Reed College, are thanked for their support of this work. Professor Frank Hauser, Drs. Bill Bhagdanov and Sue Ellenberger, and the NMR spectrometry facility at the Oregon Graduate Center are thanked for their assistance.

Registry No. 7, 1193-18-6; 8 (R = CH_3OCH_2), 108694-51-5; 9, 101514-23-2; 10, 108694-52-6; 12, 108694-53-7; 13, 108694-54-8; 15, 108694-55-9; 16, 108694-56-0; 17, 108710-75-4; 18, 108694-57-1; 19α, 108694-59-3; 19β, 108694-58-2; 20, 108694-60-6; 22, 72726-55-7; 23, 108694-61-7; 24 (isomer 1), 108741-17-9; 24 (isomer 2), 108694-70-8; 26, 108694-62-8; 28, 16544-46-0; 29 (isomer 1), 108694-63-9; 29 (isomer 2), 108741-18-0; 30 (isomer 1), 108694-64-0; 30 (isomer 2), 108694-69-5; 31 (isomer 1), 108694-65-1; 31 (isomer 2), 108741-16-8; *trans*-32, 108694-66-2; *cis*-32, 108694-71-9; 33, 108694-67-3; 34 (isomer 1), 108694-68-4; 34 (isomer 2), 108741-15-7; Me_3SiCH_2MgCl, 13170-43-9.

Chemical Synthesis of Rat Atrial Natriuretic Factor by Fragment Assembly on a Solid Support

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Received March 18, 1987

The chemical synthesis of a 26-residue atrial natriuretic peptide is reported where the assembly was carried out by using a solid-phase fragment based approach. This methodology allowed the production of the quantities necessary for safety assessment and clinical studies. A detailed description of the strategy, synthesis, purification, and associated problems is presented.

Introduction

Recently, a number of related peptides have been isolated from mammalian heart tissue which possess extremely potent vasorelaxant and diuretic properties.[1] These peptides are released from granules in the atrium in response to various stimuli, and they are thought to be a key factor in the control of water–electrolyte balance. Although the individual peptides have been given various names, they are generically known as atrial natriuretic factors(s) (ANF) or atrial natriuretic peptides(s). Despite the problems associated with isolation from tissue containing very small amounts of active material, several groups of investigators were able to characterize several peptides of varying length from rat tissue which all displayed complete sequence homology with a central "core" region, but with varying numbers of residues at the N- and C-termini.[1b-d] Almost all of the isolated peptides are active as natriuretic agents but display varying potencies. Most of these compounds also cause the relaxation of intestinal and vascular smooth muscle. We became interested in synthesizing larger amounts of one of the most potent of these peptides in order to fully determine its biological, chemical, and physical properties, as well as its possible use in a clinical setting. The 26-residue peptide 1 (rat ANF (8–33)) was chosen as our target, because it contained the minimum sequence necessary for full potency in the various tissues mentioned above.

Synthesis

Upon examination of 1 (numbering of the residues is based upon the longest ANF (33 amino acids) isolated at the Clinical Research Institute in 1983),[1b] approaches to its synthesis as well as problems that might be encountered are evident. The well-known synthetic problems[2] associated with arginine (Arg), aspartic acid (Asp), and tyrosine (Tyr) had to be considered for all possible syntheses and will be discussed in detail. The presence of several glycine (Gly) residues spaced throughout the sequence made a fragment condensation strategy worth consideration, since carboxyl activation of C-terminal Gly fragments could not lead to the problems of racemization frequently encountered in fragment couplings.[3] Fragment based syntheses rely on the assembly of several smaller peptides of high purity to provide the target peptide free of impurities that can arise in longer sequences made by the stepwise solid-phase method and that can be very difficult to remove.

Our first successful synthesis made use of minimally protected fragments that were coupled in solution by using the azide method.[4] However, in order to meet the ever increasing demand for material needed for safety evaluation and clinical testing, we required a more efficient route that was capable of yielding gram amounts of the peptide 1 in high purity. We then decided to evaluate an approach that involved the coupling of fully protected fragments onto a solid support to provide the full ANF sequence in the protected resin bound form 2 shown in Scheme I. In theory, this strategy could afford multigram amounts of resin bound peptide, which could then be processed to yield high purity material without extensive purification.

The route illustrated in Scheme I requires substantial amounts of the fragments 3, 4, and 5 in high purity, as well as the C-terminal resin-bound octapeptide 6. It seemed likely that the fragments could be synthesized by the

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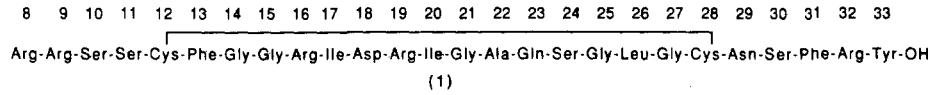
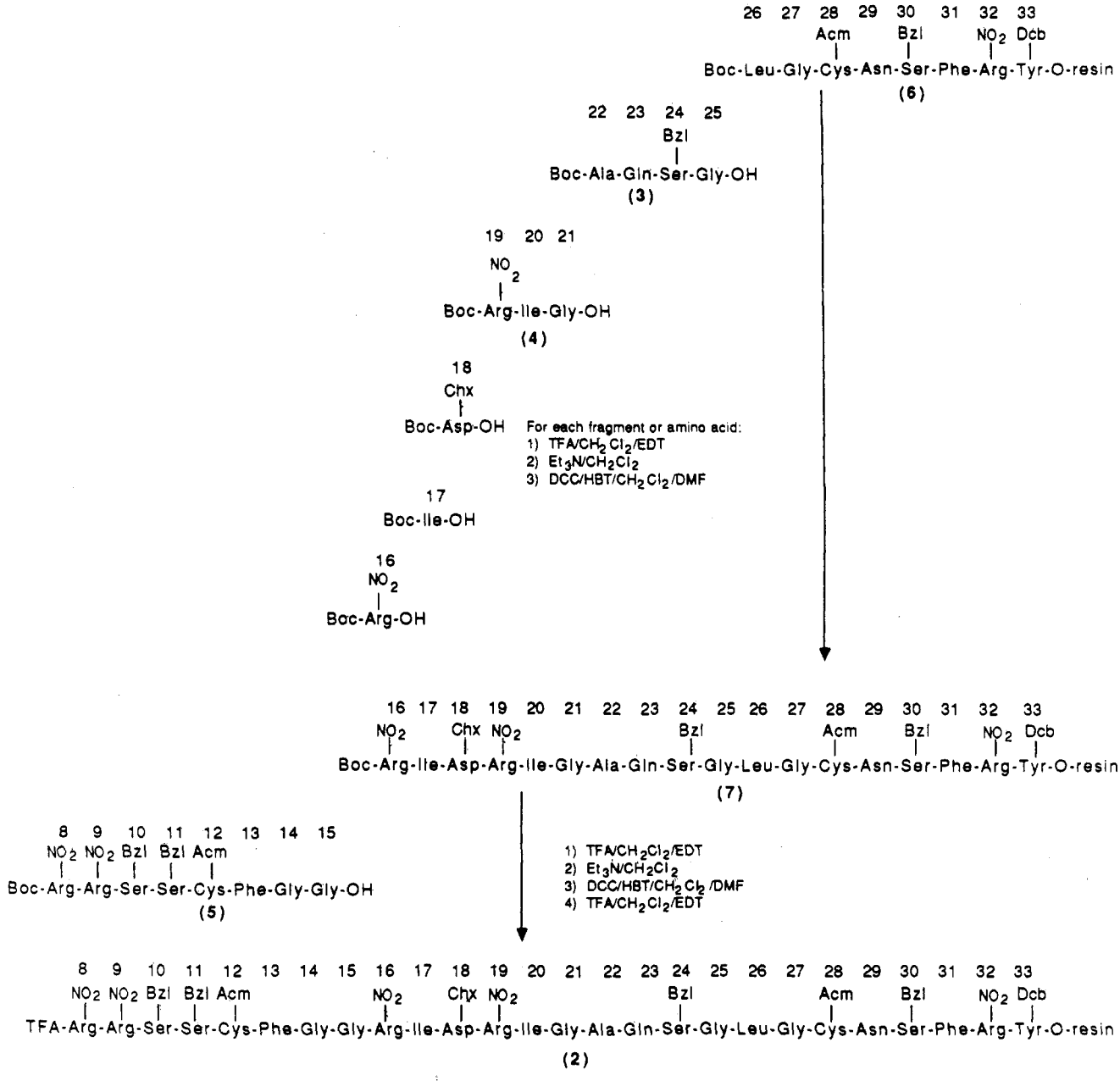


