Enzymatic Semisynthesis of a Superpotent Analog of Human Growth Hormone-Releasing Factor[†,§]

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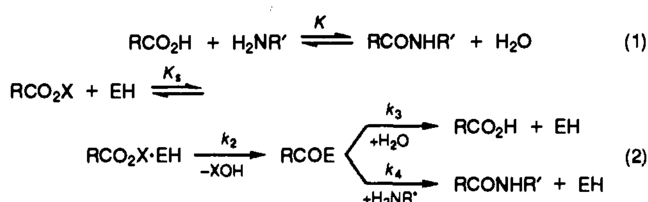
A superpotent analog of human growth hormone-releasing factor, [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]-GRF(1-29)-NH$_2$ (4), was prepared from the precursor, [Ala^{15,29}]-GRF(4-29)-OH (1), by a two-step enzymatic semisynthesis. The amidated C-terminus, essential for high biological potency, was obtained via a carboxypeptidase Y-catalyzed exchange of Ala^{29}-OH for Arg^{29}-NH$_2$ to produce [Ala^{15}]-GRF(4-29)-NH$_2$ (2). The N-terminal desNH$_2$Tyr-D-Ala moiety, which greatly increases in vivo duration of action, was then incorporated by V8 protease-catalyzed condensation of segment 2 with desNH$_2$Tyr-D-Ala-Asp(OH)-OR [R = CH_3CH_2- (3a) or 4-$NO_2C_6H_4CH_2$-(3b)]. The main focus of this report was to develop conditions to use the V8 protease-catalyzed coupling while avoiding a competing cleavage of the proteolytically-sensitive Asp^{25}-Ile^{26} bond in GRF. Conversion of 2 to 4 in couplings employing the α-ethyl ester of the acyl component 3a was limited to about 60% by competing proteolysis at Asp^{25}-Ile^{26}. This system was adequate for preparing, isolating, and fully characterizing the target analog 4 and identifying the side products. The 4-nitrobenzyl ester 3b proved to be a superior substrate, resulting in 90% conversion of 2 to 4 with no detectable loss to proteolysis and requiring significantly lesser amounts of catalyst. These results demonstrate that enzymatic semisynthesis of a biologically-active peptide amide which contains unnatural amino acids at the N-terminus can be achieved from a biosynthetic precursor in good yield and purity.

Introduction

The use of natural enzymes as efficient and selective catalysts in chemical synthesis has accelerated in step with our knowledge of their fundamental properties and the discovery of "new" enzymes isolated in ever greater quantity and purity. The proteolytic enzymes are of special interest to the peptide chemist as they represent a means of performing regioselective acylations without resorting to the usual stoichiometric reagents and their required protection/deprotection schemes. Most of the proteases also possess rigid stereoselectivity and thus provide products of the highest optical purity, even from racemic starting materials. Enzymatic peptide synthesis[1] has found use mainly for generating small oligopeptides, as in the thermolysin-catalyzed production of aspartame,[2] or for making semisynthetic modifications to larger peptides, as in the trypsin-catalyzed conversion of porcine insulin to human insulin.[3]

The two existing methods of protease-catalyzed peptide synthesis are the thermodynamically-controlled coupling (eq 1) and the kinetically-controlled coupling (eq 2):



The thermodynamically-controlled or "reverse-proteolysis" synthesis (eq 1) relies on the use of conditions which favor aminolysis over hydrolysis, including the use of a high molar excess of one of the reactants and/or less-polar water-miscible cosolvents[4] to suppress ionization of the α-carboxylic acid group. Improved yields of the aminolysis

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† This paper is dedicated to Professor Ralph F. Hirschmann on the occasion of his 70th birthday. Professor Hirschmann's accomplishments and character serve as guidelines for all who follow in his footsteps.

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§ Abbreviations are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *J. Biol. Chem.* 1989, *264*, 668-673. Other abbrevations are as follows: AAA, amino acid analysis; ABz, 2-aminobenzoyl; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; CPD-Y, carboxypeptidase Y; CZE, capillary zone electrophoresis; DMF, *N,N*-dimethylformamide; DPP IV, dipeptidyl peptidase IV; EDTA, ethylenediamine tetraacetic acid; FAB-MS, fast atom bombardment mass spectrometry; GRF, growth hormone-releasing factor; HPLC, high-performance liquid chromatography; Mal, maleyl; Nb, 4-nitrobenzyl; pNA, 4-nitroanilide; SPPS, solid-phase peptide synthesis; Su, succinimidyl; TFA, trifluoroacetic acid; TPCK, *N*-tosylphenylalanine chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane; Z, benzyloxycarbonyl.

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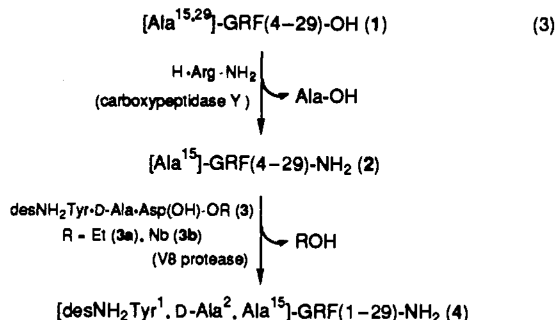
product can also be achieved by incorporating conditions which drive the reaction to completion such as precipitation or extraction into an immiscible cosolvent.

The kinetically-controlled synthesis (eq 2) is usually performed in aqueous solution (without cosolvents) and employs an active ester (RCO_2X) that rapidly forms an acyl enzyme (RCOE). This acyl enzyme is competitively deacylated by water yielding the acid (RCO_2H), and also by an added nucleophile (H_2NR') resulting in a transient accumulation of the desired peptide product (RCONHR'). Unless the protease is deactivated at the point of maximum yield, the accumulation of peptide product is only temporary. The newly-formed peptide bond is slowly hydrolyzed as the supply of ester (RCO_2X) is exhausted. This latter scheme is limited to proteases which form covalent acyl enzyme intermediates, such as the serine and thiol proteases.

