Synthesis of a Protected Peptide Corresponding to Residues 1–25 of the β-Amyloid Protein of Alzheimer's Disease

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The deposition of proteinaceous amyloid plaque in the brain is a characteristic of Alzheimer's disease. These plaques are composed of the 42 amino acid β-amyloid protein (βA4), the sequence of which has been determined. βA4 is deposited in the brain as a noncrystalline, fibrous aggregate containing regular structure. Detailed structural studies of the βA4 aggregate require pure βA4 and various labeled analogs and sequence analogs. A convergent synthetic route to the βA4 protein has been designed to meet this need. This paper reports the synthesis, by solid-phase fragment coupling, of a 25-residue protected peptide corresponding to residues 1–25 of βA4. This peptide was prepared from four protected fragments which were coupled sequentially on the Kaiser oxime resin. Deprotection of the intermediate fragments led to the discovery of aspartimide formation at one site. The protection strategy and the deprotection conditions were adjusted to eliminate the undesired rearrangement.

Introduction

The extracellular deposition of amyloid plaque in the brain is a hallmark of Alzheimer's disease (AD).[1] The major component of amyloid plaque is a 42 amino acid protein called the β-amyloid protein (βA4).[2–4] βA4 is extremely insoluble and is difficult to purify, thus pure βA4 has not been obtained from amyloid plaque.[4] Two chemical syntheses of βA4 have been reported.[5,6] However, details regarding the purity of the synthetic material are not available. We have undertaken the total synthesis of βA4 for structural studies. Our convergent strategy was designed to produce the required pure synthetic material and to allow the economical preparation of ^{13}C-labeled analogs[7] and sequence analogs. We are employing a solid-phase fragment coupling approach which requires the preparation of protected fragments of the βA4 protein. We report herein the synthesis, by fragment coupling, of a pure, protected fragment of βA4 (residues 1–25).

H_2N-D^{1}AEFRHDSGY^{10}EVHHQKLVFFAEDVG^{25}SNKGAIIGLMVGGVVIA^{42}-CO_2H

βA4

The tendency of β-amyloid to form insoluble aggregated β-sheets makes it a difficult target for stepwise solid-phase peptide synthesis. The C-terminal region of the β-amyloid protein has been shown to aggregate in organic solvents when attached to polystyrene resin.[8] Aggregation can lead to low coupling yields and an accumulation of single amino acid deletion impurities which are difficult to separate from the target protein.[9] To avert these problems, we employ the solid-phase fragment coupling strategy originally developed by E. T. Kaiser,[10,11] with modifications developed in our laboratory.[12–14] Impurities resulting from the failure of a single fragment coupling have a molecular weight and amino acid composition very different from the final product. As a result, the final purification step is easier than in a stepwise synthesis.

We have designed a convergent synthesis of the βA4 protein in which small protected peptides (<10 residues) are prepared on the oxime resin, purified, and fully characterized. Next, the small fragments are coupled on the oxime resin to produce intermediate length fragments (~20 residues) which are again purified and fully characterized. Finally, these intermediate fragments are coupled using solid-phase methods to produce the full length peptide, which is then deprotected and purified. The first two steps in this process have been carried out successfully in the synthesis of a fully protected fragment comprising residues 1–25 of βA4. In the course of our synthesis, modifications were made in the published Kaiser oxime resin procedures[10,11] to optimize yields and minimize side reactions.

Results

The strategy for the synthesis of βA4(1–25) is shown in Scheme I.

Stepwise Synthesis and Nucleophilic Cleavage of Protected Fragments. Fragments were synthesized using standard (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) activation conditions (3 equiv of aa, 3 equiv of BOP, 5.3 equiv of diisopropylethylamine (DIEA))[15] with dimethylformamide (DMF) as the coupling solvent. βA4(10–13), βA4(14–17), and βA4(18–23) were cleaved from the resin with *N*-hydroxypiperidine (HOPip) and reduced to the free acid with zinc according to published procedures.[16,17] The

