Enzymatic Oligopeptide Synthesis Using a Minimal Protection Strategy: Sequential Assembly of a Growing Oligopeptide Chain

Iqbal Gill, Rosina López-Fandiño, and Evgeny Vulfson*

Contribution from the Department of Biotechnology and Enzymology, BBSRC Institute of Food Research, Earley Gate, Whiteknights Road, Reading, Berkshire RG6 2EF, U.K.

Received August 11, 1994[⊗]

Abstract: An approach to the enzymatic synthesis of oligopeptides is described which relies upon the selectivities of the enzymes employed rather than on the differential protection of the substrates for obtaining the desired sequences. Serine and cysteine proteases were shown to posess the selectivity required to differentiate among ester substrates of similar reactivity, thereby allowing the N to C assembly of an oligopeptide by the stepwise addition of amino acid esters without the requirement for intermediate deprotection steps. The applicability of this approach to the preparation of multifunctional peptides was demonstrated by the 10-step synthesis of "delicious octapeptide" amide with an overall yield of 39%.

Enzyme-catalyzed oligopeptide synthesis, with its well-documented advantages,[1] has been extensively explored as an approach to the production of biologically active peptides.[1gi,2] However, despite considerable advances made through the application of proteases to the preparative synthesis of oligopeptides,[3] the enzymatic approach typically suffers from the same drawbacks as conventional solution phase synthesis, i.e. the necessity for intermediate *N*-deprotection or *C*-deprotection/activation of the terminal amino acid prior to elongation of the growing oligopeptide. It would be attractive to develop a methodology which would dispense with intermediate deprotection steps and allow the assembly of an oligopeptide by the stepwise addition of simple substrates, such as amino acid esters.[4] This approach would rely upon the selectivities of the esterases employed rather than on the differential protection of the substrates for obtaining the desired sequences.

In order to establish the feasibility of this methodology, one should first demonstrate that esterases are capable of effectively utilizing amino acid esters as nucleophiles in the presence of other acyl donors of similar reactivity.[5] It should be stressed that the use of reactive nucleophiles at each step is a prerequisite for the direct enzymatic coupling of the subsequent amino acid residue to the oligopeptide chain. Thus, neither amino acid amides (which are routinely used as nucleophiles in enzymatic peptide synthesis) nor *tert*-butyl esters can provide the desired continuity in the synthetic sequence.[6] Similarly, the application of the non-esterolytic protease thermolysin, although attractive for the preparation of dipeptide esters,[1g-m,2a] would necessitate an additional *C*-deprotection step prior to using this enzyme for any subsequent elongation of the peptide product. Therefore, in order to avoid incurring any additional steps in the synthesis due to intermediate deprotection, one has to rely on the use of esterases for performing the repetitive couplings. The enzymes used should also display high regioselectivities so as to facilitate the assembly of peptides containing multifunctional amino acids. This paper demonstrates that serine and cysteine proteases discriminate well among amino acid esters of similar reactivity and do indeed possess the specificity sought for the sequential assembly of oligopeptides from simple precursors with no requirement for regioselective protection.

Results and Discussion

To fully illustrate the potential of this approach, a highly functionalized target oligopeptide was required. "Delicious

[⊗] Abstract published in *Advance ACS Abstracts,* May 15, 1995.

(1) (a) Widmer, F.; Breddam, K.; Johansen, J. T. *Carlsberg Res. Commun.* **1980**, *45*, 453. (b) Fruton, J. S. *Adv. Enzymol.* **1982**, *53*, 239. (c) Chaiken, I. M.; Komoriya, A.; Ohno, M.; Widmer, F. *Appl. Biochem. Biotechnol.* **1982**, *7*, 385. (d) Jakubke, H.-D.; Kuhl, P.; Konnecke, A. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 85. (e) West, J. B.; Wong, C.-H. *J. Chem. Soc., Chem. Commun.* **1986**, 117. (f) Margolin, A. L.; Klibanov, A. M. *J. Am. Chem. Soc.* **1987**, *109*, 3802. (g) West, J. B.; Wong, C.-H. *Tetrahedron Lett.* **1987**, *28*, 1629. (h) Morihara, K. *Trends Biotechnol.* **1987**, *5*, 164. (i) Wong, C.-H.; Matos, J. R.; West, J. B.; Barbas, C. F. *Dev. Ind. Microbiol.* **1988**, *29*, 171. (j) Hill, T. G.; Wang, P.; Huston, M. E.; Wartchow, C. A.; Oehler, I. M.; Smith, M. B.; Bednarski, M. D.; Callstrom, M. R. *Tetrahedron Lett.* **1991**, *32*, 6823. (k) Wang, P.; Hill, T. G.; Wartchow, C. A.; Huston, M. E.; Oehler, L. M.; Smith, M. B.; Bednarski, M. D.; Callstrom, M. R. *J. Am. Chem. Soc.* **1992**, *114*, 378. (l) Kullmann, W. *Enzymatic Peptide Synthesis;* CRC Press: Boca Raton, FL, 1987. (m) Nakanishi, K.; Kimura, Y.; Matsuno, R. *Synthetic Peptides in Biotechnology;* Alan R. Liss Inc.: New York, 1988; p 173.

(2) (a) Bongers, J.; Heimer, E. P. *Peptides* **1994**, *15*, 183. (b) Bielka, H., Scheler, W., Eds. Proceedings of the First International Workshop on Enzymes in Peptide Synthesis. *Biomed. Acta* **1991**, *50*, S1-S270.

(3) (a) Gill, I. S.; Vulfson, E. N. *J. Chem. Soc., Perkin Trans. 1* **1992**, *6*, 667. (b) Richards, A. O.; Gill, I. S.; Vulfson, E. N. *Enzyme Microb. Technol.* **1993**, *15*, 928. (c) Gill, I.; Vulfson, E. N. *J. Am. Chem. Soc.* **1993**, *115*, 3348. (d) Kitaguchi, H.; Klibanov, A. M. *J. Am. Chem. Soc.* **1989**, *111*, 9272. (e) Barbas, C. F.; Wong, C.-H. *J. Chem. Soc., Chem. Commun.* **1987**, 533. (f) Ricca, J. M.; Crout, D. H. J. *J. Chem. Soc., Perkin Trans. 1* **1989**, *3*, 2126. (g) Gaertner, H.; Watanabe, T.; Sinisterra, J. V.; Puigserver, A. *J. Org. Chem.* **1991**, *56*, 3149. (h) Oyama, K. S.; Nishimura, S.; Nonaka, Y.; Kihara, K.; Hashimoto, T. *J. Org. Chem.* **1981**, *46*, 5242. (i) Clapés, P.; Adlercreutz, P.; Mattiasson, B. *J. Biotechnol.* **1990**, *15*, 323. (j) Chen, S.-T.; Chen, S.-Y.; Wang, K.-T. *J. Org. Chem.* **1992**, *57*, 6960. (k) Clapés, P.; Adlercreutz, P.; Mattiasson, B. *Biotechnol. App. Biochem.* **1990**, *12*, 376. (l) West, J. B.; Wong, C.-H. *J. Org. Chem.* **1986**, *51*, 2728.