Proteases can be advantageously used in the segment condensation strategy for intermediate to large peptides specifically in those cases where side-chain protection hinders solubility of the segments or where suitable glycine or proline residues are not available as coupling sites and racemization becomes a concern. Schellenberger and Jakubke have provided an excellent review[5] of the development of kinetically-controlled protease-catalyzed coupling, a technique that appears particularly well suited for segment condensation. To date, most of the studies of this enzymatic coupling method have been devoted to the synthesis of dipeptides and small peptide models, with some notable exceptions.[6]

In this paper, we report on the enzymatic semisynthesis of a superpotent analog of human growth hormone-releasing factor, GRF(1–44)-NH$_2$ (H-YADAIFTNSY^{10}-RKVLGQLSAR^{20}KLLQDIMSRQ^{30}QGESN-QERGA^{40}RARL-NH$_2$), a peptide hormone that is secreted by the hypothalamus and stimulates the release of growth hormone in the pituitary gland.[7] GRF(1–44)-NH$_2$ and the equipotent fragment, GRF(1–29)-NH$_2$,[8] are in clinical trials for the treatment of growth hormone-deficient children.[9] Preliminary studies have shown that the parent hormone and analogs may also have applications for the enhancement of growth performance in domestic livestock.[10]

We recently described[11] a GRF analog, [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]-GRF(1–29)-NH$_2$ (4), which possesses a 4–5-fold higher intrinsic potency than the parent hormone in vitro[11] and has a significantly longer duration of action in vivo.[12] The increased in vitro potency is primarily a result of the replacement of the native Gly^{15} residue by Ala which enhances the α-helicity of the peptide and maximizes the amphiphilic character of the central α-helix[13] which may lead to improved receptor affinity.[14] The longer in vivo duration of action arises from replacement of the native Tyr^1-Ala^2 by desNH$_2$Tyr^1-D-Ala^2 which confers resistance to proteolysis by dipeptidyl aminopeptidase IV (DPP IV).[15] In considering various methods for large-scale synthesis of the target analog 4, recombinant DNA synthesis is not an option as 4 contains two important features that are not afforded by existing recombinant DNA methods; namely the C-terminal amide, essential for high potency,[8] and the unnatural N-terminal residues, desNH$_2$Tyr^1-D-Ala^2. To overcome this limitation, we have devised an enzymatic semisynthetic route to 4 from the precursor, [Ala^{15,29}]-GRF(4–29)-OH (1) (eq 3).



The precursor 1, which is amenable to biosynthesis by recombinant DNA methods, was prepared for this study by the solid-phase method of peptide synthesis (SPPS). C-terminal amidation of 1 was achieved by the method of

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Breddam and co-workers[16]; i.e. carboxypeptidase Y-catalyzed exchange of Ala^{29}-OH for Arg^{29}-NH_2 using a large molar excess of H-Arg-NH_2 to produce [Ala^{15}]-GRF(4-29)-NH_2 (**2**). The N-terminal desNH$_2$Tyr^1-D-Ala^2 moiety was then incorporated by a V8 protease-catalyzed condensation of segment **2** with desNH$_2$Tyr-D-Ala-Asp(OH)-OR [R = CH_3CH_2– (**3a**) or 4-$NO_2C_6H_4CH_2$– = Nb (**3b**)], to generate the final product **4**.

Although several observations are made concerning the above transpeptidation[16] catalyzed by the serine exoprotease, carboxypeptidase Y (CPD-Y), the primary focus of this study is the kinetically-controlled segment condensation catalyzed by the V8 protease. The V8 protease (protease from the V8 strain of *Staphylococcus aureus*, endoprotease Glu-C)[17] is a serine endoprotease with a primary (P_1) specificity for Glu and, to a lesser extent, Asp. For example, the ratio of the hydrolytic coefficients (k_{cat}/K_m) for the substrates, ABz-Ala-Phe-Ala-Phe-X-Val-Tyr(NO_2)-Asp-OH (X = Glu, Asp), was found[18] to be 3000–5000 for Glu versus Asp at pH 7.8. The V8 protease has been used successfully for thermodynamically-controlled reformations of the Glu^{30}–Arg^{31} bond in the 1–47 fragment of the α-chain of hemoglobin[19] and the Glu^{302}–Val^{303} bond in several fragments of thermolysin.[20] Recently, V8 protease-catalyzed kinetically-controlled syntheses of Glu-containing dipeptides and oligopeptides have been reported.[21] The scheme in eq 2 has also been used to probe the S′-subsite specificity of the V8 protease for acyl transfers of Z-Glu-OMe to a series of amino acid and peptide nucleophiles.[22] However, the synthetic potential of the V8 protease for kinetically-controlled segment condensations in general, and for Asp–X couplings in particular, remains unexplored. The V8 protease-catalyzed segment condensation presented here is especially challenging due to the proteolytically-sensitive Asp^{25}-Ile^{26} bond in GRF. Observations are also made concerning general tactics and specific technical aspects of protease-catalyzed kinetically-controlled segment condensations.

Results and Discussion

Carboxypeptidase Y-Catalyzed Conversion of [Ala^{15,29}]-GRF(4-29)-OH (1) to [Ala^{15}]-GRF(4-29)-NH_2 (2). The first step in the semisynthesis (eq 3) is the formation of the amidated segment **2** by CPD-Y catalyzed exchange of the C-terminal Ala^{29}-OH residue of **1** for Arg^{29}-NH_2. The C-terminal amidation can also be accomplished in high yield via an α-amidating enzyme-catalyzed oxidation[23] of the glycine-extended precursor, [Ala^{15}]-GRF-(4-29)-Gly-OH. The CPD-Y transpeptidation is not a general method for the C-terminal amidation of peptides. It was developed by Breddam and co-workers[16] for the specific purpose of incorporating a C-terminal Arg^{29}-NH_2 into a GRF substrate. The model substrates Bzl-Met-Ser-X-OH (X = Ala, Leu, Arg) were tested[16] and Ala was the most favorable of the three leaving groups. Breddam and co-workers then demonstrated[16] that [Ala^{29}]-GRF-(1-29)-OH (2 mM) could be converted (87% in 150 min) to GRF(1-29)-NH_2 by CPD-Y catalysis in the presence of 1.5 M H-Arg-NH_2. We chose the CPD-Y method for the present study since it employs relatively-small amounts of readily-available enzyme from brewers' yeast. Moreover, the CPD-Y exoprotease is well suited for this type of semisynthetic modification as it will not disrupt internal peptide bonds in a polypeptide substrate as is often the case with endoproteases.

We used a lower concentration of H-Arg-NH_2 nucleophile (1.0 M) and found that a higher concentration of substrate **1** (3.8 mM) could be used at 37 °C. This slightly modified version of the original procedure[16] was used to convert **1** to **2** to an extent of 80% (62% isolated yield). The semisynthetic product **2** was purified and characterized by AAA, FAB-MS, and tryptic mapping.

The only significant side product was [Ala^{15}]-GRF(4-28)-OH (**5**) resulting from a competing hydrolysis which is virtually the only limitation on the yield of **2** under these conditions (Figure 1b). A similar hydrolysis to GRF-(1-28)-OH was also reported as the major side reaction for the [Ala^{29}]-GRF(1-29)-OH substrate.[16] We have observed that the molar ratio of **2** vs **5** (aminolysis vs hydrolysis) remains constant throughout the course of the reaction (Figure 1a) and is directly proportional to the pH of the reaction mixture in the range pH 6.5–8.5 (Figure 1c).