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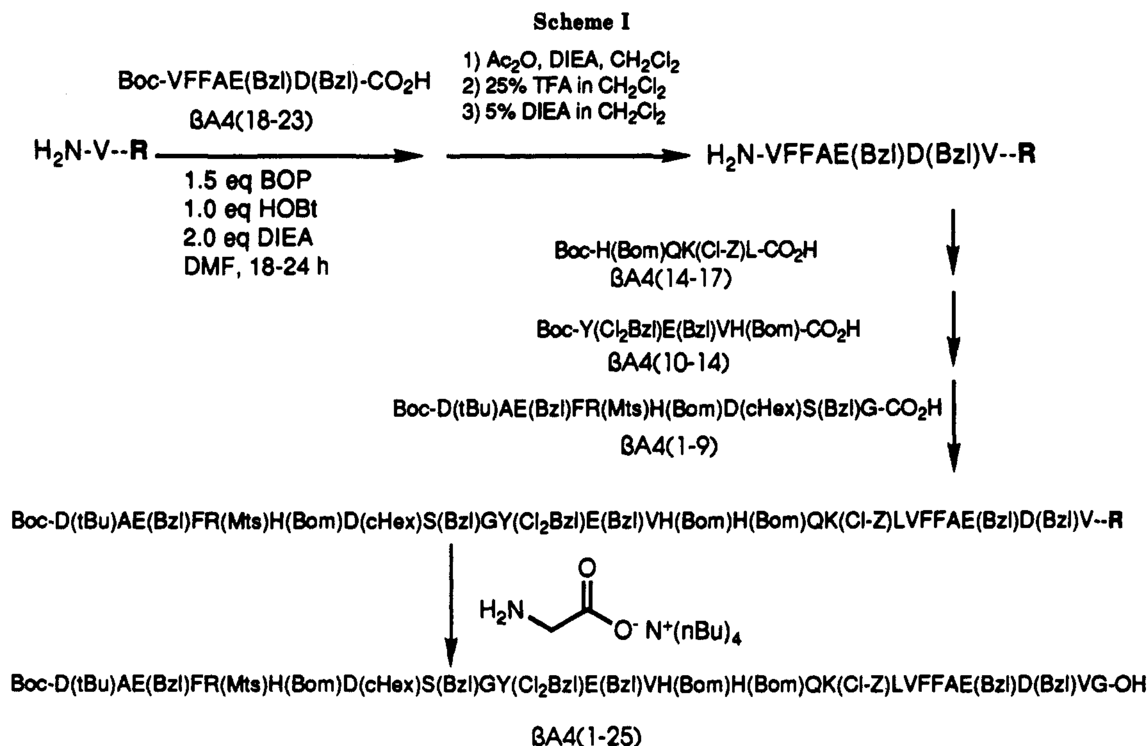
Scheme I



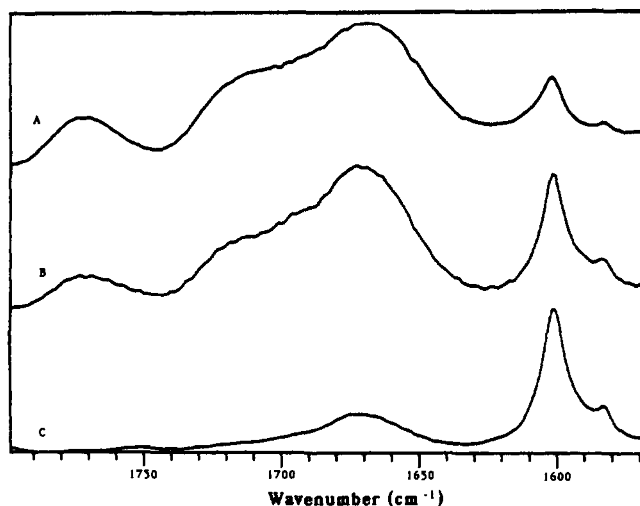


Figure 1. FTIR of Boc-H(Bom)QK(Cl-Z)L-resin as an assay of cleavage from the resin: The size of the amide I band (1655 cm^{-1}) relative to the polystyrene band (1601 cm^{-1}) is proportional to the amount of peptide on the resin. Substitution levels were determined by amino acid analysis of the resin-bound peptides: (A) 0.5 mmol/g, (B) 0.25 mmol/g (50% cleaved), (C) 0.1 mmol/g (80% cleaved).

extent of cleavage was monitored by amino acid analysis of the cleaved resin or by FTIR of the cleaved resin (Figure 1). In the event of a low yield cleavage (<80%), the cleavage procedure was repeated to remove the remaining peptide from the resin.

βA4(10–13), βA4(14–17), and βA4(18–23) were synthesized, cleaved, and purified in 68%, 63%, and 66% yields, respectively, based on resin-bound C-terminal amino acid. βA4(1–9) was initially synthesized with a purified yield of 15–20%. Increasing the concentrations of the coupling reagents (5 equiv of aa, 5 equiv of BOP, 8.9 equiv of DIEA) and adding an acetylation step after each coupling resulted in a more homogeneous crude product and a higher purified yield (40–50%).

Coupling of Protected Fragments on the Oxime Resin. All fragment coupling yields were determined by amino acid analysis of the resin-bound peptides or by a quantitative ninhydrin test.[18] In addition, coupling reactions were monitored using an HPLC assay in which a small amount of resin-bound peptide (<20 mg) was treated with HOPip (≥2 equiv, ~15 min) in DMF and the supernatant analyzed by HPLC. This method allows direct observation of both the coupled product and the uncoupled fragment. The time course of the reaction was followed in this way.

βA4(1–25) was synthesized via four fragment couplings. The coupling yields given represent the average of four syntheses. Between 0.3 and 1.5 equiv of soluble fragment were used in each coupling and yields are based on the limiting reactant. βA4(18–23) was coupled to resin-bound Val (1.5 equiv of BOP, 1 equiv of hydroxybenzotriazole (HOBt), 2 equiv of DIEA) in 75–80% yield. βA4(14–17) was coupled to resin-bound (18–24) in 80–90% yield. βA4(10–13) was coupled to resin bound (14–24) in 80–90% yield. These three couplings were carried out at 0–4 °C to minimize epimerization. Because the final fragment, βA4(1–9), has glycine at the C-terminus, this coupling was carried out at room temperature with 5 equiv of DIEA.[13] HOBt, which is normally added to suppress epimerization,[19] was omitted. The coupling yield was 80–90%. The protected peptide was cleaved from the resin with glycine tetra-*n*-butylammonium salt and purified by reverse-phase HPLC. The final purified yield of βA4(1–25) was 11% based on βA4(1–9), which was the limiting reagent. The pure protected peptide was characterized by amino acid analysis and FAB mass spectrometry (FABMS) $(M + H)^+$.