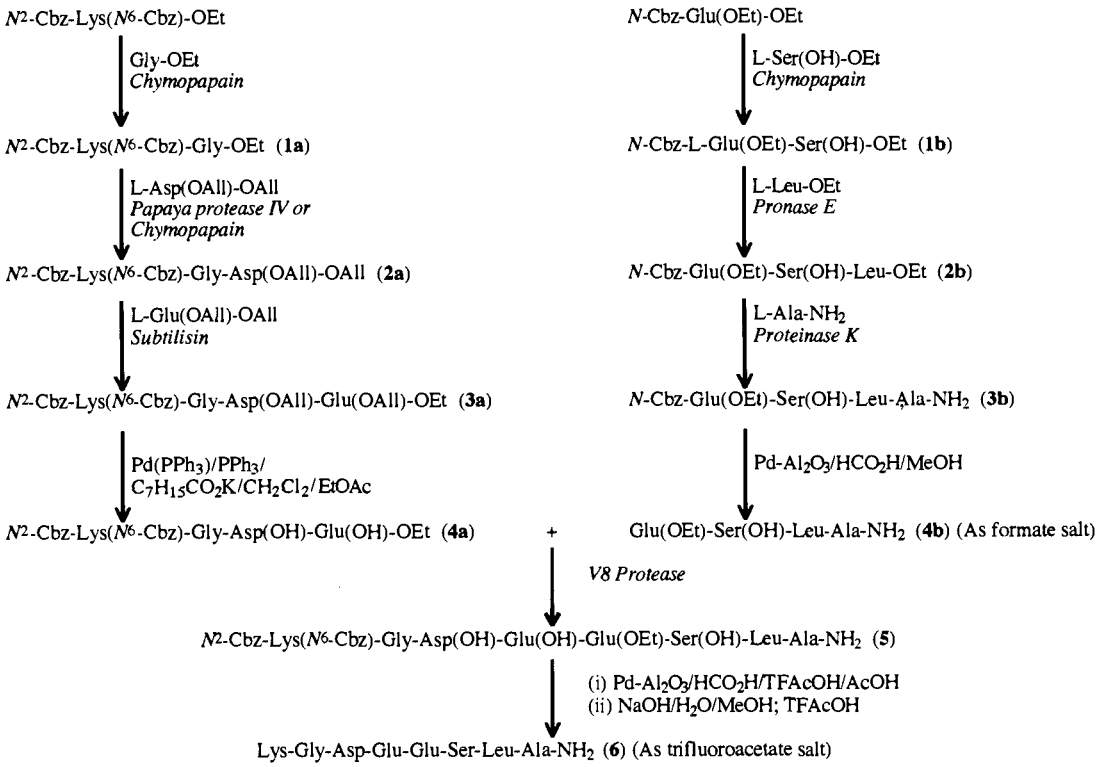
(4) Previous work suggested that good results could be obtained using amino acid esters as acyl acceptors in the protease-catalyzed synthesis of model dipeptides,[1g,2a,3l,m] despite the fact that esters are known to be poorer nucleophiles than the more widely used amides.[1g,20]

(5) A major concern regarding the use of amino acid esters as nucleophiles has been the possible formation of various oligomeric side products.[1g] However, as this paper demonstrates, this problem can be overcome by the selection of appropriate enzymes which bind the corresponding amino acid esters in the nucleophile but not in the acyl donor site with high selectivity.

(6) Although *tert*-butyl esters are good nucleophiles, their low reactivity as acyl donors in protease-catalyzed coupling reactions imposes the usual requirement for *C*-deprotection of the peptide product prior to performing the subsequent coupling and, thus, offers no advantages as compared with conventional approaches.[1g] A similar problem is faced when employing amides as nucleophiles.

0002-7863/95/1517-6175$09.00/0

Scheme 1



octapeptide", Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala,[7,8] was chosen as a suitable model for our studies, in view of the extensive requirement for regioselective protection during its conventional synthesis.[9] Since the utility of proteases for the condensation of peptide fragments had been amply demonstrated by a number of groups,[1c,g,h,2a,3a,e,h] we opted for a simple, convergent 4 + 4 strategy based on the three-step preparation of suitably protected precursors of Lys-Gly-Asp-Glu and Glu-Ser-Leu-Ala, respectively. The subsequent coupling of these fragments would yield the final octapeptide (Scheme 1), thus allowing a facile synthesis with minimal workup. An added benefit of this approach was that it allowed us to investigate the regioselectivity of esterases toward peptides containing esters of aspartic and/or glutamic acid at the N- and C-terminal positions as well as in the middle of the sequence. The *N*-Cbz-protected ethyl esters of L-Lys and L-Glu were chosen as starting materials, and the ethyl and allyl esters of the following amino acids were used for the enzymatic elongation of the oligopeptide chains.

Both of the tetrapeptides **4a** and **4b** were readily synthesized in yields of 50% and 55%, respectively (Scheme 1), using the appropriate serine and cysteine proteases.[2a,10] The dipeptides N^2-Cbz-Lys(N^6-Cbz)-Gly-OEt (**1a**) and *N*-Cbz-Glu(OEt)-Ser-OEt (**2a**) were obtained in yields of 82% and 78%, using Gly-OEt and L-Ser-OEt as nucleophiles, respectively, with free chymopapain as the catalyst (Scheme 1). The tripeptides **2a** and **2b** were then assembled in 84% and 79% yields using free papaya protease IV and immobilized pronase E, respectively.[11] Finally, the tetrapeptides **3a** and **3b**[12,13] were obtained in 76% and 92% yields using immobilized subtilisin and proteinase K.