(1)

Scheme I. Assembly of ANF Resin Peptide 2



(2)

stepwise solid-phase method followed by their removal from the resin by transesterification. Purification of the protected esters followed by saponification to give the corresponding acids would then afford the required fragments 3–5. Aspartic acid was not considered for inclusion in a fragment because the side-chain carboxyl is normally protected as an ester that would likely not be stable during the transesterification or saponification steps.[2] Therefore, residues 16, 17, and 18 were slated for stepwise incorporation as their side-chain protected N-α-Boc derivatives, which would allow the assembly of the final eight residues as the C-terminal Gly fragment 5. The overall purity of the final product should then be largely determined by the purity of the individual fragments (which can be checked directly by TLC and HPLC) and of the purity of the C-terminal octapeptide resin 6 (which cannot be directly determined).

The side-chain protecting groups for all residues were chosen to insure their stability during the synthesis of the peptide resin 2. However, it was also desirable that the final HF treatment would cleave the peptide from the resin to afford the linear peptide 8 (Scheme II) in which only the cysteine protection remained, since from our previous synthesis of ANF by solution methods[4] it had been determined that the disulfide cyclization utilizing acetamidomethyl (Acm) protection on Cys proceeded in excellent yield. In order to minimize the formation of β-aspartyl-containing impurities during resin assembly and HF treatment, the cyclohexyl ester (Chx) protection was chosen for Asp.[5] For the five Arg residues, the nitro protection for the guanido group was found to be suffi-

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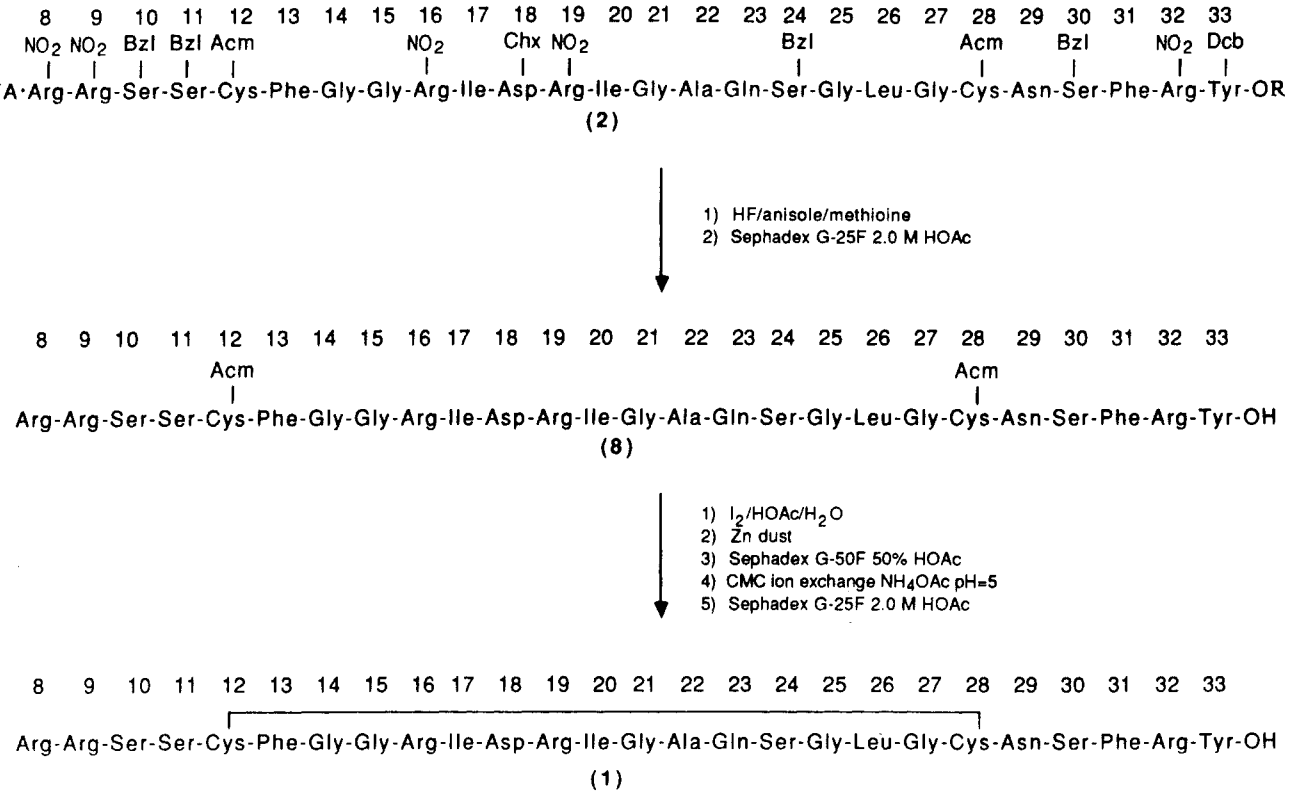
Scheme II. Synthesis of ANF (1) from Resin Bound Peptide 2



ciently stable during transesterification to permit its use for the present case. For protection of the Tyr phenol, the 2,6-dichlorobenzyl derivative was chosen because of its reported ability to suppress the formation of C-benzyl Tyr-type impurities during HF cleavages.[6]

All of the fragments were assembled on polystyrene (cross-linked with 1% or 2% *p*-divinylbenzene) resin supports. The chloromethyl-functionalized resins were treated with the cesium[7] or triethylammonium salts of the C-terminal Boc amino acids in order to attach the first amino acid of each fragment to the resin at levels of 1.1–1.3 mmol/g. Incorporation of the subsequent amino acids was carried out by using their N-α-*t*-Boc derivatives (with the aforementioned side-chain protection) by a standard Merrifield solid-phase procedure.[8] Coupling progress was monitored by using the Kaiser procedure for detection of free amino groups on peptide resins.[9]

Transesterification of the completed resin-bound fragments was performed in methanolic solutions of triethylamine. In the case of fragments **3** and **4**, the resulting methyl esters were purified by crystallization before saponification to the desired acids. Fragment **5** was not amenable to purification by crystallization, so the crude methyl ester was saponified and the acid was purified by silica gel chromatography. HPLC analyses of the fragments **3**, **4**, and **5** indicated their purity at greater than 97%. As it was not possible to check the purity of the peptide resin **6** in this manner, it was synthesized with careful monitoring of the stepwise coupling reactions to insure complete reaction. However, the purity of the resin-bound intermediate **9** was indirectly determined in connection with experiments to determine optimal HF conditions that will be discussed later.

Assembly of the fragments **3** and **4** onto the peptide resin **6** was carried out in a manner similar to that used in our normal single amino acid protocols. In the presence of neutralized peptide–resin, an excess of fragment was activated by in situ formation of its 1-hydroxybenzotriazole (HBT) ester from the fragment plus HBT with an excess of dicyclohexylcarbodiimide (DCC). The extent of coupling was determined by qualitative Kaiser analysis and the apparent pH of the reaction mixture (as determined by spotting a few microliters of the liquid phase onto moistened indicator paper) was adjusted to 6.5–7.0 by using *N*-methylmorpholine (NMM) as necessary. Removal of the N-terminal Boc group was carried out exactly as for the stepwise procedure.