Purification of Protected Peptides. Protected fragments were purified by reverse-phase HPLC using water (0.1% trifluoroacetic acid (TFA)) and acetonitrile (0.1% TFA) as the mobile phase. In some cases, trifluoroethanol (5%) was added to the acetonitrile in an effort to increase recovery from the HPLC column. Both gradient and

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isocratic elution were used to purify the peptides. βA4-(1–25) was also partially purified by gel-permeation chromatography using Sephadex LH-60 in DMF. Analysis of the fractions by HPLC indicated an overlap between βA4(1–25) and the truncated impurity βA4(10–25). Thus, a pure product could only be obtained by HPLC purification.

Deprotection of Peptides. The trial deprotections of each intermediate were performed in order to identify undesired side reactions and, in the case of βA4(1–25), to confirm the amino acid sequence of the protected peptide. Yields were not optimized.

Boc-D(tBu)AE(Bzl)FR(Mts)H(Bom)D(Bzl)S(Bzl)G-OH[20] (βA4(1–9), with a benzyl protecting group at Asp-7) was deprotected using the standard low–high trifluoromethanesulfonic acid (TFMSA)/TFA procedure.[21] FABMS of the product indicated that the product contained an aspartimide (mass was 18 mass units lower than expected). Tandem FAB–collision MS sequencing[22] located the aspartimide at position 7 (Figure 2). In the deprotection of the truncated model peptide Boc-R-(Mts)H(Bom)D(Bzl)S(Bzl)G-OH, careful control of the reaction temperature (0 °C for both the low and high steps) limited aspartimide formation to 15% of the crude product. Treatment of the aspartimide-containing peptide with aqueous NH_4OH (1 M, 1 h) completely hydrolyzed the aspartimide (molecular weight (FABMS) increased by 18 mass units).

Boc-R(Mts)H(Bom)D(cHex)S(Bzl)-OH (βA4(5–8)) was synthesized to test the utility of a cyclohexyl group at position 7. Deprotection by the same low–high TFMSA/TFA procedure used above resulted in a mixture of fully deprotected peptide (6%) and a partially deprotected peptide which retained the cyclohexyl group (94%). Eliminating ethanedithiol from the high step[23] resulted in more complete deprotection, with <5% of the cyclohexyl-Asp peptide remaining. No aspartimide was detected.

βA4(14–17) and βA4(18–23) were deprotected using low–high TFMSA/TFA[21] to provide the expected products. The deprotected peptides were purified by reverse-phase HPLC and characterized by FABMS and tandem FAB–collision MS sequencing.[22]

βA4(1–25) was deprotected by low–high TFMSA/TFA[21] with ethanedithiol omitted from the high step. The deprotected peptide was purified by HPLC and characterized by FABMS and chemical sequencing (residues 1–25). A tryptic digestion of the peptide produced two expected fragments: DAEFR (M_r 637) and LVFFAEDVG (M_r 996). The expected fragment HDSGYEVHHQK was not observed, but the closely related fragment HDSGYEVHH (M_r 1335) was observed.

Discussion

Preparation of Short Protected Fragments by Stepwise Synthesis on the Kaiser Oxime Resin. βA4(10–13), βA4(14–17), βA4(18–23) were routinely synthesized in good yield with few side products. Initial difficulties in synthesizing βA4(1–9) were overcome by increasing reagent concentrations and adding an acetylation step between couplings. Protected fragments were purified by HPLC and characterized by amino acid analysis, ^{1}H NMR, FABMS, and tandem FAB–collision MS sequencing.[22]

Solid-Phase Fragment Couplings: Preparation of βA4(1–25). All four fragment couplings proceeded in good yield to produce resin-bound βA4(1–24) (see Scheme I). Amino acid analysis of the peptide resin indicated ca. 0.1 mmol of resin-bound βA4(1–24). Nevertheless, after cleavage (~95% yield based on amino acid analysis of the resin after cleavage) and purification, the final purified yield was 0.02 mmol, 11% based on βA4(1–9). Some losses may be attributable to mechanical or handling problems such as breakdown of the resin (and loss through the fritted reaction vessel). However, the majority of the losses occur during the purification.

Size-exclusion chromatography (LH-20, LH-60), which is often the only purification method used for large, hydrophobic peptides, provided only a partial purification of βA4(1–25). Significant overlap was observed between the target 25-residue peptide and a 16-residue impurity (βA4(10–25)). However, size-exclusion chromatography can be useful for removing low molecular weight fragments from a mixture. It is also useful as an analytical tool; analytical HPLC traces of the fractions from the LH-60 purification of βA4(1–25) were compared to an analytical HPLC trace of the crude to help identify the highest molecular weight component of the crude.

Protected peptides have poor recoveries from reverse-phase HPLC columns; the problem is exaggerated for long protected peptides. Recoveries are generally better in size-exclusion chromatography, in part because DMF is used as a solvent. However, size-exclusion chromatography does not provide the high resolution purification obtained with HPLC. Thus, the utility of this fragment coupling methodology could be greatly improved by the development of new stationary phases and solvent systems for the HPLC purification of protected peptides or alternative methods for their purification.