It should be noted that the condensation of **2a** with L-Glu-(OAll)-OAll was performed in anhydrous ethanol with the aim of achieving the synthesis of the tetrapeptide N^2-Cbz-Lys(N^6-Cbz)-Gly-Asp(OAll)-Glu(OAll)-OAll and the subsequent transesterification of the C-terminal allyl ester function in one step, to yield the corresponding ethyl ester N^2-Cbz-Lys(N^6-Cbz)-Gly-Asp(OAll)-Glu(OAll)-OEt (**3a**). This would then allow the selective deprotection of **3a** to the corresponding dicarboxylic acid ethyl ester N^2-Cbz-Lys(N^6-Cbz)-Gly-Asp(OH)-Glu(OH)-OEt (**4a**), thereby tailoring this substrate to satisfy the specificity

(7) (a) Yamasaki, Y.; Maekawa, K. *Agric. Biol. Chem.* **1978**, *42*, 1761. (b) Tamura, M.; Nakatsuka, T.; Makoto, T.; Kawasaki, Y.; Kikuchi, E.; Okai, H. *Agric. Biol. Chem.* **1989**, *53*, 319.

(8) This peptide was originally isolated from extracts of beef treated with papain and was shown to impart a "beef soup" type flavor.[7a] The relatively complex structure of "delicious peptide", which to our knowledge is one of the most functionalized linear octapeptides reported to date, provided us with a good model to test the general applicability of our approach.

(9) A semiconvergent 14-step solution phase chemical synthesis has been reported in which the octapeptide was obtained with an overall yield of 9.9%.[7b] The poor final yield reflects the extensive use that was made of regioselective protection/deprotection and activation steps in order to avoid undesirable side reactions.

(10) (a) Boyer, P. D., Ed. *The Enzymes*, 3rd ed.; Academic Press: New York, 1971; Vol. III. (b) Perlmann, G. E., Lorand, L., Eds. *Methods in Enzymology. Proteolytic Enzymes;* Academic Press: New York, 1970; Vol. XIX, Part A. (c) Lorand, L., Ed. *Methods in Enzymology. Proteolytic Enzymes;* Academic Press: New York, 1976; Vol. XLV, Part B. (d) Lorand, L., Ed. *Methods in Enzymology. Proteolytic Enzymes;* Academic Press: New York, 1981; Vol. 80, Part C. (e) Kasche, V. In *Proteolytic Enzymes: A Practical Approach;* Benyon, R. J., Bond, J. S., Eds.; IRL Press: Oxford, U.K., 1989; p 125.

(11) Chymopapain also readily catalyzed the further addition of L-Asp-(OAll)-OAll to **1a**, thereby allowing the one-pot synthesis of tripeptide **2a** with an overall yield of 27%. Although **2a** could be obtained in a higher overall yield of 68% using a two-step procedure employing chymopapain/papaya protease IV, the facile one pot reaction provided an attractive, practical alternative, particularly in view of the comparatively high cost of papaya protease IV.

(12) Neither oligomeric products derived from L-Glu(OAll)-OAll nor Glu-extended derivatives of **3a** were detected during the preparation of the tetrapeptide, even though both substrates were used as their allyl esters. The absence of such side products emphasizes the high specificity of subtilisin under the conditions used.

(13) The replacement of L-Ala-NH_2 with L-Ala-OEt allowed the synthesis of the corresponding tetrapeptide ethyl ester *N*-Cbz-Glu(OEt)-Ser(OH)-Leu-Ala-OEt with a yield of 53%, using immobilized proteinase K as catalyst under the same conditions as employed for **3b**. Since Ala occupies the C-terminal position in the target octapeptide sequence (hence no extension of the oligopeptide beyond this residue was required), the synthesis of the tetrapeptide ethyl ester was not optimized further. It should be noted however that the deprotected tetrapeptide ethyl ester Glu(OEt)-Ser(OH)-Leu-Ala-OEt also served as an efficient nucleophile in the V8 protease-catalyzed fragment condensation with **4a**.

Table 1. Protease-Catalyzed Convergent (4 + 4) Synthesis of Delicious Peptide Amide

acyl donor or substrate	acyl acceptor or reagent	product	scale (mmol)	yield (%)[a]	FAB-MS[d] $(M + H)_{obsd}$	FAB-MS $(M + H)_{calcd}$
N_α-Z-L-Lys(N_ε-Z)OEt	GlyOEt	**1a**	23	82	500.2447	500.2396
N_α-Z-L-Lys(N_ε-Z)OEt	GlyOEt; L-Asp(OAll)OAll	**2a**[b]	2.0[b]	27[b]	667.2995	667.2979
1a	L-Asp(OAll)OAll	**2a**	0.26/17[c]	84/52[c]	667.2999	667.2979
2a	L-Glu(OAll)OAll	**3a**	6.3	76	824.3725	824.3718
3a	$Pd(PPh_3)_4/CH_2Cl_2$	**4a**	2.9	96	744.3048	744.3092
N-Z-L-Glu(OEt)OEt	L-Ser(OH)OEt	**1b**	30	78	425.1956	425.1923
1b	L-LeuOEt	**2b**	22	79	538.2790	538.2764
2b	L-$AlaNH_2$	**3b**	8.6	92	580.2975	580.2982
3b	Pd/HCO_2H	**4b**	6.9	97	446.2611	446.2614
4a	**4b**	**5**	1.34	84	1143.5285	1143.5210
5	Pd/HCO_2H; $NaOH/H_2O$	**6**	0.5	93	847.4172	847.4161

requirements of the serine esterase V8 protease,[14] which was used for the fragment condensation. Indeed, the required tetrapeptide **3a** was obtained as the sole product with a yield of 76% without any accompanying transesterification of the two side chain allyl ester groups. This was then readily converted to **4a** using Pd(0)-catalyzed cleavage of the side chain allyl ester functionalities. Transfer hydrogenation of **3b** with formic acid as hydrogen donor provided **4b** in almost quantitative yield for the following fragment condensation. As expected, **4a** proved to be an excellent substrate for V8 protease, and its condensation with **4b** provided the protected octapeptide amide **5** in 84% yield. Deprotection by transfer hydrogenation followed by hydrolysis then gave the octapeptide amide **6** in an overall yield of 39%.

The proteases depicted in Scheme 1 were selected on the basis of their well known substrate specificities.[1g-k,2a,10] For example, chymopapain, which displays a broad P_1 specificity, and a preference for hydrophobic groups in the P_2 site, and small, relatively hydrophilic P_1' residues, was well suited for the synthesis of dipeptides **1a**,**b**.[15,16] Similarly, papaya protease IV, with its high P_1 specificity for Gly and relaxed P_2 specificity, and proteinase K, with its preference for hydrophobic P_1 residues and smaller hydrophilic P_1' residues, were appropriate for the preparation of **2a** and **3b**, respectively. However, as modified proteases become available through the application of genetic engineering techniques,[17] it should be possible to complement or even partially replace the native enzymes with engineered mutants posessing more suitable catalytic properties. Thus, it may be feasible in the future to conduct the major part if not the entire sequence of coupling reactions using variants of a single enzyme displaying altered specificities and enhanced esterase to amidase activity ratios. The feasibility of this has been spectacularly illustrated with the protease-catalyzed semisynthesis of RNase A and three modified forms containing 4-fluorohistidine.[18] Subtiligase, a double mutant form of subtilisin BPN′ was employed as catalyst for the sequential condensation of up to seven oligopeptide fragments consisting of from nine to 27 residues. Clearly, such peptide ligases could also prove to be eminently suitable as catalysts for the stepwise, total synthesis of oligopeptides.