Although the yield of **2** increases with increasing pH (Figure 1d), there is a decline in the catalytic efficiency of the enzyme with increasing pH (Figure 1e). Thus as the pH is raised in order to approach yields of 90%, a larger amount of enzyme is required to amidate a given amount of substrate within a specific amount of time. The question of what constitutes a "catalytic" amount of enzyme is an important issue in enzymatic peptide synthesis that is often ignored. Although the enzyme catalyst can, in principle, be recovered and reused, it is generally more practical to simply use and discard a small amount of the enzyme as though it were a stoichiometric reagent. We have confirmed that conversions of $\geq$80% can be obtained with substrate to CPD-Y ratios of at least 1000:1, as originally reported,[16] and consider this a reasonably "catalytic" amount of enzyme.

We found that H-Arg-NH_2 must be present in concentrations $\geq$ 1 M ($\geq$245 mg mL^{-1}) in order to obtain optimum

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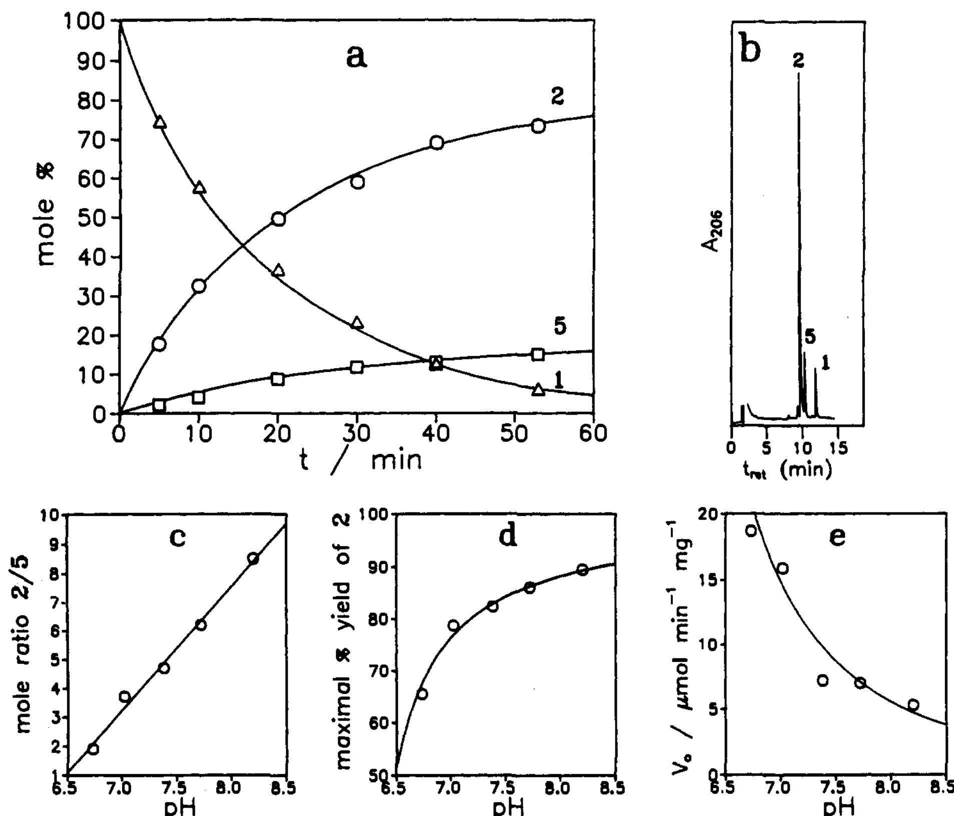

Figure 1. CPD-Y-catalyzed transpeptidation of the precursor [Ala[15,29]]-GRF(4–29)-OH (1) (14.5 mg mL^{-1}, 4.1 mM) in 1.00 M H-Arg-NH_2 to produce [Ala[15]]-GRF(4–29)-NH_2 (2) and the proteolytic side product [Ala[15]]-GRF(4–28)-OH (5) (pH 7.7, 37 °C, 0.3 mM EDTA, 0.028 mg mL^{-1} enzyme): (a) time course; (b) HPLC of the reaction mixture at the 53-min mark; (c) pH dependence of the molar ratio of 2 to 5, (d) maximal percent conversion of 1 to 2, and (e) enzyme activity, V_0 (sum of the initial rates of aminolysis and hydrolysis).

yields of 2, and these amounts of H-Arg-NH_2 become somewhat prohibitive as 1 is only soluble to the extent of about 15 mg mL^{-1} in this medium (pH 8.0, 37 °C). The solubility of 1 in 1 M H-Arg-NH_2 at pH 8.0 was improved, without greatly inhibiting the enzyme, by the use of 10–20% v:v of organic cosolvents, e.g. ethylene glycol, DMSO, EtOH, or MeOH. However, these cosolvents all decreased the ratio of aminolysis/hydrolysis (2/5) to a significant degree.

V8 Protease-Catalyzed Condensation of [Ala[15]]-GRF(4–29)-NH_2 (2) and desNH₂Tyr-D-Ala-Asp(OH)-OEt (3a) To Form [desNH₂Tyr[1],D-Ala[2],Ala[15]]-GRF(1–29)-NH_2 (4). The second step in the semisynthesis (eq 3) is the V8 protease-catalyzed condensation of the amidated segment 2 and the tripeptide ester 3 to form the target compound 4. The fate of the amino component 2 in a coupling employing a 6-fold molar excess of the ethyl ester of the acyl component 3a is shown in Figure 2a. The conversion of 2 to 4 in this system is limited to about 60%, primarily due to a competing proteolysis at Asp[25]-Ile[26] that generates the fragments [Ala[15]]-GRF(4–25)-OH (8) and [desNH₂Tyr[1],D-Ala[2],Ala[15]]-GRF(1–25)-OH (9) (Figure 2b). Subsequent enzymatic couplings of the proteolytic fragments generate the side products desNH₂Tyr-D-Ala-Asp(OH)-GRF(26–29)-NH_2 (7) and [desNH₂Tyr[1],D-Ala[2],-Ala[15]]-GRF(1–25)-[Ala[15]]-GRF(4–29)-NH_2 (10) (Figure 2b). These side products were isolated and identified by AAA. The only other significant side reaction that we could detect was an unexpected acylation of the Tris buffer by 3a to form desNH₂Tyr-D-Ala-Asp-NHC(CH_2OH)$_3$ (6), which was isolated and identified by AAA, FAB-MS [(M + H)$^+$ calcd 455, found 455], and ninhydrin testing (tested negative prior to and positive after acid hydrolysis thereby