Deprotection of Peptides: Side Reactions Can Be Identified in the Deprotection of Small Fragments. Short and intermediate length protected fragments were deprotected in order to test deprotection conditions and to complete the characterization of each fragment. Side reactions which occur during deprotection can be identified prior to the deprotection of the full length peptide. Reaction conditions can be altered to minimize these reactions.

The formation of aspartimides during acidolytic deprotection of peptides has been previously reported.[23] We observed 100% aspartimide formation in the deprotection (using a published low-high TFMSA procedure[21]) of a peptide containing an -Asp(Bzl)-Ser- sequence. Lowering the temperature during the deprotection did not eliminate aspartimide formation but reduced the amount of aspartimide formed to 15% of the product. Substitution of a cyclohexyl group for the benzyl protecting group eliminated aspartimide formation. The cyclohexyl group, however, proved to be more stable to the deprotection conditions than the benzyl group. Further adjustments in the deprotection conditions resulted in complete deprotection without aspartimide formation.

The ability to test and optimize deprotection conditions for each of the intermediate fragments is an advantage of the fragment coupling approach. An aspartimide is relatively easy to detect and locate in a nine-residue peptide containing two aspartates. In addition, the abundant

(20) Protecting groups are abbreviated as follows: Boc, *tert*-butyloxycarbonyl; Bzl, benzyl ester (Asp, Glu) or ether (Ser); Cl_2Bzl, 2,6-dichlorobenzyl ester; Mts, mesitylenesulfonyl; Bom, benzyloxymethyl; cHex, cyclohexyl ester.

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supply of the fragments relative to the full length protein allows the testing of a variety of deprotection conditions.

Conclusions

The synthesis of the protected peptide βA4(1–25) demonstrates the utility of the Kaiser oxime resin for the preparation of intermediate length (ca. 20 residues) protected peptides which can serve as intermediates in the synthesis of long peptides and proteins. However, the low purified yield for this synthesis indicates that substantial room for improvement remains.

The synthesis, characterization, and deprotection of the short fragments indicates several problems which may occur in the stepwise synthesis of the βA4 protein.[5,6] Aspartimide formation (100%) occurred during the deprotection of an Asp(Bzl)-Ser sequence. Another potential site for aspartimide formation is Asp-1, which in our synthetic strategy is protected with the sterically hindered (aspartimide suppressing[23]) *tert*-butyl group.[12] Aqueous hydrolysis of aspartimides may occur during the workup which follows acidolytic deprotection,[13] resulting in the production of an isomer (the β-aspartyl peptide)[23] of the target peptide which is not easily separable from the target peptide in commonly used molecular weight-based purification schemes. The β-aspartyl peptide cannot be identified using amino acid analysis or FABMS, the two most commonly used characterization methods.

There is considerable evidence that single deletion impurities can accumulate even in the stepwise synthesis of short peptides.[12] In a long synthesis, even when high yields are obtained for every coupling, it is inevitable that a significant portion of the crude product consists of single deletion impurities.[12] In addition, the purification methods typically used for long synthetic peptides (size-exclusion chromatography) are not adequate for the separation of single deletion impurities from the target peptide. The observed overlap between βA4(1–25) and βA4(10–25) in a size-exclusion purification is evidence of this fact. Characterization of synthetic peptides by FABMS and amino acid analysis does not prove purity. In the fragment-coupling approach, protected fragments are purified by reverse-phase HPLC and are free of deletion impurities. The assembly of the target peptide results in impurities which lack an entire fragment rather than a single amino acid. Thus the final product is more easily purified. Characterization, too, is easier because is limited number of impurities are possible and the masses of these impurities can be predicted.

Procedures for the use of the oxime resin are still being modified and improved; our efforts in this area have resulted in the production of a pure protected fragment comprising residues 1–25 of the βA4 protein of Alzheimer's disease. The strategies outlined here should enable us to produce both the pure βA4 protein and the various sequence analogs and ^{13}C-labeled analogs required for the structural characterization of this unusual protein.

Experimental Section

Equipment, Materials, and Methods. DIEA was distilled from ninhydrin under reduced pressure. Polystyrene oxime resin,[24–26] *N*-hydroxypiperidine,[27] and amino acid tetra-*n*-butylammonium salts[14] were prepared according to published procedures. Hydrolyses of peptides were performed in HCl/propionic acid (1:1 v:v) at 130 °C for 3 h. Analytical reverse-phase HPLC was carried out using Waters Deltapak C_4 100-Å and 300-Å columns (3.9 mm × 30 cm). Semipreparative reverse-phase HPLC was carried out using Waters Deltapak C_4 300-Å columns (19 mm × 30 cm), and preparative reverse-phase HPLC was carried out using a Waters C_4 Prep-pak radial compression column (57 mm × 30 cm). Gel-permeation chromatography was performed with a Sephadex LH-60 column (1.5 × 73 cm) eluting with DMF. FTIR spectra were obtained using a published procedure.[8] Proton NMR was performed using dimethyl sulfoxide-d_6 as solvent. Only resolved and/or assignable multiplets are reported. All coupling constants are reported in hertz.