This coupling protocol proved to be unsatisfactory for the attachment of the final fragment **5** onto the free N-terminal amino derivative of the hexadecapeptide resin **7**. Neither repeat couplings nor excess reagents brought about complete coupling as determined by the Kaiser test or amino acid analysis of the peptide resin product. A number of different coupling reagents and protocols were examined, including Le-Bop reagent,[10a] carbonyldiimidazole,[10b] addition of (dimethylamino)pyridine (DMAP),[10c] *p*-nitrophenol esters generated in situ by using DCC, and the use of *N*-hydroxysuccinimide (HSU) instead of HBT.[10d] Only the latter method gave acceptable coupling yields according to our analysis.

These results prompted us to investigate both the activation and acylation steps in greater detail. Coupling solutions containing activated peptide fragment were an-

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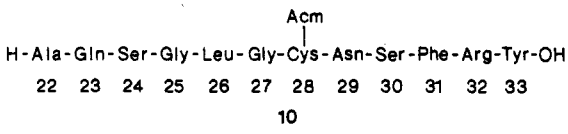
Table I. Analysis of HPLC Isolated Impurities

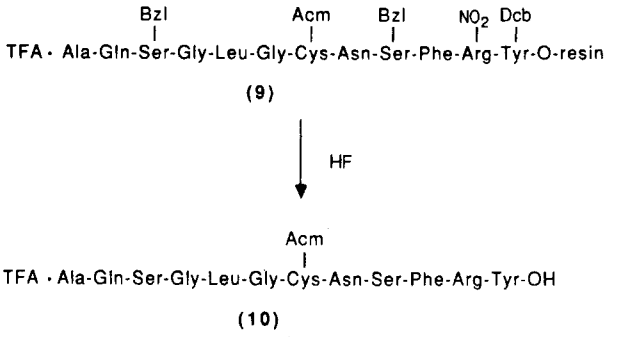


design	structure	type	key char. data	probable source	extent
A	(Asn-Asn)[29]-(22–33)	insertion	AA Anal. sequence	prolonged coupling	≤4%
B	Cys[28]-(22–33) free SH	loss of Acm	FAB-MS	partial Acm removal during HF	≤4%
C	CF_3CO-(27–33)	truncation	AA Anal. FAB-MS	incomplete washout of TFA during SPS	≤4%
D	Cys(Bzl)[28]-(22–33)	alkylation of B	FAB-MS AA Anal.	benzylation of B on prolonged HF treatment	≤7%
E	3-(Cl_2Bzl)Tyr[33]-(22–33)	rearrangement	AA Anal. FAB-MS	expected O to C migration of Cl_2Bzl	≤30%

alyzed by TLC to characterize and monitor the presence of reactive species available for reaction with the peptide–resin nucleophile. TLC systems consisting of the solvent mixtures chloroform–methanol–water and chloroform–methanol–ammonium hydroxide were used for the analysis. The expected activated species as well as the primary amide product resulting from its reaction with the ammonia solvent system were tentatively identified as spots having R_f values distinct from that of fragment **5**. From these experiments it was concluded that activation of the carboxylic acid occurred by using either HBT or HSU; however, in the ammonia system it was apparent that the active HBT species reacted much more readily with water than with ammonia. For the HSU-active ester, reaction with ammonia was faster than its reaction with water. This led us to conclude that the desired acylation of the peptide resin vs. inactivation by adventitious water was more favorable for the HSU-active species than for HBT-active esters. This reaction selectivity in favor of water over amine has been observed previously for acylimidazoles.[11] Advantages of *N*-hydroxysuccinimide esters over hydroxybenzotriazole esters have been observed during acylations of poor or sterically hindered nucleophiles such as thiaproline and pipecolic acid.[12]

Subsequent steps in the synthesis (Scheme II) involved treatment of the protected peptide resin with anhydrous HF to remove the peptide from the support as well as to remove all protecting groups except for those on cysteine, followed by disulfide cyclization to afford the ANF (8-33) (**1**). Due to the large amount of resin to be processed, use of the preferred S_N2 conditions[2b] for HF reactions was impractical. The HF reaction conditions were studied in some detail by using the C-terminal dodecapeptide resin intermediate **9** as a model to determine optimal conditions for yield and purity (Scheme III). A number of HF reactions were carried out by using various scavengers and reaction conditions, and the major impurities were isolated by using HPLC and silica gel chromatography. Table I shows the yield (HPLC) and identity (from AA analysis & FAB mass spectra) of the significant impurities. The major impurity was determined to be the well-known 3-(2,6-dichlorobenzyl)tyrosine[2a] containing impurity E arising

Scheme III



from HF-catalyzed migration of the Tyr protecting group. The unexpectedly high yield of this compound may be due in part to its close proximity to the resin. The only impurity that could be attributed to the peptide resin **6** was the Asn insertion compound A. Because our source of Boc-Asn was shown to be free of any dipeptide contaminant, we feel that this insertion reaction is a result of excess HBT causing the slow removal of the Boc group during extended coupling periods (16–24 h), which then allows the second Boc-Asn to be incorporated.[17] When shorter coupling times were used, none of the insertion product could be observed. The two impurities B and D seem to arise from the slight instability of the Acm group during HF treatment. The purified C-terminal dodecapeptide **10** (in which the only protection was the Acm group on Cys) was treated with 9:1 HF–anisole for 1 h at 0 °C in order to determine the inherent stability of the Acm group toward HF. The total loss of Acm was 2.1% as determined by Ellman analysis[13] of the crude product. Examination of the HPLC profiles of several small-scale HF reaction mixtures led to the use of *m*-cresol and methionine[18] as optimal scavengers.

The best conditions found for the HF treatment of the C-terminal dodecapeptide **9** were applied to the resin-bound ANF (8-33) (**2**) followed by partial purification on Sephadex G-25F using 2.0 M acetic acid. The column fractions were analyzed by using TLC and HPLC, and fractions containing significant amounts of the major product were combined, concentrated, and lyophilized. In a typical experiment, 2.0 g (0.28 mmol) of peptide resin **2** was treated with 36 mL of anhydrous HF (after swelling for 1 h in a mixture of 4.0 mL of *m*-cresol and 1.0 g of methionine) and worked up by precipitation with ether and

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leaching with aqueous acetic acid. The crude peptide solution was placed directly on Sephadex to afford 400–500 mg (43–54%) of the linear 26-amino acid peptide 8, which had an average purity of 85% by HPLC. Ellman analysis of this material indicated that partial removal of the Acm protecting groups had occurred, which could account for one or more of the impurities observed in the HPLC profile. A major byproduct that was tentatively identified as the 3-(2,6-dichlorobenzyl)-Tyr[33] impurity (by analogy to E in Table I) was largely removed in earlier eluting fractions and was estimated to represent about 15% of the crude peptidal material.

Cyclization of linear peptide 8 with iodine in aqueous acetic acid followed by partial purification on Sephadex G-50F was carried out according to the protocol developed for the corresponding N-terminal Boc-protected compound from the solution synthesis.[4] A typical procedure afforded approximately 900 mg (84%) of ANF 1, which analyzed to an average purity of 91% by HPLC.