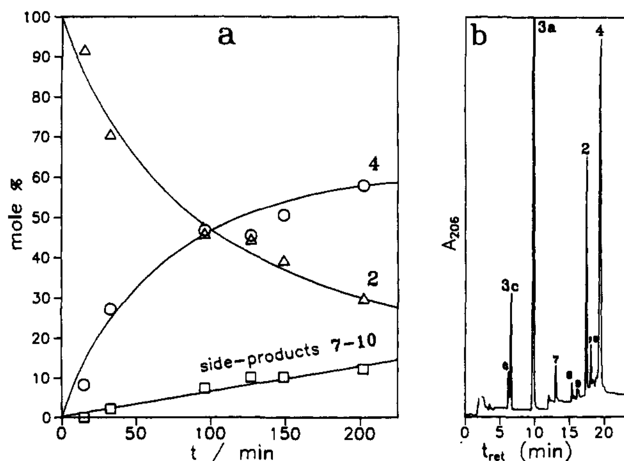

Figure 2. V8 protease-catalyzed condensation of [Ala[15]]-GRF-(4–29)-NH_2 (2) (26.5 mg mL^{-1}, 7.2 mM) and desNH₂Tyr-D-Ala-Asp(OH)-OEt (3a) (16.3 mg mL^{-1}, 43 mM) in 20% DMSO (0.14 M Tris, pH 8.2, 37 °C, 1.3 mg mL^{-1} enzyme) to produce the target analog [desNH₂Tyr[1],D-Ala[2],Ala[15]]-GRF(1–29)-NH_2 (4): (a) time course with respect to the amino component 2 (i.e. mole % = $[n]/[2]_0$); (b) HPLC of the reaction mixture at the 203-min mark. See Experimental Section for HPLC conditions. Numerical labels correspond to those in the synthetic scheme (eq 3) and desNH₂-Tyr-D-Ala-Asp-OH (3c), desNH₂Tyr-D-Ala-Asp-NHC(CH_2OH)$_3$ (6), desNH₂Tyr-D-Ala-Asp-GRF(26–29)-NH_2 (7), [Ala[15]]-GRF(4–25)-OH (8), [desNH₂Tyr[1],D-Ala[2],Ala[15]]-GRF(1–25)-OH (9), [desNH₂Tyr[1],D-Ala[2],Ala[15]]-GRF(1–25)-[Ala[15]]-GRF-(4–29)-NH_2 (10).

confirming an amide linkage). Although this side reaction depletes the acyl component 3a, it is a relatively minor loss if the concentration of Tris is kept to a minimum, particularly in comparison to the competing deacylation by water to form the tripeptide acid desNH₂Tyr-D-Ala-

Asp(OH)-OH (**3c**). A certain degree of hydrolysis of the acyl component in this type of enzymatic coupling is unavoidable.

We find that Tris does not adversely effect the solubility of 2 as do phosphate buffers, for instance, which tend to "salt-out" the peptides 2 and 4. It is very important to employ as high an initial concentration of the amino component as possible so that it competes effectively with water as a nucleophile. Solubility can be especially troublesome with larger polypeptide substrates such as 2 and is a major obstacle to applying this technique to polypeptide substrates. For comparison, concentrations of 100–500 mM are typically employed for small amino acid and oligopeptide nucleophiles.

Ideally, one could employ a pH stat to maintain the desired pH with small increments of NaOH.[5] However, implementing such a device on a small scale is difficult compared to the use of buffers. We should also note that simple monovalent anions such as Cl^-, NO_3^-, and CH_3COO^- actually act as competitive binding inhibitors of the V8 protease[18,24] and their use in buffers for reactions catalyzed by this enzyme should be avoided. To eliminate the use of salts which could reduce the solubility of the peptides and/or inhibit the enzyme, we have taken the approach of using Tris base and the weakly acidic substrates 2 and **3a** themselves as the conjugate acids to buffer the reaction mixture. It may be noted that 2 was used as the hexakistrifluoroacetate salt, and these trifluoroacetate counter ions (43 mM) may exert a significant inhibitory effect on the V8 protease in this reaction mixture.

We noted that the rate of coupling at Asp^3 (formation of 4) decreases during the course of the reaction, as the concentration of the nucleophile 2 is depleted, whereas the competing side reactions at Asp^{25} (formation of side products 7–10) proceed at a fairly constant rate (Figure 2a). The maximal percent conversion of 2 to 4 is reached when the these two rates are equal. Thus, despite the fact that 30% of the initial 2 and most of the initial **3a** remain at the 200-min mark, the rate of coupling vs proteolysis has decreased to the point that the concentration of 4 begins to slowly decrease. Since the rate of coupling increases with the initial concentration of nucleophile, $[2]_0$, in a pseudo-first-order fashion, whereas the zeroth-order proteolysis at Asp^{25}-Ile^{26} does not increase with increasing $[2]_0$, the yield of 4 can be enhanced by simply employing a higher $[2]_0$. Unfortunately, the solubility of 2 is limited to about 10 mM under these conditions. Although the solubility of 2 is much higher at lower pH, this variable cannot be altered since the pH must be kept reasonably near the narrow pH optimum of the enzyme and the pK_a of the α-NH_3^+ of 2, in order for 2 to be in the reactive deprotonated form. Proteolysis at Asp^{25}-Ile^{26} was lessened somewhat by using a large molar excess of the acyl component **3a** to competitively inhibit binding of the enzyme to Asp^{25} and by using higher amounts of DMSO cosolvent to supress hydrolysis. However, these measures necessitate an excessive use of **3a** and conditions that degrade the catalytic efficiency of the protease.

While far from ideal, the system in Figure 2a was sufficient to prepare and characterize the target peptide [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]-GRF(1–29)-NH$_2$ (4) (50% isolated yield of 4). Purified semisynthetic 4 was found to be identical to a purified SPPS standard of 4 by the criteria of analytical HPLC, CZE, and tryptic fingerprinting (Figure 3). The semisynthetic analog also had the predicted *m/z* by FAB-MS and full potency in an in vitro rat pituitary cell bioassay (see Experimental Section).

V8 Protease-Catalyzed Condensation of [Ala^{15}]-GRF(4–29)-NH$_2$ (2) and desNH$_2$Tyr-D-Ala-Asp(OH)-ONb (3b) To Form [desNH$_2$Tyr^1,D-Ala^2,Ala^{15}]-GRF(1–29)-NH$_2$ (4). We next turned our attention to improving this enzymatic coupling with respect to the following criteria: (1) maximize the percent conversion of 2 to 4; (2) minimize the amount of V8 protease required to effect this conversion in a reasonable amount of time (ca. 1–24 h), i.e. use a "catalytic" amount of enzyme; and (3) minimize consumption of tripeptide acyl component 3.

Schellenberger and Jakubke have summarized many of the general principles that are important in optimizing kinetically-controlled couplings and have also developed several quantitative methods of studying the fundamental kinetics of these systems.[5] The V8 protease-catalyzed coupling of 2 and **3a** suffers mainly from the yield-limiting proteolysis at Asp^{25}-Ile^{26}. Unfortunately, nearly all of the experimental studies of kinetically-controlled enzymatic couplings to date have been on the synthesis of dipeptide and oligopeptide model compounds with only a single enzyme binding site. The only competing proteolysis in the synthesis of a dipeptide, for instance, is the relatively minor "back-hydrolysis" of the newly-formed peptide bond.