General Procedure for the Synthesis of Protected Peptides. Synthesis of Protected Peptides. Boc-D(tBu)AE(Bzl)FR(Mts)H(Bom)D(cHex)S(Bzl)G-OH (βA4(1–9)). Boc-D(tBu)AE(Bzl)FR(Mts)H(Bom)D(cHex)S(Bzl)-resin was synthesized according to a modified version of our standard procedure[12] (5 equiv of BOP, 5 equiv of aa, and 8.9 equiv of DIEA used for each coupling). An acetylation step (10 equiv of acetic anhydride, 5 equiv of DIEA) was performed after each coupling. The peptide was cleaved with the tetra-*n*-butylammonium salt of glycine.[14] The combined filtrate from the cleavage was evaporated to a solid residue and dissolved in DMF. The solution was purified by HPLC [prep: 37/63 H_2O/CH_3CN, (0.1% TFA), R_v = 1040 mL]. FABMS: 1753.1 (M + H)$^+$ (calcd 1752.8). Amino acid analysis: D 2.0 (2), A 1.0 (1), E 1.0 (1), F 1.2 (1), R 1.1 (1), H 1.2 (1), S 0.5 (1), G 2.1 (1). ^{1}H NMR: 1.34 ppm (s, 18 H, Boc and Asp(t-Bu)), 3.6 (d, 2 H, J = 5.3), 3.75 (d, 2 H, J = 5.5), 4.45 (s, 2 H, Ser(Bzl) or His(Bom)), 4.53 (s, 2 H, Ser(Bzl) or His(Bom)), 5.05 (s, 2 H, Glu(Bzl)), 5.64 (d, 1 H, J = 11, His(Bom)), 5.72 (d, 1 H, J = 11, His(Bom)), 6.89 (s, 1 H, His(Bom) 4 H), 7.8 (d, 1 H, J = 8), 7.9 (d, 1 H, J = 8), 8.0 (d, 1 H, J = 8), 8.55 (d, 1 H, J = 8), 9.2 (s, 1 H, His(Bom) 2H). Sequence was confirmed by tandem FAB-collision MS.[22] Yield: 45% based on Gly-resin.

Boc-Y(Cl_2Bzl)E(Bzl)VH(Bom)-OH (βA4(10–13)). Protected βA4(10–13)-resin was synthesized according to our standard procedure,[12] cleaved with HOPip, and reduced to the free acid.[15] The peptide was purified by HPLC [prep: 65 mL/min of 55/45 H_2O/CH_3CN, (0.1% TFA), 3 min isocratic followed by linear gradient to 5/95 (9 min)]. FABMS: 1015 (M + H)$^+$, 1017 (M + H, ^{37}Cl)$^+$ (calcd 1014.4). Amino acid analysis: Y 0.4 (1), E 1.2 (1), V 1.3 (1), H 1.3 (1). ^{1}H NMR: 0.84 ppm (dd, 6 H, J = 7, 7, Val CH_3), 1.27 (s, 9 H, Boc), 1.8 (m, 1 H, Val βH), 1.95 (m, 2 H, Glu βH), 2.4 (m, 2 H, Gly γH), 2.66 (dd, 1 H, J = 13, 10, Tyr(Cl_2Bzl) or His(Bom) βH), 2.88 (dd, 1 H, J = 13, 3.5, Tyr(Cl_2Bzl) or His(Bom) βH), 3.06 (dd, 1 H, J = 16, 9, Tyr(Cl_2Bzl) or His(Bom) βH), 3.26 (dd, 1 H, J = 16, 4.5, Tyr(Cl_2Bzl) or His(Bom) βH), 4.1 (m, 2 H), 4.3 (m, 1 H), 4.54 (s, 2 H, His(Bom)), 4.65 (m, 1 H), 5.07 (s, 2 H, Glu(Bzl) or Tyr(Cl_2Bzl)), 5.16 (s, 2 H, Glu(Bzl) or Tyr(Cl_2Bzl)), 5.67 (d, 1 H, J = 11, His(Bom)), 5.72 (d, 1 H, J = 11, His(Bom)), 6.95 (m, 3 H), 7.2 (d, 2 H, J = 8.5), 7.3–7.6 (m), 7.8 (d, 1 H, J = 8), 8.0 (d, 1 H, J = 8), 8.5 (d, 1 H, J = 8), 9.14 (s, 1 H, His(Bom) 2H). Sequence was confirmed by tandem FAB–collision MS.[22] Yield: 68% based on His-resin.