Final purification was carried out by cation exchange chromatography on carboxymethyl cellulose (CMC) using aqueous ammonium acetate at pH 5 as the eluant. The separation could be carried out by using a stepped gradient elution or by a carefully controlled isocratic elution. Fractions were analyzed by HPLC and those of sufficient purity were combined, placed directly on Sephadex G-25F, and eluted with 2.0 M acetic acid to remove the ammonium acetate. This ion exchange method was particularly attractive since it was capable of affording gram amounts of ANF 1 from a single CMC chromatography in purities over 97% as determined by HPLC. Attempted purification by preparative HPLC was hampered by the difficulty of separating close running impurities which were only resolved by using carefully controlled analytical HPLC conditions. The successful analysis of ANF by reversed phase HPLC was critically dependent upon the choice of column and use of extremely pure solvents.

One of the impurities that was present in amounts up to 2.0% after cyclization was partially resolved on CMC and was determined to be an Asn-29 insertion impurity (see Table I) by sequencing and amino acid analysis. HPLC spiking experiments showed the absence of both mono- and diiodo Tyr impurities as well as the absence of any of the linear precursor 8. Ellmann analysis indicated that the amount of free sulfhydryl was less than 1.0 mol %. The absence of ammonium salt was determined by Nessler's reagent[14] and heavy metals were analyzed by using spectrographic analysis.

Characterization of the product 1 was carried out by using the following analytical techniques: amino acid analysis, sequencing, optical rotation, behavior on HPLC and TLC, elemental analysis, thermogravimetric analysis, gas-phase chromatography for acetic acid content, and Karl Fisher titration for the amount of water.

The full biological activity of this material has been demonstrated in detailed animal studies and has been reported elsewhere.[15] Also, initial results from clinical trials involving human subjects have confirmed its activity in man.[16]

Summary

With use of the methodology described above, approximately 100 g of peptide resin 2 was processed from ca. 50 separate HF reactions and accompanying gel filtration columns. Up to 10 g of ANF 1 has been obtained from this material which was used for safety assessment and clinical studies. The availability of large amounts of this material has been of critical importance to our current level of understanding of the biological effects of ANF and its implications for human and animal health.

Experimental Section

General. All amino acids and derivatives were obtained from Bachem unless otherwise indicated. Commercially available solvents were used without further purification, with the exception of *N,N*-dimethylformamide from which approximately 5% was removed by distillation at reduced pressure to remove volatile impurities. All solutions were made up on a volume to volume basis unless otherwise indicated. HPLC was carried out at 40 °C by using a Vydac C-18, 5 μm, 300 Å, 15 × 0.4 cm column (column A), a Vydac C-4, 10 μm, 80 Å, 25 × 0.4 cm column (column B), or a Waters μ-Bondapack 30 × 0.4 cm column (column C) with UV detection at 210 nm using the following linear gradients. System I, solvent A is 1000:1 H_2O–trifluoroacetic acid (TFA) and solvent B is 1000:1 acetonitrile–TFA (90:10 A/B to 25:75 A/B over 50 min at 1.0 mL/min); system II, 95:5 A/B to 70:30 A/B over 1 h at 1.0 mL/min; system III, 95:5 A/B to 30:70 A/B over 30 min at 2.0 mL/min; system IV, solvent C is 1000:1 H_2O–85% phosphoric acid adjusted to pH 3.2 with aqueous trimethylamine and solvent D is acetonitrile (95:5 C/D to 5:95 C/D over 30 min at 2.0 mL/min). TLC was performed on Analtech silica gel GF 250-μm glass plates using *tert*-butyl hypochlorite followed by starch–KI for visualization. Solvent systems for TLC were system V, 95:5:0.5 $CHCl_3$–CH_3OH–H_2O; system VI, 70:30:3 $CHCl_3$–CH_3OH–H_2O; system VII, 80:20:2 $CHCl_3$–CH_3OH–H_2O, system VIII, 85:15:1.5 $CHCl_3$–CH_3OH–H_2O; system IX, 15:5:1:2 ethyl acetate–pyridine–HOAc–H_2O; system X 45:20:6:24 butanol–pyridine–HOAc–H_2O. Proton and carbon NMR experiments were performed on a Nicolet 360-MHz or a Varian 400-MHz instrument in Me_2SO-d_6 or D_2O with tetramethylsilane or sodium 3-(trimethylsilyl)propionate-d_4, respectively, as internal standards. Melting points are uncorrected. Fast atom bombardment mass spectroscopy was carried out on a modified Finnigan instrument using Xe gas.

Boc-Tyr(Cl_2Bzl)-*O*-resin (11). A solution of 100.0 g (227 mmol) of N-*t*-Boc-*O*-(2,6-dichlorobenzyl)tyrosine in 250 mL of absolute EtOH was treated with a solution of 36.2 g (111 mmol) of Cs_2CO_3 (Alfa) in 640 mL of absolute EtOH. The solvent was removed at reduced pressure to give a solid mass, which was broken up and dried for 18 h at 0.1 mm. This material was dissolved in 400 mL of freshly degassed DMF, evaporated to dryness at reduced pressure, and redissolved in 500 mL of degassed DMF. This solution was added to a mixture of 125 g (162.5 mequiv) of preswollen chloromethylpolystyrene resin (1% cross-linked, 1.3 mequiv/g, Lab Systems Inc.) in 150 mL of degassed DMF. The resulting mixture was slowly rotated on a rotary evaporator at 50 °C for 18 h, 100 mL of degassed DMF added, and rotation continued for 6 h. The mixture was filtered and the resin was washed with three 100-mL and one 200-mL portions of degassed DMF. Approximately 6% of the wet resin was removed, and the remainder was washed according to the following protocol: 2 × 375 mL 1:2 (v/v) DMF/H_2O, 4 × 250 mL H_2O, 2 × 250 mL 1:1 DMF/H_2O, 4 × 250 mL CH_3OH. The washed resin was suspended in 1.2 L of CH_2Cl_2 in a separatory funnel, the lower part drained to remove fine particles, and the procedure repeated twice with the addition of 500 mL of CH_2Cl_2. The resin was filtered and washed with 500- and 200-mL portions of CH_3OH, 1.0 L of CH_2Cl_2, and four 250-mL portions of CH_3OH. The resin was dried to constant weight at 0.1 mm for 82 h. The dried resin was suspended in 500 mL of degassed DMF and treated with a suspension of 23 g (120 mmol) of cesium acetate (Aldrich) in 500 mL of degassed DMF at 60 °C, and the mixture was rotated on a rotary evaporator at 50 °C for 31 h. The resin was filtered and washed according to the following scheme: 1 × 250 mL DMF, 2 × 500 mL 1:2 (v/v) DMF/H_2O, 4 × 300 mL H_2O, 1 × 500 mL 1:1 (v/v) CH_3OH/H_2O, 3 × 250 mL CH_3OH, and 1 × 250 mL CH_2Cl_2. After removing the fine particles as described above, the resin was washed as follows: 2 × 250 mL CH_3OH, 1 × 1.0 L CH_2Cl_2, and 6 × 250 mL CH_3OH. Drying the resin at 0.1 mm for 64 h yielded 164 g of 11: aaa (aaa = amino acid analysis) Tyr 0.479 mmol/g; ca (ca = combustion analysis) nitrogen = 1.49 wt %, chlorine = 6.08 wt %.