In conclusion, the results obtained clearly demonstrate the feasibility of sequential oligopeptide synthesis in the N to C direction via the stepwise addition of amino acid esters. The direct enzymatic coupling of the subsequent amino acid ester to the growing oligopeptide chain eliminates the requirement for intermediate *C*-deprotection/activation, the necessity for which has considerably limited conventional approaches. A wide range of serine and cysteine esterases was shown to discriminate among substrates of similar reactivity, thereby allowing one to obtain the target sequences in high preparative yields. The nature of the target octapeptide, which contains a number of multifunctional amino acid residues, suggests that this approach should be generally applicable to the preparation of a wide range of biologically active sequences. In addition, some of the emerging genetically engineered[17,18] and chemically modified[19] proteases such as subtiligase and methylated α-chymotrypsin should be ideally suited as catalysts for the implementation of this stepwise strategy. Furthermore, larger peptides should be readily accessible through the condensation of suitable fragments synthesized in this manner.

Experimental Section

Enzymes and Chemicals. Subtilisin (EC 3.4.21.14, ca. 10 U mg^{-1} solid), pronase E (EC undefined, ca. 4 U mg^{-1} solid), proteinase K (EC 3.4.21.14, ca. 8.8 U mg^{-1} solid), partially purified chymopapain (EC 3.4.22.6, ca. 2.1 U mg^{-1} solid), chromatographically purified chymopapain (EC 3.4.22.6, ca. 1.0 U mg^{-1} solid), V8 protease (EC 3.4.21.19, ca. 830 U mg^{-1} solid), N^2,N^6-di-Cbz-L-lysine, ethyl glycinate hydrochloride, O^1,O^4-diallyl L-aspartate tosylate salt, O^1,O^5-diallyl L-glutamate tosylate salt, dihydrogen *N*-Cbz-L-glutamate, ethyl L-serinate hydrochloride, ethyl L-leucinate hydrochloride, L-alaninamide hydrobromide, dithiothreitol, and Vydac 218TP/40-63 μm were obtained from Sigma Chemical Co. (Dorset, England). Endopeptidase Gly-C (papaya proteinase IV) was supplied by Novabiochem Ltd (Nottingham, England). Thionyl chloride, formic acid, Degussa type E211 palladium on alumina catalyst (5% w/w), 2-ethylhexanoic acid, triphenylphosphine, tetrakis(triphenylphosphine)palladium(0), and trifluoroacetic acid were purchased from Aldrich Chemical Co. (Dorset, England). Celite

(14) V8 protease only accepts acyl donors which contain glutamic/aspartic residues with unprotected side chains in the P_1 site.[21] Therefore, it was neccessary to employ a tetrapeptide of the type N^2-Cbz-Lys(N^6-Cbz)-Gly-Asp(OX)-Glu(OH)-OY where the side chain of the C-terminal glutamic residue was present as the free acid.

(15) According to the conventional nomenclature,[1g] P_1 (P_n) and P_1' (P_n') refer to amino acid residues situated on the carboxyl and amino sides, respectively, of the cleavable peptide bond.

(16) Although chymopapain can accept a wide range of amino acids in the S_1 site, it does at the same time display a distinct affinity for Lys in the P_1 position, especially where small peptide substrates are concerned.[10] This together with the concurrent binding of the N^1-Cbz group of the acyl donor to the S_2 site can explain why the enzyme was not observed to catalyze the further addition of Gly-OEt to **1a** during the preparation of this dipeptide.

(17) (a) Wong, C.-H. *Trends Biotechnol.* **1992**, *10*, 378. (b) Abrahamsen, L.; Tom, J.; Burnier, J.; Butcher, K. A.; Kossiakoff, A.; Wells, J. A. *Biochemistry* **1991**, *30*, 4151. (c) Bonneau, P. R.; Graycar, T. P.; Estell, D. A.; Jones, J. B. *J. Am. Chem. Soc.* **1991**, *113*, 1026. (d) Carter, P.; Abrahamsen, L.; Wells, J. A. *Biochemistry* **1991**, *30*, 6142. (e) Zhong, Z.; Liu, L.-C.; Dinterman, L. M.; Finkelman, M. A. J.; Mueller, W. T.; Rollence, M. L.; Whitlow, M.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113*, 683. (f) Wong, C.-H.; Schuster, M.; Wang, P.; Sears, P. *J. Am. Chem. Soc.* **1993**, *115*, 5896.

(18) Jackson, D. Y.; Burnier, J.; Clifford, Q.; Stanley, M.; Tom, J.; Wells, J. A. *Science* **1994**, *266*, 243.

(19) (a) Nakatsuka, T.; Sasaki, T.; Kaiser, E. *J. Am. Chem. Soc.* **1987**, *109*, 3808. (b) Wu, Z.-P.; Hilvert, D. *J. Am. Chem. Soc.* **1989**, *111*, 4513. (c) West, J. B.; Scholten, J.; Stolwich, N. J.; Hogg, J. L.; Scott, A. I.; Wong, C.-H. *J. Am. Chem. Soc.* **1988**, *110*, 3709. (d) West, J. B.; Hennen, W. J.; Lalonde, J. L.; Wong, C.-H. *J. Am. Chem. Soc.* **1990**, *112*, 5313.

(20) Bizzozero, S. A.; Baumann, W. K.; Dutler, H. *Eur. J. Biochem.* **1982**, *122*, 251.

(21) (a) Houmard, J.; Drapeau, G. R. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 3506. (b) Drapeau, G. R. *Methods Enzymol.* **1977**, *47*, 189.

(GLC grade, 30−80 mesh) was obtained from BDH Ltd (Dorset, England). HPLC grade methanol and water were purchased from Fisons Ltd (Loughborough, England). All of the chemicals and solvents used were of the highest available purity.

Substrates. Ethyl N^2,N^6-di-Cbz-L-lysinate (N^2-Cbz-L-Lys(N^6-Cbz)-OEt) and O^1,O^5-diethyl *N*-Cbz-L-glutamate (*N*-Cbz-L-Glu(OEt)-OEt) were prepared from the corresponding free acids using the thionyl chloride method. The free bases of the amino acid esters were prepared by neutralizing concentrated aqueous solutions of the hydrochloride or tosylate salts with aqueous sodium hydroxide, followed by extraction with ethyl acetate/ether and rotary evaporation. The free base of alaninamide was prepared by neutralizing an ethanolic suspension of the hydrobromide salt with ethanolic sodium hydroxide, followed by rotary evaporation and extraction with ethanol/acetonitrile.