Boc-H(Bom)QK(Cl-Z)L-OH (βA4(14–17)). Protected βA4(14–17)-resin was synthesized according to the standard procedure, cleaved with HOPip, and reduced to the free acid.[16] The peptide was purified by HPLC [prep: 37/63 H_2O/CH_3CN, (0.1% TFA), R_v = 618 mL]. FABMS: 913.6 (M + H)$^+$ (calcd 912.4). Amino acid analysis: H 1.2 (1), Q 1.9 (1), K 0.9 (1), L 1.0 (1). ^{1}H NMR: 0.80 (d, 3 H, J = 6.5, Leu CH_3), 0.86 (d, 3 H, J = 6.5, Leu CH_3), 1.31 (s, 9 H, Boc), 1.5 (m, 2 H), 1.55 (m, 2 H), 1.6 (m, 2 H), 2.1 (m, 2 H), 2.95 (m, 3 H), 3.18 (dd, 1 H, J = 7.5, 4), 3.85, (m, 1 H), 4.2–4.4 (m, 6 H), 4.57 (s, 2 H, His(Bom)), 5.06 (s, 2 H, Lys(Cl-Z)), 5.72 (s, 2 H, His(Bom)), 6.76 (d, 1 H, J = 8.5, His(Bom) NH), 6.78 (s, 1 H, His(Bom) 4H), 7.15–7.5 (m), 7.72 (d, 1 H, J = 8), 8.05–8.2 (m), 9.19 (s, 1 H, His(Bom) 2H). Sequence was confirmed by tandem FAB–collision MS.[22] Yield: 63% based on Leu-resin.

Boc-VFFAE(Bzl)D(Bzl)-OH (βA4(18–23)). Protected βA4(18–23)-resin was synthesized using the standard procedure. The peptide was cleaved with HOPip, reduced to the free acid,[16] and purified by HPLC [semiprep: 38/62 H_2O/CH_3CN (5% trifluoroethanol), (0.1% TFA), R_v = 285 mL]. FABMS: 1007 (M + H)$^+$, 1029 (M + Na)$^+$ (calcd 1006.4). Amino acid analysis: V

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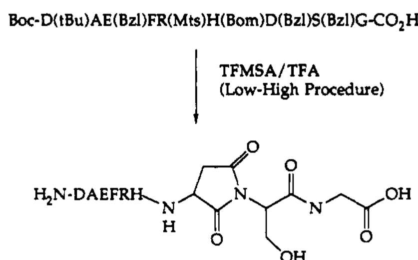

Figure 2.

0.9 (1), F 2.0 (2), A 1.2 (1), E 1.0 (1), D 0.9 (1). ^{1}H NMR: 0.60 (d, 3 H, J = 7, Val CH_3), 0.64 (d, 3 H, J = 7, Val CH_3), 1.20 (d, 3 H, J = 7, Ala βH), 1.35 (s, 9 H, Boc), 2.40 (t, 2 H, J = 8), 4.3 (m, 3 H), 4.55 (m, 3 H), 5.05 (s, 2 H, Glu(Bzl) or Asp(Bzl)), 5.09 (s, 2 H, Glu(Bzl) or Asp(Bzl)), 6.60 (d, 1 H, J = 9, Val NH), 7.1–7.4 (m), 7.78 (d, 1 H, J = 8), 8.01 (d, 1 H, J = 8), 8.14 (d, 1 H, J = 8), 8.17 (d, 1 H, J = 8), 8.31 (d, 1 H, J = 8). Sequence was confirmed by tandem FAB–collision MS.[22] Yield: 66% based on Asp-resin.

Synthesis of βA4(1–25). βA4(18–24)-resin. V-resin (3.08 g, 1.2 mmol) was acetylated (10 equiv of acetic anhydride, 5 equiv of DIEA, 6 h, rt), deprotected (25% TFA/CH_2Cl_2, 30 min, rt), neutralized (5% DIEA/CH_2Cl_2, 3 × 1 min), and coupled with Boc-VFFAE(Bzl)D(Bzl)-OH (400 mg, 0.4 mmol) using BOP (0.6 mmol), HOBt (0.4 mmol), and DIEA (0.8 mmol) in DMF (8 mL) for 14 h at 0–4 °C. Coupling yield as determined by amino acid analysis was 75–80% based on βA4(18–23).

βA4(14–24)-resin. (18–24)-resin (from above) was acetylated, deprotectd, neutralized, and coupled with Boc-H(Bom)QK(Cl-Z)L-OH (365 mg, 0.4 mmol) using BOP (0.6 mmol), HOBt (0.4 mmol), and DIEA (0.8 mmol) in DMF (8 mL) for 25 h at 0–4 °C. Coupling yield as determined by amino acid analysis was 80–90% based on (18–24)-resin.

βA4(10–24)-resin. (14–24)-resin (from above) was acetylated, deprotected, neutralized, and coupled with Boc-Y(Cl_2Bzl)E-(Bzl)VH(Bom)-OH (400 mg, 0.4 mmol) using BOP (0.6 mmol), HOBt (0.4 mmol), and DIEA (0.8 mmol) in DMF (8 mL) for 14 h at 0–4 °C. Coupling yield as determined by amino acid analysis was 80–90% based on (14–24)-resin.

βA4(1–24)-resin. (10–24)-resin (from above) was acetylated, deprotected, neutralized, and coupled with Boc-D(tBu)AE-(Bzl)FR(Mts)H(Bom)D(cHex)S(Bzl)G-OH (275 mg, 0.16 mmol) using BOP (0.6 mmol) and DIEA (2.1 mmol) in DMF (8 mL) for 14 h. Coupling yield as determined by amino acid analysis was 80–90% based on βA4(1–9). Quantitative analysis indicated 0.6 mmol of Arg/g of resin.