Boc-Leu-Gly-Cys(Acm)-Asn-Ser(Bzl)-Phe-Arg(NO_2)-Tyr(Cl_2Bzl)-*O*-resin (6). The solid-phase synthesis was carried

out starting from 50.0 g (38 mmol) of 11 by using the following protocol. The Boc amino acids used were Boc-Arg(NO_2)-OH (Chemalog), Boc-Phe-OH, Boc-Ser(Bzl)-OH, Boc-Asn-OH, Boc-Cys(Acm)-OH, Boc-Gly-OH, and Boc-Leu-OH. All volumes used were 500 mL unless otherwise indicated. Boc removal was accomplished by using 1:2 (v/v) TFA/CH_2Cl_2 (with the addition of 1:19 (v/v) ethanedithiol after the incorporation of cysteine) in three treatments of 1, 2, and 25 min, followed by three 2-min washes with CH_2Cl_2. Neutralization was carried out with 1:9 (v/v) triethylamine/$CHCl_3$ using treatments of 2 and 4 min, followed by five 2-min washes with CH_2Cl_2. Coupling reactions were carried out in the order listed above by treating the neutralized resin with a solution of 100 mmol of the Boc amino acid and 15 g (100 mmol) of 1-hydroxybenzotriazole (HBT) in 180 mL of 1:2 (v/v) DMF/CH_2Cl_2, followed by the addition of 100 mL of a 1.0 M solution of dicyclohexylcarbodiimide (DCC) in CH_2Cl_2. In the case of Boc-Asn, 30 g (200 mmol) of HBT was used. The mixture was mixed by gentle rocking for approximately 2 h. After washing the resin for 2 min with DMF, followed by two 2-min treatments with 1:4 (v/v) CH_3OH/CH_2Cl_2 and three 2-min washes with CH_2Cl_2, the coupling yield was estimated by using the Kaiser test on a small sample of resin. Incomplete couplings were repeated by using the same procedure, beginning with the neutralization step. The completed peptide–resin was dried to constant weight under reduced pressure to afford 85.3 g (86%) of **6**: aaa avg = 0.384 mmol/g, Arg 1.004 (1), Asx 1.082 (1), Gly 1.051 (1), Leu 0.971 (1), Tyr 0.950 (1), Phe 0.942 (1).

Boc-Ala-Gln-Ser(Bzl)-Gly-*O*-resin (13). Starting from 46.0 g (50.6 mmol) of Boc-Gly-*O*-resin (**12**) (prepared by using the triethylammonium salt method from 2% cross-linked resin), the solid-phase synthesis was carried out using the following protocol. The Boc amino acids used were Boc-Ser(Bzl)-OH, Boc-Gln-OH, and Boc-Ala-OH. All volumes were 450 mL unless otherwise stated. The resin was capped before each TFA deblocking and after the final coupling with acetic anhydride and pyridine, each 1 M in CH_2Cl_2. Boc removal was executed by using 1:2 (v/v) TFA/CH_2Cl_2 in two treatments of 2 min and one of 25 min, followed by three 2-min washes with CH_2Cl_2. Neutralization was performed with 1:9 (v/v) triethylamine/CH_2Cl_2 by using treatments of 2 and 4 min, followed by four 2-min washes with CH_2Cl_2 and one 2-min DMF wash. Couplings were carried out under the following conditions (DCC was employed as a 1.0 M solution in CH_2Cl_2): the neutralized resin was rocked gently with a solution of 15.3 g (100 mmol) of HBT and 100 mmol of the Boc amino acid in 180 mL of 1:2 (v/v) CH_2Cl_2/DMF plus 100 mL of DCC solution for 16 h, followed by washing with one 2-min treatment of DMF, two 2-min treatments with 1:4 CH_3OH/CH_2Cl_2, and three 2-min treatments of CH_2Cl_2. In the case of Boc-Gln, 30.6 g (200 mmol) of HBT was used. In the case of Boc-Ala, an initial coupling was performed for 2 h without HBT in 180 mL of CH_2Cl_2 and washing with three 2-min treatments of CH_2Cl_2 followed by the normal protocol described above beginning with neutralization. The completed peptide–resin was dried to constant weight under reduced pressure to afford 61.7 g (82%) of **13**; aaa avg = 0.675 mmol/g, Glx 0.957 (1), Gly 1.053 (1), Ala 0.990 (1).

Boc-Ala-Gln-Ser(Bzl)-Gly-OCH_3 (14). A mixture of 50 g (29.6 mmol) of **13** and 1.8 L of 1:9 (v/v) triethylamine/CH_3OH was stirred under nitrogen for 1 h. After filtration, the resin was washed with 500 mL of CH_3OH, which was combined with the filtrate and concentrated to dryness at reduced pressure. The residue was redissolved in 500 mL of CH_3OH and reconcentrated to dryness to give a pale yellow solid that was crystallized from 120 mL of CH_3OH. The crystals were washed with a minimum amount of cold CH_3OH and dried to a constant weight at 0.1 mm to give 16.7 g (99%) of **14**: mp 198–200 °C; FABMS M + H = 566; aaa avg = 1.735 mmol/g, Ser 1.026 (1), Glx 0.980 (1), Gly 1.003 (1), Ala 0.991 (1); TLC (system V) R_f 0.41; HPLC (system I, column B) 95%, t_R 20 min.

Boc-Ala-Gln-Ser(Bzl)-Gly-OH (3). A mixture of 5.3 g (9.4 mmol) of **14** and 500 mL of 1:2:2 (v/v/v) triethylamine/CH_3OH/H_2O was stirred for 3 h during which time dissolution occurred. The solvents were removed at reduced pressure to give a colorless glassy solid that was dissolved in 25 mL of H_2O and acidified to pH 3.0 with 2.5 M HCl. The resulting precipitate was filtered and washed with a minimum amount of cold water. After drying to constant weight at 0.1 mm, 4.05 g (78.4%) of **3** was obtained as a colorless solid: mp 203 °C dec; aaa avg = 1.73 mmol/g, Ser 1.040 (1), Glx 1.005 (1), Gly 0.971 (1), Ala 0.983 (1); FABMS M + H = 552; TLC (system VI) R_f 0.23; HPLC (system IV, column C) 97.5%, t_R 15 min.

Boc-Arg(NO_2)-Ile-Gly-*O*-resin (15). Starting from 50.4 g (60 mmol) of **12**, the solid-phase synthesis was carried out by using the following protocol. All volumes were 500 mL unless otherwise stated. Boc removal was executed by using 1:2 (v/v) TFA/CH_2Cl_2 in two treatments of 2 and 25 min, followed by three 2-min washes with CH_2Cl_2. Neutralization was performed with 1:9 (v/v) triethylamine/CH_2Cl_2 using treatments of 2 and 4 min, followed by four 2-min washes with CH_2Cl_2. Couplings were carried out under the following conditions (DCC was employed as a 1.0 M solution in CH_2Cl_2): Ile, 90 mmol of Boc-Ile-OH in 110 mL of CH_2Cl_2 plus 90 mL of DCC solution for 30 min, followed by a neutralization/washing cycle (as above) and recoupling with 60 mmol in 1:2 (v/v) CH_2Cl_2/dimethylacetamide (DMA) plus 60 mL of DCC solution for 1 h; Arg, 90 mmol of Boc-Arg(NO_2)-OH in 110 mL of 1:2 (v/v) CH_2Cl_2/DMA plus 12.2 g (90 mmol) of HBT and 90 mL of DCC solution for 1 h, followed by a neutralization/washing cycle (as above) and recoupling with 60 mmol in 110 mL of 1:2 CH_2Cl_2/DMA plus 8.1 g (60 mmol) of HBT and 60 mL of DCC solution for 1 h (3.3 mL (30 mmol) of *N*-methylmorpholine added after 30 min). Each coupling or recoupling was followed by two 2-min washes with CH_2Cl_2 (or DMA in those cases where CH_2Cl_2/DMA mixtures were used in the coupling) and three 2-min washes with 1:4 (v/v) CH_3OH/CH_2Cl_2. The completed peptide–resin was dried to constant weight under reduced pressure to afford 68.5 g (75%) of **15**: aaa avg = 0.662 mmol/g, Arg (Orn) [1.145] (1), Gly 0.991 (1), Ile, 1.009 (1).