Immobilized Enzymes. The enzymatic reactions were conducted using either suspended or immobilized enzymes (IME's) as catalysts. Subtilisin, pronase E, and proteinase K were immobilized on Celite at loadings of 40, 40, and 20 mg g^{-1}, respectively, as described previously.[3b] The following enzyme solutions were used at 0.9 mL g^{-1} for immobilization purposes: (i) subtilisin, 40 mg mL^{-1} in 100 mM CHES (pH 9.0) containing 50 mM sodium sulphate, 10 mM calcium acetate, and 0.5 mM EDTA; (ii) proteinase K, 22 mg mL^{-1} in 200 mM CAPSO (pH 9.5) containing 5 mM calcium acetate and 0.5 mM EDTA; (iii) pronase E, 44 mg mL^{-1} in 100 mM CHES (pH 9.0) containing 10 mM calcium acetate.

Analytical Procedures. The reactions were monitored by reverse phase HPLC, using an LDC Milton Roy CM4000 ternary gradient pump together with a Spectra Physics SP8450 UV/vis detector, LDC Marathon autosampler, and a Hewlett Packard 35900 Chemstation for data acquisition and integration. Samples were quenched with pure methanol, centrifuged at 12 000 rpm for 5 min, and then analyzed using a 0.46 × 15 cm Hichrom RPB/5 μm column, maintained at 45 °C and at a flow rate of 1.0 mL min^{-1} and with detection at 220 or 257 nm. Methanol and 8:2 water:methanol, both containing 0.05% v/v of phosphoric acid, were used as the mobile phases.

Preparative medium-pressure, reverse phase liquid chromatrography (MP-RPLC) was carried out using a 2.6 × 46 cm Büchi jacketed medium-pressure column connected to a Büchi 688 preparative chromatography pump and an LDC Milton Roy SpectroMonitor D. The column was slurry packed under vacuum with Vydac 218TP/40-63 μm and maintained at 22 °C. Samples were injected using a 40 mL loop, and purifications were carried out at a flow rate of 12 mL min^{-1}, with detection at 257 nm. Isocratic or step gradient elution was employed, using methanol and water as eluents. The required fractions were concentrated by rotary evaporation at 30 °C, then freeze dried.

IR spectra were recorded on a Perkin Elmer 1600 FTIR spectrometer using a diffuse reflectance cell with potassium bromide as the matrix (64 scans, 4.0 min, 0.1 cm^{-1} resolution, 2.0% band and base threshold). ^{1}H, ^{13}C, DEPT, ^{1}H−^{1}H COSY, and ^{1}H−^{13}C COSY spectra were recorded on a Jeol EX270 FT spectrometer at 35 °C, using DMSO-d_6 as the solvent. Proton spectra were recorded at 270.0 MHz and carbon-13 spectra at 67.8 MHz with broad-band proton decoupling, both using a DMSO field lock. ^{1}H−^{1}H COSY spectra were recorded in symmetrized mode. FAB-MS was carried out on a Kratos MS9/50TC spectrometer using a xenon gun operating at 5−7 kV or on a VG AUTOSPEC spectrometer using a cesium gun operating at 30 kV. The samples were dissolved in either 5% v/v acetic acid or UHQ water, then applied to a copper probe smeared with glycerol or thioglycerol or dispersed directly in glycerol before application. Sodium acetate was added to the sample where it was desired to observe sodium adducts. Spectra were recorded at 100 or 0.1 mmu in positive ionization mode, using polyglycerol ions as reference. Elemental analyses were carried out on a Perkin Elmer 2400 Series II CHNS/O analyzer. Optical rotations were measured on an optical activity digital polarimeter, at 589.3 nm, 20 °C, using a 0.5 cm (0.15 mL) cell, using either methanol or 1:1 methanol:DMSO as the solvent. Melting points were measured on a Stuart Scientific SMP-1 apparatus, using sealed capillaries, and are uncorrected.