βA4(1–25). (1–24)-resin (from above) was cleaved with glycine tetra-*n*-butylammonium salt.[13] The combined filtrate from the cleavage was evaporated to a crude oil. A portion of the crude product was dissolved in DMF and applied to a Sephadex LH-60 column (1.5 × 73 cm). βA4(1–25) eluted at 48–54 mL. Analytical HPLC of the gel-purified fractions indicated the presence of a deletion impurity which was identified as Ac-(βA4 10–25)-OH by FABMS: 2796 (M + H)$^+$ (calcd 2795). The semipure material was subsequently purified by HPLC. The remaining crude product was dissolved in 1:1 DMSO:DMF (v/v) and purified by HPLC [semiprep: 25 mL/min 65/35 H_2O/CH_3CN, (0.1% TFA), 3 min isocratic followed by linear gradient to 5/95 (25 min)] FABMS: 4493.4 (M + H)$^+$ (calcd 4492.5) Amino acid analysis: D 2.7 (3), E/Q 4.3 (4), S 1.0 (1), G 4.2 (2), H 2.1 (3), R 0.9 (1), A 2.0 (2), Y 1.0 (1), V 2.9 (3), L 1.3 (1), F 2.3 (3), K 1.1 (1). Yield: 81 mg, 11% based on βA4(1–9) (limiting fragment).

General Procedure for the Deprotection of Peptides.[28] **"Low" Stage.** The peptide (10–20 μmol) was cooled to 0 °C; *m*-cresol (100 μL) and dimethyl sulfide (300 μL) were added. TFA (500 μL, degassed and cooled to 0 °C before use) and TFMSA (100 μL, stored at −20 °C before use) were added. The reaction mixture was stirred under Ar at 0 °C and was allowed to come to room temperature over a period of 3 h. The mixture was evaporated under vacuum to an oil and the peptide precipitated with cold diethyl ether (5–10 mL). The peptide was isolated by centrifugation, washed with ether, and dried under vacuum. **"High" Stage.** Thioanisole (100 μL) and ethanedithiol (50 μL) were added to the dried peptide; the mixture was stirred at 0 °C for 10 min. TFA (1.0 mL, degassed and cooled to 0 °C before use) was added and the mixture was stirred for 10 min. TFMSA (100 μL, stored at −20 °C before use) was added, and the mixture was stirred under Ar at 0 °C for 1.5 h. TFA was evaporated under vacuum and the peptide precipitated by addition of cold diethyl ether (10 mL).

Deprotection of Boc-D(tBu)AE(Bzl)FR(Mts)H(Bom)D-(Bzl)S(Bzl)G-OH. The peptide was deprotected according to the standard procedure. FABMS of crude product: 1015.4 (calcd 1032.4, M + H − 18 indicates aspartimide). Tandem FAB–collision MS sequencing[22] indicated aspartimide at Asp-7.

Deprotection of Boc-R(Mts)H(Bom)D(Bzl)S(Bzl)G-OH. Protected Boc-R(Mts)H(Bom)D(Bzl)S(Bzl)-resin was synthesized using the standard procedure, cleaved with glycine tetra-*n*-butylammonium salt,[14] and purified by HPLC [semiprep 60/40 H_2O/CH_3CN (5% trifluoroethanol), (0.1% TFA), R_v = 270 mL]. The peptide was deprotected according to a modified standard procedure in which the temperature was 0 °C for the duration of the low step. FABMS of crude product: 571 (M + H)$^+$, 553 (M + H − 18, aspartimide) (calcd 570.2). The aspartimide (553) peak was 18% of (M + H)$^+$ (571) peak.

Deprotection of Boc-R(Mts)H(Bom)D(cHex)S(Bzl)-OH (βA4(5–8)). Protected βA4(5–8)-resin was synthesized using the standard procedure, cleaved with HOPip, reduced to the free acid,[16] and purified by HPLC [semiprep 60/40 H_2O/CH_3CN (5% trifluoroethanol), (0.1% TFA), R_v = 240 mL]. The peptide was deprotected according to a modified standard procedure in which the temperature was 0 °C for the duration of the low step. FABMS: 596.3 (M + H + cyclohexyl group)$^+$ (calcd 513). The fully deprotected (514) peak was 7% of cyclohexyl (596.3) peak. The peptide was deprotected a second time according to a modified standard procedure in which the temperature was 0 °C for the duration of the low step and ethanedithiol was eliminated from the high step. FABMS of crude product: 514 (M + H)$^+$ (calcd 513). The cyclohexyl − Asp (596) peak was <5% of fully deprotected (514) peak.

Deprotection of Boc-H(Bom)QK(Cl-Z)L-OH (βA4(14–17)). The peptide was deprotected using a modified standard procedure in which the low step was omitted. FABMS of crude product: 525 (M + H)$^+$ (calcd 524.3). Tandem FAB–collision MS sequencing[22] confirmed the correct sequence.

Deprotection of Boc-VFFAE(Bzl)D(Bzl)-OH (βA4(18–23)). The peptide was deprotected using a modified standard procedure in which the low step was omitted. The deprotected peptide was purified by reverse-phase HPLC [semiprep: 15 mL/min 80/20 H_2O/CH_3CN (5% trifluoroethanol), (0.1% TFA), 3 min isocratic followed by linear gradient to 40/60 (15 min)]. FABMS: 727 (M + H)$^+$ (calcd 726.3) Tandem FAB–collision MS sequencing[22] confirmed the correct sequence.