Boc-Arg(NO_2)-Ile-Gly-OCH_3 (16). A mixture of 30 g (19.9 mmol) of **15** and 1.2 L of 1:9 (v/v) triethylamine/CH_3OH was stirred under nitrogen for 1 h. After filtration, the filtrate was concentrated to dryness at reduced pressure, redissolved in 400 mL of CH_3OH, and reconcentrated to dryness to give a colorless solid. This material was crystallized by being dissolved in 120 mL of CH_3OH, diluted with 600 mL of ethyl acetate, and concentrated to 350 mL by boiling on a hot plate. After standing at −20 °C for 18 h, the crystals were filtered, washed with a minimum of cold ethyl acetate, and dried to constant weight at reduced pressure to afford 10.6 g (99%) of **16** as colorless crystals: mp 145–147 °C; FABMS M + H = 504; aaa avg = 1.868 mmol/g, Arg (Orn) 0.987 (1), Gly 1.039 (1), Ile 0.974 (1); TLC (system V) R_f 0.41; HPLC (system IV, column C) 94%, t_R 15 min.

Boc-Arg(NO_2)-Ile-Gly-OH (4). A mixture of 7.17 g (13.4 mmol) of **16** and 700 mL of 1:2:2 (v/v/v) triethylamine/CH_3OH/H_2O was stirred for 5 h during which time dissolution occurred. The solvents were removed at reduced pressure to give a colorless glassy solid that was triturated with 350 mL of H_2O and acidified to pH 3.0 with 2.5 M HCl. The resulting precipitate was stirred for 30 min in an ice bath, filtered, and washed with a minimum amount of cold water. After drying to constant weight at 0.1 mm, 6.88 g (96.5%) of **4** was obtained as a colorless solid: mp 201–203 °C; aaa avg = 1.879 mmol/g, Arg (Orn) 1.010 (1), Gly 1.006 (1), Ile 0.984 (1); FABMS M + H = 490; TLC (system VII) R_f 0.5; HPLC (system III, column B) 98.5%, t_R 10 min.

Boc-Arg(NO_2)-Ile-Asp(Chx)-Arg(NO_2)-Ile-Gly-Ala-Gln-Ser(Bzl)-Gly-Leu-Gly-Cys(Acm)-Asn-Ser(Bzl)-Phe-Arg-(NO_2)-Tyr(Cl_2Bzl)-*O*-resin (7). Starting from 23.0 g (8.83 mmol) of **6**, the solid-phase synthesis was carried out by using the following protocol. All volumes were 250 mL unless otherwise stated. Resin **6** was capped with a solution of acetic anhydride and pyridine, each 1.0 M in CH_2Cl_2 for 15 min, followed by washing four times with CH_2Cl_2. Boc removal was executed by using 1:2 (v/v) TFA/CH_2Cl_2 containing 1:50 (v/v) ethanedithiol in three treatments of 1, 2, and 25 min, followed by three 2-min washes with CH_2Cl_2. Neutralization was performed with 1:9 (v/v) triethylamine/CH_2Cl_2 by using treatments of 2 and 4 min, followed by four 2-min washes with CH_2Cl_2 and one 2-min DMF wash. Couplings were carried out by rocking the resin gently under the following conditions, where washing refers to two 2-min treatments with DMF (or CH_2Cl_2 for those couplings carried out in CH_2Cl_2), two 2-min treatments with 1:4 (v/v) CH_3OH/CH_2Cl_2, and two 2-min treatments with CH_2Cl_2: (1) a solution of 8.3 g (14.4 mmol) of **3** in 80 mL of DMF plus 20 mL of DCC (DCC was employed as a 1.0 M solution in CH_2Cl_2) and 2.3 g (15 mmol) of HBT, 19

h, washed as above; (2) a solution of 7.3 g (13.7 mmol) of **4** in 80 mL of DMF plus 20 mL of DCC solution and 2.3 g (15 mmol) of HBT, 18.5 h; plus 0.55 mL (5 mmol) of *N*-methylmorpholine (NMM), 24 h; plus 0.55 (5 mmol) of NMM, 7.5 h; plus 1.52 g (3.1 mmol) of **4**, 3.1 mL of DCC solution, 0.34 mL (3.1 mmol) of NMM and 0.47 g of HBT, 16.5 h; washed and capped as above; (3) a solution of 6.4 g (20 mmol) of Boc-Asp(Chx)-OH (**17**) (Peninsula) in 70 mL of DMF plus 20 mL of DCC solution and 3.06 g (20 mmol) of HBT, 54 h; washed and recoupled with 6.4 g (20 mmol) of **17** in 70 mL of CH_2Cl_2 plus 20 mL of DCC solution, 30 min; plus 40 mL of DMF, 4 h; washed, neutralized, and recoupled with 3.2 g (10 mmol) of **17** in 60 mL of 1:2 (v/v) CH_2Cl_2/DMF plus 10 mL of DCC solution and 1.53 g (10 mmol) of HBT, 18.5 h; washed and capped as above; (4) a solution of 4.8 g (20 mmol) of Boc-Ile-OH (**18**) in 50 mL of CH_2Cl_2 plus 20 mL of DCC solution, 3.5 h; washed and recoupled with 4.8 g (20 mmol) of **18** in 60 mL of 1:2 (v/v) CH_2Cl_2/DMF plus 20 mL of DCC solution and 3.06 g (20 mmol) of HBT, 18 h; washed and recoupled with 2.4 g (10 mmol) of **18** in 60 mL of 1:2 (v/v) CH_2Cl_2 plus 10 mL of DCC solution and 1.53 g (10 mmol) of HBT, 6.5 h; washed and capped as above; (5) a solution of 6.4 g (20 mmol) of Boc-Arg(NO_2)-OH (**19**) in 40 mL of DMF plus 20 mL of DCC solution and 3.06 g (20 mmol) of HBT, 17.5 h; plus 0.55 mL (5 mmol) of NMM, 1.5 h; washed, neutralized, and recoupled with 6.4 g (20 mmol) of **19** in 40 mL of DMF plus 20 mL of DCC solution, 3.06 g (20 mmol) of HBT, 17.5 h; plus 0.55 mL (5 mmol) of NMM, 1.5 h; washed, neutralized, and recoupled with 6.4 g (20 mmol) of **19** in 40 mL of DMF plus 20 mL of DCC solution, 3.06 g (20 mmol) of HBT, 3 h; plus 0.55 mL (5 mmol) of NMM, 1.5 h; washed as above. The completed peptide–resin was washed with three 2-min washes of CH_3OH and dried to constant weight under reduced pressure to give 31.4 g (87%) of **7**: aaa avg = 0.246 mmol/g, Arg (Orn) 2.945 (3), Asx 2.023 (2), Glx 1.024 (1), Gly [2.823] (3), Ala 0.999 (1), Ile [1.783] (2), Leu 1.003 (1), Tyr 1.007 (1), Phe 0.999 (1).

Boc-Gly-*O*-resin (20). A solution of 61.3 g (350 mmol) of Boc-Gly-OH in 500 mL of absolute EtOH was added to a solution of 57 g (175 mmol) of Cs_2CO_3 (Alfa) in 800 mL of EtOH and allowed to stand for 1 h, and the solvent was removed at reduced pressure to give a colorless solid. This solid was broken up and dried for 48 h at 0.1 mm to constant weight. A solution of this material in 500 mL of degassed dimethylacetamide (DMA) was added to a mixture of 192.3 g (250 mequiv) of preswollen and twice washed (degassed DMA) chloromethylpolystyrene resin (1% cross-linked, 1.3 mequiv/g, Lab Systems) in 1.1 L of degassed DMA. The resulting mixture was slowly rotated on a rotary evaporator at 50 °C for 20 h and filtered, and the resin was washed according to the following protocol: 3 × 600 mL DMA, 3 × 900 mL 1:3 (v/v) CH_3OH/CH_2Cl_2, 2 × 1.2 L CH_2Cl_2, 3 × 400 mL CH_3OH, 4 × 500 mL 1:1 (v/v) CH_3OH/H_2O, 3 × 400 mL CH_3OH, 3 × 1.2 L CH_2Cl_2. The resin was dried to constant weight at 0.1 mm for 96 h, reswollen in 1 L of DMA, and filtered. A solution of 50 g (260 mmol) of cesium acetate (Aldrich) in 1.2 L of DMA was added to the swollen resin and the mixture rotated on the rotary evaporator at 50 °C for 20 h. The resin was filtered and washed according to the following scheme: 2 × 700 mL DMA, 3 × 600 mL 1:1 (v/v) CH_3OH/H_2O, 3 × 1.2 L CH_2Cl_2, 2 × 600 mL 1:1 (v/v) CH_3OH/H_2O, 4 × 500 mL CH_3OH. Drying the resin to constant weight at 0.1 mm for 48 h afforded 210 g of **20**: aaa Gly 0.873 mmol/g; ca nitrogen = 1.45 wt %.