Ethyl N^2,N^6-Di-Cbz-L-lysylglycinate (1a). Ethyl glycinate hydrochloride (Gly-OEt·HCl, 8.36 g, 60 mmol) was suspended in 100 mL of ethanol containing 2.0 mL of water. An ethanolic solution of sodium hydroxide (1.0 M, 58.8 mL) was slowly added with stirring, followed by 0.4 g each of powdered potassium carbonate and potassium bicarbonate. After stirring for 20 min, the volume was adjusted to 200 mL with ethanol and the mixture filtered through Celite. This was mixed with ethyl N^2,N^6-di-Cbz-L-lysinate (N^2-Cbz-L-Lys(N^6-Cbz)-OEt, 10.0 g, 23 mmol) dissolved in 40 mL of ethanol. A solution of chymopapain (0.30 g of purified enzyme in 6.0 mL of 0.25 M CHES buffer (pH 9.0) containing 10 mM EDTA) was slowly added to the substrate solution while stirring vigorously, followed by dithiothreitol (1.25 g) dissolved in ethanol (4.0 mL). After stirring for 20 h at 37 °C, HPLC analysis showed that 68% of dipeptide had been formed. At this point, more chymopapain (0.15 g in 2.0 mL of buffer) and dithiothreitol (0.5 g in 1.0 mL of ethanol) were added and the mixture stirred for a further 20 h, by which time the conversion had increased to 89%. The reaction mixture was then filtered through Celite and rotary evaporated to dryness. The solid obtained was washed successively with 3 × 50 mL of 15:1 petroleum ether:diethyl ether, 3 × 50 mL of pure diethyl ether, 2 × 20 mL of ice cold 0.05 M aqueous potassium bicarbonate, 3 × 20 mL of ice cold 0.05 M aqueous citric acid, 2 × 20 mL of water, and then finally 2 × 20 mL of 8:2 water:ethanol. After drying, the title compound was obtained as a microcrystalline white solid (9.21 g, 82% yield, >99% pure): mp 87−89 °C; $[\alpha]_{D,20}$ −12.0° (*c* 0.1, methanol); FTIR (KBr powder, DR), 3310.7 (broad), 3063.8, 3032.4, 2973.8, 2952.9, 1743.0, 1693.1, 1650.0, 1538.0, 1743.0, 1693.1, 1650.0, 1538.0, 1498.2, 1479.4, 1462.3, 1453.9, 1442.5, 1404.9, 1374.9, 1350.6, 1334.5, 1312.3, 1284.1, 1259.3, 1231.9, 1212.1, 1284.1, 1132.1, 1120.1, 1070.3, 1041.5, 1027.9, 1020.2, 892.0, 868.2, 776.3, 738.9 cm^{-1}; ^{1}H NMR (DMSO-d_6, 270 MHz, 35 °C), δ 1.17 (t, 3H, J = 7.0 Hz, OCH_2CH_3), 1.25−1.44 (m, 4H, Lys-H_γ + Lys-H_δ), 1.54 (m, 1H, Lys-$H_{\beta1}$), 1.63 (m, 1H, Lys-$H_{\beta2}$), 2.97 (dt, 2H, $J_{\delta,\epsilon}$ = 6.0 Hz, $J_{\epsilon,NH\zeta}$ = 6.2 Hz, Lys-H_ϵ), 3.76 (dd, 1H, $J_{\alpha1,\alpha2}$ = 17.1 Hz, $J_{\alpha,1NH}$ = 5.9 Hz, Gly-$H_{\alpha1}$), 3.87 (dd, 1H, $J_{\alpha1,\alpha2}$ = 17.1 Hz, $J_{\alpha2,NH}$ = 5.4 Hz, Gly-$H_{\alpha2}$), 4.02 (dt, 1H, $J_{\alpha,\beta}$ = 7.3 Hz, $J_{\alpha,NH\alpha}$ = 6.8 Hz, Lys-H_α), 4.07 (q, 2H, J = 7.0 Hz, OCH_2CH_3), 5.00 (s, 2H, Z_ϵ-CH_2), 5.02 (s, 2H, Z_α-CH_2), 7.16 (m, 10H, $J_{\epsilon,NH\zeta}$ = 6.2 Hz, Lys-NH_ζ-Z_ζ), 7.28 (m, 10H, $J_{\alpha,NH\alpha}$ = 6.8 Hz, Lys-NH_α-Z_α), 7.30−7.40 (m, 10H, Arom-*H*), 8.24 (dd, 1H, $J_{\alpha1,NH}$ = 5.9 Hz, $J_{\alpha2,NH}$ = 5.4 Hz, Lys-N*H*-Gly) ppm; ^{13}C NMR (DMSO-d_6, 67.8 MHz, 35 °C), δ 13.912 (OCH_2CH_3), 22.555 (Lys-γ), 28.970 (Lys-δ), 31.468 (Lys-β), 40.416 (Lys-ϵ), 40.614 (Gly-α), 54.414 (Lys-α), 60.236 (OCH_2CH_3), 65.016 (Z_ζ-CH_2), 65.304 (Z_α-CH_2), 127.585/128.196 (arom), 155.832/155.976/169.560/172.382 (carbonyl) ppm; FAB-MS (glycerol matrix, 8 keV Xe) (M + H) calcd for $C_{26}H_{34}N_3O_7$ 500.2396, obsd 500.2447. Elemental analysis. Calcd for $C_{26}H_{33}N_3O_7$: C, 62.50; H, 6.66; N, 8.41. Found: C, 62.44; H, 6.64; N, 8.36.

$O^{1.3}$,$O^{4.3}$-Diallyl $N^{2.1}$,$N^{6.1}$-Di-Cbz-L-lysylglycyl-L-aspartate (2a). Tripeptide **2a** was synthesized using either endopeptidase Gly-C (papaya protease IV) or purified chymopapain as catalyst:

(i) Synthesis using endopeptidase Gly-C as catalyst: Ethyl N^2,N^6-di-Cbz-L-lysylglycinate (**1a**, 0.13 g, 0.26 mmol) and O^1,O^4-diallyl L-aspartate (L-Asp(OAll)-OAll, 0.11 g, 0.52 mmol) were dissolved in 2.0 mL of ethanol containing 10% v/v of CHES buffer (0.20 M, pH 9.0, containing 10 mM EDTA) and 0.5% w/v of dithiothreitol. A solution of papaya protease IV (2.0 mg in 20 μL of 0.20 M CHES buffer (pH 9.0) containing 10 mM EDTA) was added slowly with stirring, and the mixture was stirred at 37 °C for 2 h, when HPLC analysis showed that 91% of the tripeptide had been formed. The reaction mixture was diluted with 1:1 methanol:ethanol (4.0 mL), filtered through Celite, then rotary evaporated to a syrup. This was then triturated with 0.05 M aqueous citric acid containing 0.2 M sodium chloride, and the solid obtained filtered off and washed successively with 2 × 2 mL of 0.05 M aqueous citric acid, 2 × 2 mL of 4:1 water:ethanol, then finally 2 × 5 mL of ether. After drying, the product was obtained as a white solid (0.15 g, 84% yield, >98% pure).

(ii) Synthesis using purified chymopapain as catalyst: Ethyl N^2,N^6-di-Cbz-L-lysylglycinate (**1a**, 8.48 g, 17 mmol) and O^1,O^4-diallyl L-aspartate (7.24 g, 34 mmol) were dissolved in a mixture of 116 mL of ethanol and 45 mL of CHES buffer (0.25 M, pH 9.0, containing 10 mM EDTA). A solution of chymopapain (0.20 g of crude enzyme in 6.0 mL of 0.25 M CHES buffer (pH 9.0) containing 10 mM EDTA) was added slowly with stirring, followed by 0.85 g of dithiothreitol dissolved in 3.0 mL of ethanol. After stirring for 4 h at 37 °C, HPLC analysis showed that 58% of tripeptide had been formed, and at this