This problem of the kinetically-controlled enzymatic synthesis of a peptide containing more than one site of proteolysis has been addressed recently, for the first time, by Schellenberger and co-workers.[25] They designed an α-chymotrypsin catalyzed coupling of Mal-Leu-OR (R = Me, Bzl, Nb) to Phe-pNA and found that the relative rates of coupling, to form Mal-Leu-Phe-pNA, vs the competing hydrolysis at the highly-sensitive Phe–pNA anilide bond were dramatically influenced by the nature of the ester leaving group, R. These workers found that the 4-nitrobenzyl ester was a superior substrate for α-chymotrypsin compared to the methyl ester (ca. 300-fold higher k_{cat}/K_m). Thus Mal-Leu-ONb permitted a much higher extent of peptide synthesis than Mal-Leu-OMe in the presence of the competing proteolysis at the Phe–pNA bond.

We prepared desNH$_2$Tyr-D-Ala-Asp(OH)-ONb (**3b**) and studied the segment condensation with 2 using V8 protease catalysis. In contrast to the coupling of 2 and the ethyl ester **3a** (Figure 2), coupling of 2 and the 4-nitrobenzyl ester **3b** (Figure 4) occurs so much more rapidly than proteolysis at Asp^{25}–Ile^{26} that no side products were detected (Figure 4b). DMF was used as the cosolvent in place of DMSO since the solubility of **3b** was poor in the DMSO system used for **3a**. Use of **3b** in place of **3a** improves the coupling on the basis of all three criteria: (1) the higher rate of synthesis versus proteolyis gave a superior yield (90% conversion of 2 to 4 using **3b** versus 60% using **3a**); (2) the required amount of enzyme was reduced (200:1 w:w enzyme:2 using **3b** versus 20:1 using **3a**; and (3) consumption of acyl component was minimized (3.3:1 molar ratio of **3b**:2 versus 6:1 **3a**:2).

The 4-nitrobenzyl ester **3b** also provides an advantage over the ethyl ester **3a** in that the UV absorption of HONb

(24) Houmard, J. Kinetic Investigation of the Staphylococcal Protease-Catalyzed Hydrolysis of Synthetic Substrates. *Eur. J. Biochem.* **1976**, *68*, 621–627.

(25) Schellenberger, V.; Görner, A.; Könnecke, A.; Jakubke, H.-D. Protease-Catalyzed Peptide Synthesis: Prevention of Side Reactions in Kinetically Controlled Reactions. *Peptide Res.* **1991**, *4*, 265–269.

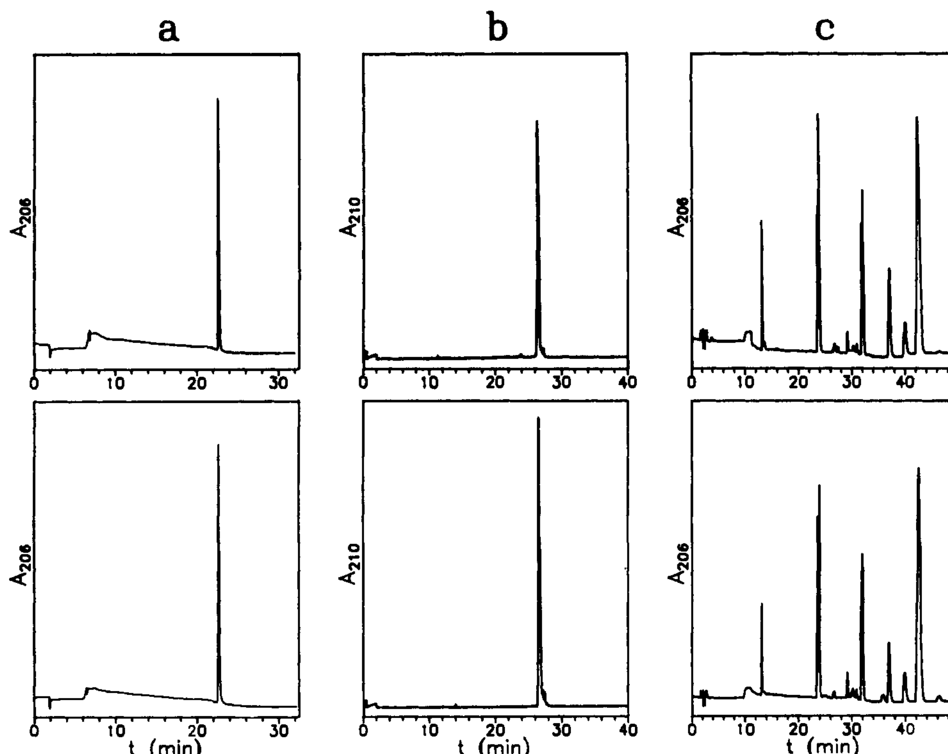

Figure 3. Comparison of semisynthetic [desNH$_2$Tyr[1],D-Ala[2],Ala[15]]-GRF(1-29)-NH_2 (4) (upper panels) to a SPPS standard (lower panels) by (a) analytical HPLC, (b) CZE, and (c) HPLC tryptic mapping. HPLC was performed on a Waters μBondapak C_{18} column (0.4 × 30 cm): eluants, (A) 0.025% TFA and (B) 0.025% TFA/CH_3CN; flow rate, 1.5 mL min^{-1}; gradients, (a) 0 to 43% B in 20 min and (c) 0 to 28% B in 30 min. See Experimental Section for CZE conditions and tryptic digest conditions.

alcohol (11) provides a convenient way of monitoring the molar quantities of the reactants and products by HPLC (see Experimental Section for details).

A simple extrapolation of the data in Figure 4a suggests that if 2 can be converted to 4 in 90% yield in 30 min using a substrate to enzyme ratio (S:E) of 200:1 then one should, for instance, be able to obtain the same result in 150 min at 1000:1 S:E. Although this type of extrapolation is correct in principle, it is necessary to confirm it experimentally since enzymes are sometimes inactivated to a significant extent, either reversibly or irreversibly, when used at low concentrations. Indeed, we have thus far been unable to increase the S:E for this coupling beyond 200:1. For example, when couplings that were otherwise identical to the coupling in Figure 4 were initiated at 500:1 and 1000:1 S:E product was generated at the predicted rates but synthesis ceased rather abruptly at about 45% and 30% conversion of 2 to 4. There are numerous possible reasons for the inactivation of the catalyst in these reaction mixtures at lower enzyme concentrations, including a slow exponential denaturation of the enzyme, product inhibition, or possibly some type of turn-over-dependent loss of activity.

Conclusions

Both steps of the semisynthesis in eq 3 have been accomplished on the milligram scale with good yields and the final product 4 was shown to be chemically and biologically equivalent to the chemically synthesized peptide. The first step, C-terminal amidation of the precursor 1 via CPD-Y-catalyzed transpeptidation,[16] is a relatively good process in terms of yield and efficiency of the enzyme. The main shortcoming of this step at present is the low solubility of the precursor in the 1 M H-Arg-NH_2, which requires the use of large amounts of H-Arg-NH_2. The second stage, introduction of desNH$_2$Tyr-D-Ala at the N-terminus via the V8 protease-catalyzed condensation of 2 and **3b**, was also achieved in high yield. Although this step could be further improved by decreasing the amount of enzyme required, the catalytic efficiency achieved for the Asp-bearing substrate **3b** is remarkably good considering that the primary specificity of the V8 protease is for Glu-bearing substrates.