Boc-Arg(NO_2)-Arg(NO_2)-Ser(Bzl)-Ser(Bzl)-Cys(Acm)-Phe-Gly-Gly-*O*-resin (21). The solid-phase synthesis was performed by using 48.3 g (42 mmol) of **20** and was carried out by using the following protocol. The Boc amino acids used were Boc-Gly-OH (Chemalog), Boc-Phe-OH, Boc-Cys(Acm)-OH, Boc-Ser(Bzl)-OH twice, and Boc-Arg(NO_2)-OH twice. All volumes used were 500 mL unless otherwise indicated. Boc removal was accomplished with 1:2 (v/v) TFA/CH_2Cl_2 (with the addition of 1:99 (v/v) ethanedithiol after the incorporation of cysteine) in two treatments of 2 and 25 min, followed by three 2-min washes with CH_2Cl_2. Neutralization was performed with 1:9 (v/v) triethylamine/CH_2Cl_2 by washing for periods of 2 and 4 min, followed by three 2-min washes with CH_2Cl_2. Coupling reactions were carried out according to the following protocol with exceptions noted in brackets. The neutralized resin was treated with a solution of 75 mmol of the Boc amino acid in the minimum volume of CH_2Cl_2 required for efficient mixing (150–350 mL), followed by the addition of 75 mL of a 1.0 M solution of DCC in CH_2Cl_2. [For reasons of solubility it was necessary to use a mixture of 15 mL of DMF and 135 mL of CH_2Cl_2 for Boc-Cys(Acm) and 250 mL of 1:1 (v/v) DMF/CH_2Cl_2 for the first Boc-Arg(NO_2). After the Boc-Gly coupling, the resin was washed with CH_2Cl_2 and DMF, followed by a recoupling for 1.5 h using 50 mmol of Boc-Gly and 6.76 g (50 mmol) of HBT in 50 mL of DMF with the addition of 50 mL of DCC solution. A similar recoupling protocol was required for the complete incorporation of the last Arg residue, using 75 mmol of Boc-Arg(NO_2) and 10.1 g (75 mmol) of HBT in 300 mL of 1:1 (v/v) DMF/CH_2Cl_2 and 75 mL of DCC solution for 1 h with the addition of 4.1 mL of *N*-methylmorpholine after 30 min.] The coupling mixtures were mixed by gentle rocking for 15 min, except as noted above. After the couplings, the resin was washed with CH_2Cl_2 (except for the Boc-Gly recoupling where DMF was used, and for the Boc-Arg(NO_2) couplings where 1:1 (v/v) DMF/CH_2Cl_2 was used) followed by three 2-min treatments of 1:4 (v/v) CH_3OH/CH_2Cl_2, and three 2-min treatments with CH_2Cl_2. The completed peptide-resin was dried to constant weight under reduced pressure to give 90.0 g (83%) of **21**; aaa avg = 0.389 mmol/g, Arg 1.957 (2), Gly 2.034 (2), Phe 1.009 (1).

Boc-Arg(NO_2)-Arg(NO_2)-Ser(Bzl)-Ser(Bzl)-Cys(Acm)-Phe-Gly-Gly-OH (5). A stirred suspension of 60.0 g (23.3 mmol) of **21** in 900 mL of anhydrous methanol was treated with 100 mL of triethylamine for 1 h, at which time the mixture was filtered and the collected solid washed three times with 300 mL of methanol. The resin was retreated as above for 5 h and washed as above with methanol. The ester was leached from the mixture with three 300-mL portions of DMF. The combined DMF filtrates were concentrated at reduced pressure to a thick residue which was stirred in 250 mL of methanol at 4 °C overnight. The solid was collected by filtration, washed twice with 100 mL of methanol, and dried to constant weight under reduced pressure to give 10.8 g of crude methyl ester. Two further retreatments of the resin for 24 and 48 h with methanolic triethylamine followed by leaching the ester with DMF and workup as above gave an additional 11.2 g and 5.6 g of crude ester, respectively. All three samples were shown to be 90% pure by HPLC (system IV, column C, t_R 20 min) and were combined to give a total of 27.6 g. A sample of 21.3 g of this material was dissolved in 760 mL of 1:1 (v/v) THF–H_2O and treated with 190 mL of triethylamine. Saponification was complete after 7 h as determind by TLC (system VIII). The reaction was worked up by concentration at reduced pressure to a gel-like consistency, then dispersing to homogeneity with 1.0 L of H_2O, and finally acidifying under vigorous mechanical stirring with 2.5 M HCl to pH <2 which caused the semisolid gel to become a fine precipitate. After stirring for 0.5 h, the solid was filtered and washed three times with H_2O and suspended in approximately 600 mL of H_2O, and the slurry was lyophilized to give 18.1 g of crude acid. This material was purified in portions by silica gel chromatography as follows: 8.15 g of the crude acid was dissolved in a solution of 80 mL of pyridine, 16 mL of acetic acid, and 24 mL of H_2O, diluted with 240 mL of ethyl acetate (final ratio 15:5:1:1.5 EtOAc–Py–HOAc–H_2O), and introduced onto a column of 1100 g of silica gel (230–400 mesh) packed in a 15:5:1:1 solution of the same solvents. The column was eluted with 8.0 L of a 15:5:1:1.5 solution followed by sufficient 15:5:1:1.75 solution to elute the main component. Fractions free of impurities as determined by TLC were pooled and concentrated at reduced pressure, and the residue was dissolved in DMF and reconcentrated to remove any remaining acetic acid and pyridine. This evaporation procedure was repeated twice, and the residual oil was treated with 250 mL of H_2O, triturated, filtered, washed with water, and dried at reduced pressure to afford 5.90 g (49%) of **5** as a colorless solid: mp 215–225 °C dec; aaa avg = 0.671 mmol/g, Arg (Orn) 1.89 (2), Ser [1.58] (2), Gly 2.06 (2), Phe 1.05 (1); TLC (system IX) R_f 0.4; HPLC (system IV, column C) 99.8% t_R 14 min.