point, the reaction mixture was diluted with methanol (200 mL), filtered through Celite, then rotary evaporated to a syrup. This was dissolved in 15 mL of 2:1 methanol:water and purified by RP-MPLC (single application on a 2.6 × 46 cm column packed with Vydac 218TP/40-63 μm; isocratically eluted with 65% methanol + 35% water, 12 mL min^{-1}, 22 °C), to give the title compound as a white solid (5.83 g, 52% yield, >99% pure). Tripeptide **2a** was also prepared using a chymopapain-catalyzed one-pot reaction starting from N^2-Cbz-L-Lys-(N^6-Cbz)-OEt. Dipeptide **1a** was first prepared (2.0 mmol scale) as described above, except that a 2-fold excess of Gly-OEt was employed and a buffer concentration of 5% v/v was used. After 20 h, the buffer concentration was increased to 15% v/v and 2.0 equiv of L-Asp(OAll)-OAll was added, together with a further portion of chymopapain. After the mixture was stirred for a further 4 h, the yield of **2a** had reached 34% and the product was purified by RP-MPLC as described above to give a white solid (0.36 g, 27% yield, >98% pure): mp 116−118 °C; $[\alpha]_{D,20}$ −7.9° (*c* 0.1, methanol); FTIR (KBr powder, DR) 3312.7 (broad), 3064.7, 3033.1, 2939.2 (broad), 2892.1, 2862.1, 1738.1, 1691.6, 1667.0, 1639.2, 1537.6 (broad), 1454.4, 1442.8, 1386.1, 1335.5, 1295.7, 1259.9, 1246.3, 1232.8, 1185.3, 1148.3, 1082.7, 1056.1, 1022.3, 1148.3, 1082.7, 921.1, 845.8, 776.4, 752.4, 736.5 cm^{-1}; ^1H NMR (DMSO-d_6, 270 MHz, 35 °C), δ 1.23−1.45 (m, 4H, Lys-H_γ + Lys-H_δ), 1.53 (m, 1H, Lys-$H_{\beta1}$), 1.61 (m, 1H, Lys-$H_{\beta2}$), 2.74 (dd, 1H, $J_{\alpha,\beta1}$ = 6.1 Hz, $J_{\beta1,\beta2}$ = 16.3 Hz, Asp-$H_{\beta1}$), 2.86 (dd, 1H, $J_{\alpha,\beta2}$ = 6.6 Hz, $J_{\beta1,\beta2}$ = 16.3 Hz, Asp-$H_{\beta2}$), 2.97 (dt, 2H, $J_{\delta,\epsilon}$ = 6.0 Hz. $J_{\epsilon,NH\zeta}$ = 6.4 Hz, Lys-H_ϵ), 3.70 (dd, 1H, $J_{\alpha1,\alpha2}$ = 15.3 Hz, $J_{\alpha1,NH}$ = 6.0 Hz, Gly-$H_{\alpha1}$), 3.78 (dd, 1H, $J_{\alpha1,\alpha2}$ = 15.3 Hz, $J_{\alpha2,NH}$ = 5.6 Hz, Gly-$H_{\alpha2}$), 3.97 (dt, 1H, $J_{\alpha,\beta}$ = 7.4 Hz, $J_{\alpha,NH\alpha}$ = 6.6 Hz, Lys-H_α), 4.52−4.60 (m, 4H, Asp-α-OCH_2-CH=CH_2 + Asp-β-OCH_2CH=CH_2), 4.73 (ddd, 1H, $J_{\alpha,\beta1}$ = 6.1 Hz, $J_{\alpha,\beta2}$ = 6.6 Hz, $J_{\alpha,NH}$ = 8.2 Hz, Asp-H_α), 4.98 (s, 2H, Z_ϵ-CH_2), 5.03 (s, 2H, Z_α-CH_2), 5.17−5.34 (m, 4H, Asp-α-OCH$_2$CH=CH_2 + Asp-β-OCH$_2$CH=CH_2), 5.80−5.97 (m, 2H, Asp-α-OCH$_2$C*H*=CH_2 + Asp-β-OCH$_2$C*H*=CH_2), 7.15 (t, 1H, $J_{\epsilon,NH\zeta}$ = 6.4 Hz, Lys-NH_ζ-Z_ζ), 7.26−7.42 (m, 11H, Arom-*H* + Lys-NH_α-Z_α), 8.13 (dd, 1H, $J_{\alpha1,NH}$ = 6.0 Hz, $J_{\alpha2,NH}$ = 5.6 Hz, Lys-N*H*-Gly), 8.26 (d, 1H, $J_{\alpha,NH}$ = 8.2 Hz, Gly-N*H*-Asp) ppm; ^{13}C NMR (DMSO-d_6, 67.8 MHz, 35 °C) δ 22.627 (Lys-γ), 28.970 (Lys-δ), 31.270 (Lys-β), 35.691 (Asp-β), 40.416 (Lys-ϵ), 40.602 (Gly-α), 48.395 (Asp-α), 54.684 (Lys-α), 64.729 (Asp-β-OCH$_2$-CH=CH_2), 65.016 (Asp-α-OCH$_2$CH=CH_2), 65.124 (Z_ζ-CH_2), 65.376 (Z_α-CH_2), 117.684 (Asp-β-OCH$_2$CH=CH_2), 117.791 (Asp-α-OCH$_2$-CH=CH_2), 127.171/127.585/127.854/128.016/128.213 (arom), 132.041 (Asp-β-OCH$_2$C*H*=CH_2), 132.275 (Asp-α-OCH$_2$C*H*=CH_2), 155.994/168.680/169.417/170.010/172.166 (carbonyl) ppm; FAB-MS (glycerol matrix, 8 keV Xe) (M + H) calcd for $C_{34}H_{43}N_4O_{10}$ 667.2979, obsd 667.2999. Elemental analysis. Calcd for $C_{34}H_{42}N_4O_{10}$: C, 61.23; H, 6.35; N, 8.41. Found: C, 61.26; H, 6.28; N, 8.37.

