$.

Ht31(493 – 515): The peptide Ht31(493 – 515) was obtained from **4** (product of enzymatic ligation) after the removal of the N^{α}-Boc protecting group. For this purpose, 3.6 mg tetracosapeptide **4** was treated with a mixture of TFA/H_2O/triethylsilane (95:2.5:2.5) (0.1 mL) at 5 °C for 40 min. The product was precipitated with dry diethyl ether, washed several times with diethyl ether, and dried, yielding 3.2 mg of homogeneous peptide Ht31(493 – 515) (as determined by gradient HPLC and high-voltage paper electrophoresis). MS (FAB): *m/z*: 2532.3 $[M+H]^+$.

Enzymatic segment condensation: Octapeptide phenyl ester **2a** (10 mg, 0.01 mmol) dissolved in DMSO (320 μL) was added to a solution of hexadecapeptide **3** (10 mg, 0.005 mmol) in 0.2 M sodium veronal buffer (pH 8.5; 465 μL) alkalized with 1 M NaOH (10 μL) to pH 9 (the apparent pH value was determined with precise indicator paper). After the addition of α chymotrypsin (TLCK-treated; 0.1 mg) dissolved in 1 mM HCl (5 μL), the mixture was stirred at room temperature for 30 min. The progress of the reaction was followed by gradient HPLC (Vydac C-18, 25 × 0.4 cm column, 0.1% TFA/acetonitrile eluent, flow rate 1 mL min^{-1}, detection at 280 and 222 nm). After termination of the reaction with 2% TFA (1 mL), the product (peptide **4**) was isolated in two runs by reversed-phase HPLC (Vydac C-18, 25 × 1 cm column, flow rate 3 mL min^{-1}, same gradient system, detection at 280 nm), yielding 4.3 mg (30%) of **4**. Amino acid analysis: Asp 2.23, Ser 1.22, Glu 4.24, Gly 1.25, Ala 6.16, Val 2.37, Ile 2.27, Leu 1.12, Tyr 1.03, Lys 1.07, Arg 1.03; MS (FAB): *m/z*: 2633 $[M+H]^+$. The same reaction conditions using octapeptide 4-guanidinophenyl ester **2b** as the acyl component gave similar results. In this case, the pH value of the medium was kept below 9 to avoid nonenzymatic ester hydrolysis of **2b**.

Electrophysiology: Single guinea pig ventricular myocytes were isolated by standard enzymatic procedures as described previously.[13] Myocytes were whole-cell voltage-clamped at 30 °C by means of an EPC-8 patch clamp amplifier (HEKA, Germany). Patch pipettes (GC150TF-10, Clark Electromedical Instruments, UK) had initial resistances between 1 and 2 MΩ when filled with the pipette solution. Membrane currents were low-pass-filtered at 200 Hz, sampled at 1 kHz, and recorded and analyzed with the ISO2 software package (MFK, Germany). Solutions were chosen so as to isolate the cAMP-dependent chloride current. Cells were superfused with a solution containing 144 mM NaCl, 5 mM $NiCl_2$, 4 mM $BaCl_2$, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES (pH 7.4, adjusted with NaOH). The pipette solution included 105 mM tetraethylammonium aspartate, 30 mM NaCl, 20 mM NaOH, 3 mM $MgCl_2$, 6 mM EGTA, 16 mM HEPES, 10 mM MgATP (pH 7.3, adjusted with tetraethylammonium hydroxide). Thus, the equilibrium potential for chloride was −40 mV. Membrane currents were recorded at +40 mV and normalized to cell membrane capacitance. Under these experimental conditions, the outward current activated by forskolin was the cAMP-dependent chloride current as verified by its pharmacological and biophysical properties.[13]

Forskolin and the Ht31(493 – 515) peptide were prepared as 10^{-2} M stock solutions in ethanol and DMSO, respectively, and diluted to the final concentrations in the bath or pipette solution as indicated in the text. PKI was directly dissolved in the pipette solution.

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[14] Abbreviations: Boc = *tert*-butoxycarbonyl, Bzl = benzyl, cAMP = cyclic adenosine 3′,5′-monophosphate, DCC = *N*,*N*′-dicyclohexylcarbodiimide, DIC = *N*,*N*-diisopropylcarbodiimide, DIEA = *N*,*N*-diisopropylethylamine, Fmoc = fluoren-9-ylmethoxycarbonyl, EGTA = ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HBTU = *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, HEPES = *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid], HOBt = 1-hydroxybenzotriazole, PKA = cAMP-dependent protein kinase or protein kinase A, PKI = cAMP-dependent protein kinase inhibitor peptide (5 – 24), Obzl = benzyl ester, OGp = 4-guanidinophenyl ester, OPh = phenyl ester, TFA = trifluoroacetic acid, TLCK = N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, Z = benzoxycarbonyl.

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