Arg(NO_2)-Arg(NO_2)-Ser(Bzl)-Ser(Bzl)-Cys(Acm)-Phe-Gly-Gly-Arg(NO_2)-Ile-Asp(Chx)-Arg(NO_2)-Ile-Gly-Ala-Gln-Ser(Bzl)-Gly-Leu-Gly-Cys(Acm)-Asn-Ser(Bzl)-Phe-Arg(NO_2)-Tyr(Cl_2Bzl)-*O*-resin (2). The solid-phase synthesis was carried out in the following manner: All volumes were 120 mL unless otherwise indicated. Boc removal was accomplished

by treating 12.0 g (2.95 mmol) of 7 for 2, 2, and 25 min with 1:2 (v/v) TFA/CH_2Cl_2 containing 1:49 (v/v) ethanedithiol, followed by five 2-min washes with CH_2Cl_2. Neutralization was carried out with 1:9 (v/v) triethylamine/CH_2Cl_2 for periods of 2, 2, and 4 min, followed by five 2-min washes with CH_2Cl_2 and one DMF wash. Coupling of the fragment was performed by treating the neutralized resin with a solution of the activated N-terminal fragment prepared as follows: a stirred solution of 6.9 g (5.27 mmol) of **5** and 606 mg (5.27 mmol) of *N*-hydroxysuccinimide (recrystallized) in 15 mL of degassed DMF was cooled in an ice–water bath for 5 min, and 5.27 mL of a 1 M solution of DCC in CH_2Cl_2 was added in one portion. The ice bath was removed, and the thick mixture was stirred for 4 h. After adding this solution to the resin, 159 mg (1.3 mmol) of recrystallized (ethyl acetate) 4-(dimethylamino)pyridine (DMAP) was added, and the mixture was rocked gently for 22 h. Then 1.0 mL of DCC solution was added and mixing continued for 26 h at which time a solution of 159 mg (1.3 mmol) of DMAP in 3.0 mL of degassed DMF was added and mixing continued for an additional 64 h. The resin was washed twice with DMF for 2 min each, followed by six 2-min washes with CH_2Cl_2. After removing the Boc protection using the TFA solution described above for 3- and 25-min periods followed by six 2-min washes with CH_2Cl_2, the peptide–resin was dried to constant weight at reduced pressure to afford 19.5 g (91%) of **2**: aaa avg = 0.138 mmol/g, Arg (Orn) 5.047 (5), Asx 2.285 (2), Glx 1.157 (1), Gly 5.010 (5), Ala 1.120 (1), Ile 1.685 (2), Leu 0.962 (1), Tyr 1.056 (1), Phe 1.678 (2).

Arg-Arg-Ser-Ser-Cys(Acm)-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys(Acm)-Asn-Ser-Phe-Arg-Tyr-OH (8). A Kel-F reaction vessel was charged with a slurry of 2.0 g (0.28 mmol) of **2** and 1.0 g (6.7 mmol) of L-methionine in 4.0 mL of *m*-cresol (Aldrich), which was stirred slowly for 1 h. The reaction vessel was attached to a Kel-F manifold and cooled in a dry ice/acetone cold bath for approximately 10 min, and approximately 45 mL of anhydrous HF (Matheson) was condensed into the stirred mixture. The cold bath was replaced with an ice–water bath, the mixture was stirred in the cold for 75 min, and the HF was removed in the cold under reduced pressure (water aspirator for 70 min followed by vacuum pump for 1 h). After triturating the residue with 50 mL of ether for 15 min in the cold, the mixture was filtered and washed with two 30-mL portions of ether and dried briefly under reduced pressure. The peptide was leached from the resin by stirring and filtering with 15 mL of 1:1 (v/v) acetic acid/H_2O. Purification was achieved by applying the combined filtrates onto a Sephadex G-25F column (5 × 100 cm) and eluting with 2.0 M acetic acid. Product purity was checked by assaying the fractions (22 mL) by TLC (system IX) and HPLC (system II, column A). Fractions of appropriate purity were combined and evaporated to dryness at reduced pressure. Material from four identical runs was lyophilized from 150 mL of H_2O to afford 1.765 g (41%) of **8**: aaa avg = 0.259 mmol/g, Arg (Orn) 4.918 (5), Asx 2.038 (2), Ser [3.476] (4), Glx [1.164] (1), Gly [4.369] (5), Ala 1.052 (1), Ile 1.968 (2), Leu 1.032 (1), Tyr 1.017 (1), Phe 1.976 (2); TLC (system X) R_f 0.3; HPLC (system II, column A) 86%, t_R 50 min.

Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr-OH, Disulfide Form (1). A solution of 1.39 g (5.5 mmol) of I_2 in 620 mL of 80% HOAc was added rapidly to a briskly stirred solution of 1.21 g (0.3 mmol) of **8** in 37 mL of 50% HOAc. After 2 h the reaction mixture was cooled in an ice–water bath for 5 min and treated with 4.9 g of zinc dust until decolorization of the I_2 was complete. The mixture was filtered, and the filtrate was concentrated at reduced pressure to a volume of about 25 mL, diluted with an equal volume of H_2O, and charged onto a 5 × 100 cm column of Sephadex G-50F in 50% HOAc. The column was eluted with 50% HOAc, and the fractions were pooled on the basis of HPLC analysis (system II, column A), combined, concentrated at reduced pressure, and lyophilized from H_2O to give 0.92 g of crude **1**. A solution of 1.76 g of this material in 50 mL of 0.05 M NH_4OAc (pH adjusted to 5.0 with acetic acid) was applied to a 300-mL (4.0 cm diameter) column of carboxymethylcellulose (CMC) (Whatman) equilibrated with 0.3 M NH_4OAc (prepared by diluting 76.5 mL of concentrated NH_4OH (29% NH_3) and 68.6 mL of glacial acetic acid to 4.0 L with degassed H_2O and acidifying to pH 5.0 with additional acetic acid). The column was eluted with 4 L of 0.3 M NH_4OAc (pH 5.0, prepared as above) and 22-mL fractions were collected over a period of 24 h. Those fractions showing greater than 97% purity by HPLC (system II, column A) were combined, applied directly to a Sephadex G-25F column (5 × 100 cm), and eluted with 2.0 M acetic acid to remove NH_4OAc. The fractions containing product were combined, evaporated to dryness under reduced pressure, and lyophilized from 125 mL of H_2O to give 890 mg (40.6%) of **1** as a colorless solid: aaa avg = 0.286 mmol/g, Arg 5.04 (5), Asx 2.05 (2), Ser 4.06 (4), Glx 1.00 (1), Gly 4.90 (5), Ala 1.00 (1), Ile 1.93 (2), Leu 1.02 (1), Tyr 1.00 (1), Phe 2.00 (2); TLC (system X) R_f 0.33; HPLC (system II, column A) 97.3%, t_R 49 min; GC, acetic acid 6.9%; H_2O, Karl Fischer titration 8.1%; specific rotation $[\alpha]^{26}_D$ +47.3°; cumulative sequence preview ≤3% at Ser^{30} cycle. Combustion analysis (based on tetraacetate minus water). Calcd: C, 49.17; H, 6.66; N, 19.42; S, 2.07. Found: C, 49.11; H, 6.80; N, 19.32; S, 2.42.

Acknowledgment. We thank Sue Fitzpatrick for the amino acid analyses, Carl Homnick for his help with HPLC problems, Bud Rodkey and Dr. Carl Bennett for the sequencing work, Dr. Byron Arison, Dr. Steve Pitzenberger, Dr. Sandor Varga, and Joan Murphy for NMR studies, Jane Wu and Bill Reuter for analytical services, and Cheryl Gerson for typing the manuscript.

New Approaches to the Synthesis of *trans*-Alkene Isosteres of Dipeptides

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Received March 12, 1987

Two new syntheses of protected dipeptide analogues bearing a trans carbon–carbon double bond in place of the amide linkage are reported. One route is a linear synthesis employing the rearrangement of an allylic selenide to a protected allylic amine. The second route is convergent and uses the Julia olefin synthesis in a key step. The latter route is fully stereocontrolled and has been used to prepare protected *trans*-alkene isosteres of the dipeptides TyrAla, PhePhe, LeuPhe, and LeuLeu.

Introduction

Studies of structure–function relationships in the field of biologically active peptides have largely focused on the effects of changes in side chain residues. This is experimentally advantageous, since peptide synthesis is now routine, but does not permit the study of questions regarding the role played by the amide backbone. Changing the amide backbone is in general more synthetically challenging, but, despite this, numerous amide replacements have been pursued.[1] It has been suggested that the

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0022-3263/87/1952-3759$01.50/0