We have also made a number of observations about the problems encountered in protease-catalyzed semisynthesis with intermediate-length peptide substrates as opposed to the more widely studied amino acid and model peptide substrates. The low solubility of the peptides in the reaction medium and competing proteolyses at sites distant from the site of coupling are the main problems that require further study.

A major finding in this study is that the 4-nitrobenzyl ester, desNH$_2$Tyr-D-Ala-Asp(OH)-ONb proved to be a very advantageous substrate for the V8 protease in this semisynthesis. It suggests that the approach of Schellenberger and co-workers,[25] i.e. finding and employing an acyl ester of very high specificity (k_{cat}/K_m), may have broad implications for the technique of protease-catalyzed kinetically-controlled peptide synthesis in general. The present semisynthesis illustrates this principle. The V8 protease was originally chosen for this semisynthesis because its stringent primary specificity for Glu(Asp) would permit a reasonably successful segment condensation since only a single internal peptide bond at Asp[25]–Ile[26] in the GRF peptide substrate might be affected. However, this potential drawback was found to be unfounded from the results obtained in this semisynthesis using the 4-nitrobenzyl ester **3b**. If the specificity of the enzyme for the acyl donor can be raised several orders of magnitude higher than its specificity for any of the internal

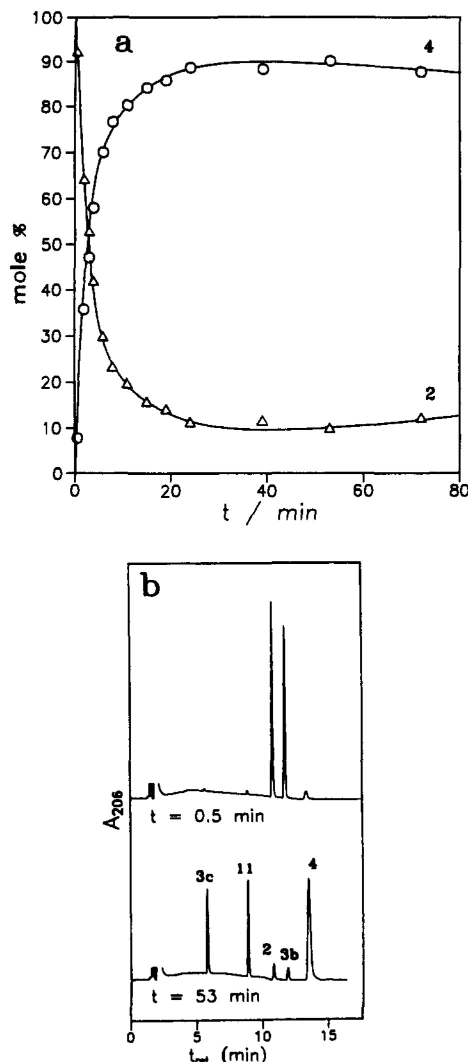

Figure 4. V8 protease-catalyzed condensation of [Ala[15]]-GRF-(4–29)-NH_2 (**2**) (16.0 mg mL^{-1}, 4.32 mM) and desNH$_2$Tyr-D-Ala-Asp(OH)-ONb (**3b**) (7.03 mg mL^{-1}, 14.4 mM) in 15% DMF (25 mM Na_3PO_4/H_3PO_4, pH 8.1, 37 °C, 0.08 mg mL^{-1} enzyme) to produce [desNH$_2$Tyr[1],D-Ala[2],Ala[15]]-GRF(1–29)-NH_2 (**4**): (a) time course showing the fate of the amino component **2** (i.e. mole % = $[n]/[2]_0$); (b) HPLC of the reaction mixture at the indicated times. The numerical labels correspond to those in the synthetic scheme (eq 3) and desNH$_2$Tyr-D-Ala-Asp-OH (**3c**), HONb (**11**). See Figure 3 for HPLC column and conditions (gradient, 10 to 40% B in 10 min).

peptide cleavage sites by use of an appropriate ester leaving group, coupling can proceed cleanly and in high yield, with high catalytic efficiency, even if the substrate contains multiple competing proteolytic cleavage sites. These studies demonstrate that the use of the V8 protease with a peptide fragment possessing a C-terminal 4-nitrobenzyl ester offers important opportunities for coupling large peptide segments.

We are currently conducting experiments to determine the catalytic constants (k_{cat} and K_m) of the V8 protease for a series of desNH$_2$Tyr-D-Ala-Asp(OH)-OR esters in order to arrive at a better understanding of how the ester leaving group R influences the kinetics of this segment condensation.

Experimental Section

Protected amino acids were purchased from Bachem Inc. (Torrance, CA) and other reagents and solvents were of the highest available commercial purity. All pH values were measured with a glass electrode. Mass spectra were recorded in the fast atom bombardment (FAB) mode. Amino acid analyses (AAA) were performed on a Beckman Model 121M Amino Acid Analyzer. Peptides were hydrolyzed for AAA in 6 N HCl, 1% thioglycolic acid, for 24 h at 110 °C. Tryptic digests containing 1.0 mg mL^{-1} peptide and 0.1 mg mL^{-1} bovine trypsin (TPCK-treated, 209 U mg^{-1}, Millipore Corp., Freehold, NJ) in 0.5 M NH_4HCO_3 (pH 8.0) were allowed to stand at room temperature for 20 h, acidified with dilute TFA and then applied to the HPLC column.

Analytical HPLC was carried out on a Laboratory Data Control Constametric IIG equipped with a Gradient Master and Spectromonitor III UV variable wavelength detector. Preparative HPLC was carried out on a Waters Delta Prep 3000 with a Waters 484 tunable absorbance detector. Capillary zone electrophoresis (CZE) was performed on a Spectra PHORESIS 1000 instrument (Spectra Physics, San Jose, CA) equipped with a fast-scanning variable-wavelength UV–vis detector. A fused-silica capillary with polyimide outer coating (95 cm × 50 μm i.d.) was used. Sample injection was by electromigration at 15 kV for 2 s and separation was performed at a constant 25 kV (263 V cm^{-1}), 25 °C, in 0.12 M Na_2HPO_4/H_3PO_4 buffer, pH 2.5.

The peptides, [Ala[15,29]]-GRF(4–29)-OH (**1**), [Ala[15]]-GRF(4–29)-NH_2 (**2**), and [desNH$_2$Tyr[1],D-Ala[2],Ala[15]]-GRF(1–29)-NH_2 (**4**) were prepared by solid-phase peptide synthesis (SPPS) as previously described.[11] These peptides were characterized by analytical HPLC, amino acid analysis, and FAB-MS.