Deprotection of βA4(1–25). The peptide was deprotected using a modified standard procedure in which the temperature was 0 °C for the duration of the low step and ethanedithiol was eliminated from the high step. The peptide was purified by HPLC [Vydac C_4 300-Å column (4.6 mm × 25 cm), 2.0 mL/min 70/30 H_2O/CH_3CN (5% trifluoroethanol), (0.1% TFA), 4 min isocratic followed by linear gradient to 3/97 (21 min)]. FABMS: 2932.3 (M + H)$^+$ (calcd 2931.4). Chemical sequencing (25 cycles) confirmed the correct sequence.

Tryptic Digestion of Deprotected βA4(1–25). Deprotected βA4(1–25) from above (100 nmol) was dissolved in 0.1 mM NH_4HCO_3 (500 μL, pH 8.5). Trypsin (5 μg in 50 μL of 10 mM HCl) was added and the solution was incubated in a 37 °C constant temperature bath. A second aliquot of trypsin (5 μg) was added after 2 h. After 4 h, the sample was frozen and lyophilized. The sample was then dissolved in water and lyophilized three times to remove NH_4CO_3. The mixture of tryptic fragments was analyzed by FABMS: 637 (DAEFR), 996 (LVFFAEDVG), 1080 (HDSGYEVHH).

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Syntheses, Structures, and Enzymatic Evaluations of Hemiacylcarnitiniums, a New Class of Carnitine Acyltransferase Inhibitors

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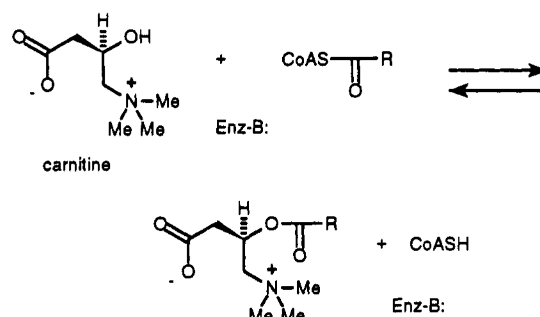
The syntheses of (2*S*,6*R*)-6-(carboxymethyl)-2-hydroxy-2,4,4-trimethylmorpholinium chloride (hemiacetylcarnitinium, HAC), (2*S*,6*R*)-6-(carboxymethyl)-2-ethyl-2-hydroxy-4,4-dimethylmorpholinium bromide (hemipropanoylcarnitinium, HPrC), and (2*S*,6*R*)-6-(carboxymethyl)-2-hydroxy-4,4-dimethyl-2-phenylmorpholinium chloride monohydrate (hemibenzoylcarnitinium, HBC) are described. The crystal structure of HAC is reported and compared with crystal structures of HPrC, HBC, carnitine·HCl, acetylcarnitine·HCl, and acetylcarnitine·HCl·H_2O. HAC, HPrC, and HBC inhibit carnitine acetyltransferase (CAT) activity from multiple biological sources. The best inhibitor, HAC, has K_i of 69 ± 5 μM with rat liver peroxisomal CAT. HAC binds more strongly than the natural substrate (and isomer), acetylcarnitine. HAC also strongly inhibits, K_i = 92 ± 14 μM, CAT from rat heart mitochondria. HPrC inhibits pigeon breast CAT with a K_i of 200 ± 30 μM. HBC inhibits pigeon breast CAT, rat heart mitochondrial CAT, rat liver mitochondrial CAT, and rat liver peroxisomal carnitine octanoyltransferase (COT), with values of K_i of 3500 ± 500, 2400 ± 70, 1670 ± 70, and 1100 ± 100 μM, respectively. Racemic 6-(carboxymethyl)-2-hydroxy-2-pentadecyl-4,4-dimethylmorpholinium bromide (hemipalmitoylcarnitinium, HPC) strongly inhibits (K_i = 2 ± 0.3 μM) beef liver mitochondrial CPT_i. In summary, hemiacylcarnitiniums show promise as a general class of carnitine acyltransferase inhibitors.

The carnitine acyltransferases comprise a family of proteins that differ with respect to subcellular localization, substrate specificity, and sensitivity to inhibitors.[1] The molecular basis for this protein diversity remains unexplained, but the recent mappings of multiple distinct gene sequences[2-4] confirm the cellular synthesis of distinct polypeptides. Insufficient data are available to address the question of active site homology among these enzymes. The evaluation of putative active-site-directed inhibitors against a spectrum of carnitine acyltransferases reveals similarities and differences among these enzymes, and it provides information that is needed to design specific inhibitors for each protein.

Carnitine acetyltransferase (CAT), carnitine octanoyltransferase (COT), and carnitine palmitoyltransferase (CPT) catalyze acyl transfer between carnitine and coenzyme A (CoASH). CAT, CPT, and carnitine–acylcarnitine translocase regulate the transport of activated acyl groups across the inner mitochondrial membrane, while peroxisomes and microsomes contain COT activity. The three-dimensional structures of these proteins are unknown.

Conformationally rigid inhibitors can probe the structure of enzymic binding sites. This idea originates in medicinal



chemistry;[5,6] e.g., hemicholiniums[7] 1 are analogues of choline. Similarly, we make carnitine analogues, which we term hemiacylcarnitiniums,[8,9] 2. Racemic hemiacetyl-

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