V8 protease (EC 3.4.21.19) was obtained from Sigma Chemical Co. (protease from the V8 strain of *Staphylococcus aureus*, type XVII-B) and had a specific activity of 770 U mg^{-1} (1 U = amount of enzyme that hydrolyzes 1 μmol min^{-1} Boc-Glu-αOPh at pH 7.8, 37 °C). Carboxypeptidase Y (CPD-Y, EC 3.4.16.1) was a generous gift from Carlbiotech Limited A/S, Copenhagen.

In vitro bioassays[14,26] measured growth hormone release in cultured rat anterior pituitary cells. Potencies are relative to GRF(1–44)-NH_2 (relative potency = 1.00).

desNH$_2$Tyr-D-Ala-Asp(OH)-OEt (3a). H-Asp(OH)-OEt was prepared via ethanolysis of the cyclic anhydride of Asp as described.[27] A mixture of H-Asp(OH)-OEt (1.00 g, 6.2 mmol) in 10 mL of water and Boc-D-Ala-OSu (1.95 g, 6.8 mmol) in 10 mL of ethylene glycol dimethyl ether was stirred at 25 °C for 1.5 h and at 4 °C overnight. The reaction mixture was acidified to pH 3.5 with solid citric acid and evaporated to dryness and the residue taken up in water (100 mL) and lyophilized. The resulting Boc-D-Ala-Asp(OH)-OEt was treated with 50% TFA/CH_2Cl_2 (60 mL) for 20 min at 25 °C, evaporated to dryness, and twice taken up in CH_2Cl_2 (60 mL) and evaporated. The residue was dissolved in water (50 mL), solid $NaHCO_3$ added to pH 7.4, followed by desNH$_2$Tyr-OSu (1.95 g, 7.4 mmol) in ethylene glycol dimethyl ether (100 mL), and the mixture was stirred at 4 °C overnight.

The reaction mixture was evaporated, dissolved in 10% CH_3CN, acidified with TFA to pH 2.5, and filtered (0.45 μm). The crude product was purified by HPLC [YMC Phenyl column (2.2 × 30 cm)]: eluants, (A) 0.025% TFA and (B) 0.025% TFA/CH_3CN; gradient, 0 to 35% B in 90 min; flow rate, 15 mL min^{-1}; detection, 210 nm. Product containing fractions were pooled and lyophilized to give 1.25 g (53%) of **3a**. AAA: Asp 1.00 (1), Ala 0.99 (1). FAB-MS: $(M + H)^+$ calcd 381.4, found 381.3. ^1H NMR (DMSO-d_6): δ 1.15 (m, 6 H), 2.34 (t, J = 7.9 Hz, 2 H), 2.65 (t, J = 7.9 Hz, 2 H), 4.04 (qd, J = 7.1 Hz, 2 H), 4.29 (q, J = 7.4 Hz, 1 H), 4.47 (qd, J = 6.5 Hz, 1 H), 6.64 (d, J = 8.4 Hz, 2 H), 6.97 (d, J = 8.4 Hz, 2 H), 8.05 (d, J = 7.6 Hz, 1 H), 8.34 (d, J = 7.6 Hz, 1 H).

desNH$_2$Tyr-D-Ala-Asp(OH)-ONb (3b). Z-Asp(OBu^t)-OH·H_2O (21.3 g, 62 mmol), 1-hydroxybenzotriazole–water (10.5 g, 78 mmol), and 1,3-dicyclohexylcarbodiimide (13.0 g, 63 mmol) were dissolved in 75:25 (v:v) CH_2Cl_2/DMF and the mixture stirred at 25 °C for 2 h. After the urea was removed by filtration, 4-nitrobenzyl alcohol (19.1 g, 125 mmol) and 4-(dimethylamino)-

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(27) Kovacs, J.; Kovacs, H. N.; Ballina, R. Glutamic and Aspartic Anhydrides. Rearrangement of N-Carboxyglutamic 1,5-Anhydride to the Leuchs' Anhydride and Conversion of the Latter to Pryoglutamic Acid. *J. Am. Chem. Soc.* **1963**, *85*, 1839–1844.

pyridine (0.77 g, 6.3 mmol) were added and the reaction mixture stirred at 25 °C for 3 h. After adding N,N-diisopropylethylamine (10.9 mL, 63 mmol), the reaction mixture was evaporated to a thick syrup. The crude Z-Asp(OBu^t)-ONb was deprotected in 33% HBr/CH_3COOH and the hydrobromide salt neutralized as described[28] to yield 8.4 g (50%) of crude H-Asp(OH)-ONb. The crude H-Asp(OH)-ONb (1.0 g) was coupled to Boc-D-Ala-OSu followed by desNH_2Tyr-OSu as described above for the preparation of desNH_2Tyr-D-Ala-Asp(OH)-OEt.

The resulting **3b** was purified by HPLC [YMC ODS Basic column (4.7 × 30 cm)]: eluants, (A) 0.1% TFA and (B) 0.1% TFA/CH_3CN; gradient, 5 to 45% B in 60 min; flow rate, 50 mL min^{-1}; detection, 210 nm. Product-containing fractions were pooled and lyophilized to give 0.37 g (19%) of **3b**. FAB-MS: $(M + H)^+$ calcd 488.5, found 488.1. ^1H NMR (DMSO-d_6): δ 1.14 (d, J = 6.8 Hz, 3 H), 2.34 (m, 2 H), 2.67 (m, 3 H), 2.80 (m, 1 H), 4.33 (q, J = 6.8 Hz, 2 H), 4.70 (qd, J = 6.4 Hz, 2 H), 5.28 (s, 2 H), 6.64 (d, J = 8.0 Hz, 2 H), 6.96 (d, J = 8.0 Hz, 2 H), 7.63 (d, J = 8.0 Hz, 2 H), 7.93 (d, J = 7.6 Hz, 1 H), 8.23 (d, J = 8.0 Hz, 2 H), 8.43 (d, J = 7.6 Hz, 1 H).

CPD-Y-Catalyzed Semisynthesis of [Ala^{15}]-GRF(4-29)-NH_2 (2). The method of Breddam and co-workers[16] was used as follows: A solution of 5.93 M H-Arg-NH_2·2HCl and 1.6 mM EDTA (600 μL) was slowly added to a solution of 1·5TFA (50.0 mg, 14.3 μmol) in 2.00 mL of water at 45 °C. After equilibrating to 37 °C, the above mixture was titrated to pH 8.0 with 3 M NaOH (475 μL) and then diluted to 3.78 mL. The reaction was started by adding 24.4 μL of aqueous CPD-Y (20.5 mg mL^{-1}) and maintained at 37 °C. The progress of the reaction was monitored by quenching 1-μL aliquots in 200-μL portions of 0.1% TFA and analyzing by HPLC [Vydac 218TP1022 C_{18} column (0.46 × 25 cm)]: eluants, (A) 0.025% TFA and (B) 0.025% TFA/CH_3CN; gradient, 38 to 44% B in 10 min; flow rate, 1.5 mL min^{-1}; detection, 206 nm.

The reaction was stopped at the 70-min mark by adding glacial acetic acid (1.0 mL) and purified by HPLC [YMC ODS-Basic (4.7×30 cm)]: eluants, (A) 0.1% TFA and (B) 0.1% TFA/CH_3CN; gradient, 20 to 50% B in 90 min; flow rate, 50 mL min^{-1}; detection, 210 nm. Product-containing fractions were pooled and lyophilized to give 33.0 mg (62%) of 2·6TFA. AAA: Asx 2.0 (2), Thr 1.0 (1), Ser 2.7 (3), Glx 2.0 (2), Ala 2.9 (3), Val 0.8 (1), Met 1.0 (1), Ile 1.8 (2), Leu 4.1 (4), Tyr 1.0 (1), Phe 1.0 (1), Lys 2.2 (2), Arg 3.4 (3). FAB-MS: $(M + H)^+$ calcd 3023.6, found 3023.8.

pH Dependence of the CPD-Y-Catalyzed Transpeptidation. H-Arg-NH_2·2HCl (0.374 g, 1.53 mmol) was dissolved in 0.42 mL of 0.7 M NaOH, 1 mM EDTA. Five 80-μL portions of the above H-Arg-NH_2 stock solution were titrated to the desired pH with 3 M NaOH and then diluted to 153 μL each. The reaction mixtures were prepared by mixing 15-μL portions of each of the five 1.8 M H-Arg-NH_2 solutions with 10 μL of 40 mg mL^{-1} 1·5TFA and 5 μL of 0.16 mg mL^{-1} CPD-Y at 37 °C. The pH values were remeasured (pH 6.73, 7.02, 7.72, 8.20) and the progress of each reaction mixture at 37 °C was monitored by HPLC as described above.

V8 Protease-Catalyzed Segment Condensation of 3a and 2 To Form [desNH_2Tyr^1,D-Ala^2,Ala^{15}]-GRF(1-29)-NH_2 (4). The ester **3a** (8.1 mg, 21.3 μmol) was suspended in 100 μL of water and dissolved by titrating at pH 8.5 with 1 M Tris base (70 μL), and the solution was diluted to a final volume of 350 μL. The above solution of **3a** (252 μL, 15.3 μmol) was added to a solution of 2·6TFA (9.5 mg, 2.6 μmol) in 72 μL of DMSO. After the above mixture was equilibrated to 37 °C, the reaction was initiated by adding 36 μL of aqueous V8 protease (5.30 mg mL^{-1}, 147 U) and maintained at 37 °C. The progress of the reaction was monitored by quenching 1-μL aliquots in 200-μL portions of 0.1% TFA and analyzing by HPLC [Waters μBondapak C_{18} column (0.4 × 30 cm)]: eluants, (A) 0.025% TFA and (B) 0.025% TFA/CH_3CN; gradient, 10 to 42% B in 15 min; flow rate, 1.5 mL min^{-1}; detection, 206 nm.

The reaction was stopped at the 3.3-h mark by adding glacial acetic acid (250 μL) and purified by HPLC [Vydac 218TP1022 column (2.2 × 25 cm)]: eluants, (A) 0.1% TFA and (B) 0.1% TFA/CH_3CN; gradient, 20 to 40% B in 60 min; flow rate, 25 mL min^{-1}; detection, 210 nm. Product-containing fractions were pooled and lyophilized to give 5.0 mg (49%) of 4·5TFA. AAA: Asx 3.4 (3), Thr 1.0 (1), Ser 2.7 (3), Glx 2.2 (2), Ala 4.0 (4), Val 0.9 (1), Met 0.9 (1), Ile 1.9 (2), Leu 4.0 (4), Tyr 1.1 (1), Phe 1.1 (1), Lys 1.9 (2), Arg 3.3 (3). FAB-MS: $(M + H)^+$ calcd 3357.9, found 3358.1. Relative potency: semisynthetic 4.33 ± 0.35, SPPS standard 4.70 ± 0.45.

V8 Protease-Catalyzed Segment Condensation of 3b and 2 To Form 4. The ester **3b** (2.23 mg, 4.57 μmol) was dissolved in 23.8 μL DMF, followed by 7.5 μL of 0.5 M Na_3PO_4 and 121 μL of water. The segment **2** (4.00 mg, 1.08 μmol) was dissolved in 18.8 μL of DMF, followed by 120 μL of the above **3b** solution, titrated to pH 8.0 with 6.5 μL of Na_3PO_4, and diluted to 240 μL with water. The reaction was initiated by equilibrating the above **2**–**3b** mixture at 37 °C and adding 10 μL of 2.0 mg mL^{-1} V8 protease and monitored by HPLC as described above (gradient, 10–40% B in 10 min).

A second reaction mixture containing no enzyme, but otherwise identical to the above reaction mixture, was prepared and kept at 37 °C for 12 h. Only a slow hydrolysis of **3b** to give equimolar **3c** and **11** was observed ($t_{1/2}$ = 55 h). Thus, in the absence of enzyme, **3b** did not undergo any detectable aminolysis by the various amino groups in **2**.

Calculations. For the CPD-Y-catalyzed transpeptidations, molar quantities of **1**, **2**, and **5** (Figure 1) were taken as proportional to the areas under the respective HPLC peaks (A_n) monitored at 206 nm divided by the number of peptide bonds (i.e. the absorbance at 206 nm is taken as proportional to the number of peptide bonds): $[1] = [1]_0A_1/[A_1 + A_2 + (25/24)A_5]$; $[2] = [1]_0A_1/[A_1 + A_2 + (25/24)A_5]$; $[5] = [1]_0 - [1] - [2]$. Molar quantities for the peptides arising from the amino component **2** in the V8 protease-catalyzed couplings of **2** to **3a** (Figure 2) and **2** to **3b** (Figure 4a) were estimated in a similar fashion: $[4] = [2]_0A_4/[A_4 + (28/25)A_2 + A_{\text{side products}}]$, etc.

The UV absorption of HONb at 206 nm provides a means of determining the concentrations of all species in the case of the segment condensation of **3b** and **2** (Figure 4b) as follows: Upon complete hydrolysis of a sample of **3b**, the HPLC peak corresponding to **3b** disappears and is replaced by peaks corresponding to **3c** and **11** with a ratio of areas of 1.10 (A_{3c}/A_{11} = 1.10) and a combined area ($A_{3c} + A_{11}$) approximately equal to the area of original 3b peak. Thus, for example: $[4] = [3b]_0(1.10A_{11} - A_{3c})/[1.10A_{11} + (1.10/2.10)A_{3b}]$; $[3c] = [3b]_0A_{3c}/[1.10A_{11} + (1.10/2.10)\cdot A_{3b}]$; $[3b] = [3b]_0 - [4] - [3c]$; $[2] = [2]_0 - [4]$.

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