$O^{4.3}$,$O^{5.4}$-Diallyl $O^{1.4}$-Ethyl $N^{2.1}$,$N^{6.1}$-di-Cbz-L-lysylglycyl-α-L-aspartyl-L-glutamate (3a). $O^{1.3}$,$O^{2.3}$-Diallyl $N^{2.1}$,$N^{6.1}$-di-Cbz-L-lysylglycyl-L-aspartate (**2a**, 4.2 g, 6.3 mmol) and O^1,O^5-diallyl L-glutamate (L-Glu(OAll)-OAll, 4.29 g, 18.9 mmol) were dissolved in 150 mL of anhydrous ethanol. Immobilized subtilisin (20.0 g) was added, and the mixture incubated in a reciprocal shaker at 37 °C for 40 h, when HPLC analysis showed that 83% of the tripeptide had been formed. The reaction mixture was filtered through Celite, the immobilized enzyme washed thoroughly with 1:1 methanol:ethanol, and the filtrate rotary evaporated to dryness at 30 °C. The semisolid obtained was dissolved in 15 mL of 3:1 methanol:water and purified by RP-MPLC (single application on a 2.6 × 46 cm column packed with Vydac 218TP/40-63 μm; step gradient elution with 60% methanol + 40% water followed by 70% methanol + 30% water, 12 mL min^{-1}, 22 °C), to give the title compound as a white solid (3.97 g, 76% yield, > 98% pure): mp 114−116 °C; $[\alpha]_{D.20}$ −16.7° (*c* 0.1, methanol); FTIR (KBr powder, DR) 3294.1 (broad), 3067.4, 3044.6, 3036.0, 2939.9, 2863.7, 2769.5, 1731.7, 1707.8, 1688.4, 1670.2, 1659.4, 1654.2, 1639.1, 1594.2, 1537.8, 1482.3, 1453.8, 1429.5, 1379.9, 1358.2, 1343.2, 1326.0, 1260.7, 1199.5, 1174.5, 1105.9, 1024.7, 1006.0, 990.5, 951.5, 932.0, 856.9, 803.0, 776.4, 770.5, 731.9, 716.0, 711.8 cm^{-1}; ^1H NMR (DMSO-d_6, 270 MHz, 35 °C) δ 1.23 (t, 3H, *J* = 7.2 Hz, Glu-α-OCH$_2$C*H*$_3$), 1.30−1.56 (m, 4H, Lys-H_γ + Lys-H_δ), 1.60 (m, 1H, Lys-$H_{\beta1}$), 1.67 (m, 1H, Lys-$H_{\beta2}$), 1.96 (m, 1H, Glu-$H_{\beta1}$), 2.07 (m, 1H, Glu-$H_{\beta2}$), 2.49 (dd, 2H, $J_{\beta1,\gamma}$ = $J_{\beta2,\gamma}$ = 7.6 Hz, Glu-H_γ), 2.66 (dd, 1H, $J_{\alpha,\beta1}$ = 7.9 Hz, $J_{\beta1,\beta2}$ = 15.7 Hz, Asp-$H_{\beta1}$), 2.85 (dd, 1H, $J_{\alpha,\beta2}$ = 5.3 Hz, $J_{\beta1,\beta2}$ = 15.7 Hz, Asp-$H_{\beta2}$), 3.03 (dt, 2H, $J_{\delta,\epsilon}$ = 6.2 Hz, $J_{\epsilon,NH\zeta}$ = 6.3 Hz, Lys-H_ϵ), 3.78 (d, 2H, $J_{\alpha,NH}$ = 4.7 Hz, Gly-H_α), 4.04 (m, 1H, Lys-H_α), 4.13 (q, 2H, *J* = 7.2 Hz, Glu-α-OCH_2CH$_3$), 4.33 (m, 1H, Glu-H_α), 4.60 (m, 4H, Asp-β-OCH_2CH=CH_2 + Glu-γ-OCH_2CH=CH_2), 4.77 (m, 1H, Asp-H_α), 5.06 (d, 1H, J_{a1a2} = 12.5 Hz, Z_α-CH_{a1}CH$_{a2}$), 5.07 (s, 2H, Z_ϵ-CH_2), 5.13 (d, 1H, J_{a1a2} = 12.5 Hz, Z_α-CH$_{a1}$CH_{a2}), 5.15−5.38 (m, 4H, Asp-β-OCH$_2$-CH=CH_2 + Glu-γ-OCH$_2$CH=CH_2), 5.86−6.04 (m, 2H, Asp-β-OCH$_2$C*H*=CH_2 + Glu-γ-OCH$_2$C*H*=CH_2), 7.22 (t, 1H, $J_{\epsilon,NH\zeta}$ = 6.2 Hz, Lys-NH_ζ-Z_ζ), 7.31−7.52 (m, 10H, Arom-*H* + Lys-NH_α-Z_α), 8.16−8.25 (m, 3H, Lys-N*H*-Gly + Gly-N*H*-Asp + Asp-N*H*-Glu) ppm; ^{13}C NMR (DMSO-d_6, 67.8 MHz, 35 °C) δ 13.840 (Glu-α-OCH$_2$CH$_3$), 22.627 (Lys-γ), 25.754 (Glu-β), 28.970 (Lys-δ), 29.617 (Glu-γ), 31.216 (Lys-β), 36.140 (Asp-β), 40.434 (Lys-ϵ), 42.070 (Gly-α), 48.988 (Asp-α), 51.360 (Glu-α), 54.792 (Lys-α), 60.488 (Glu-α-OCH$_2$CH$_3$), 64.297/64.531 (Asp-β-OCH$_2$CH=CH_2 + Glu-γ-OCH$_2$CH=CH_2), 65.016 (Z_ζ-CH_2), 65.429 (Z_α-CH_2), 117.558/117.594 (Asp-β-OCH$_2$CH=CH_2 + Glu-γ-OCH$_2$CH=CH_2), 127.585/128.213 (arom), 132.418/132.544 (Asp-β-OCH$_2$C*H*=CH_2 + Glu-γ-OCH$_2$C*H*=CH_2), 155.958/156.048/168.608/169.542/170.261/171.088/171.735/172.382 (carbonyl) ppm; FAB-MS (glycerol matrix, 8 keV Xe) low resolution 824 (M + H); 690 (M + H − CBZ); FAB-MS accurate mass (M + H) calcd for $C_{41}H_{54}N_5O_{13}$ 824.3718, obsd 824.3725. Elemental analysis. Calcd for $C_{41}H_{53}N_5O_{13}$: C, 59.75; H, 6.49; N, 8.50. Found: C, 59.81; H, 6.51; N, 8.48.

$O^{1.4}$-Ethyl Dihydrogen $N^{2.1}$,$N^{6.1}$-Di-Cbz-L-lysylglycyl-α-L-aspartyl-L-glutamate (4a). All of the following operations were carried out in the absence of light, under nitrogen, using oven dried glassware, and solvents were dried over 3 Å molecular seives and previously sparged with nitrogen. $O^{4.3}$,$O^{5.4}$-Diallyl $O^{1.4}$-ethyl $N^{2.1}$,$N^{6.1}$-di-Cbz-L-lysylglycyl-α-L-aspartyl-L-glutamate (**3a**, 2.40 g, 2.9 mmol) and potassium 2-ethylhexanoate (1.84 g, 10.2 mmol) were dissolved in 10 mL of 2:1 dichloromethane:ethyl acetate. A solution of tetrakis(triphenylphosphine)palladium(0) (84 mg, 73 μmol, 2.5 mol %) and triphenylphosphine (38 mg, 145 μmol, 5.0 mol %) dissolved in 3 mL of 1:1 dichloromethane:ethyl acetate was added, and the mixture was stirred in the absence of light at 20 °C for 4 h, by which time deprotection was complete. The reaction mixture was evaporated to dryness at 20 °C, and the residue was dissolved in water (20 mL) and extracted with 3 × 15 mL of toluene. The aqueous layer was acidified with 0.3 M aqueous sulphuric acid, and the precipitate formed was filtered off and washed successively with 2 × 20 mL of 0.05 M aqueous citric acid, 2 × 20 mL of water, 2 × 20 mL of 8:2 water:ethanol, 2 × 20 mL of 8:2 ether:ethanol, then finally 3 × 20 mL of ether. After drying, the product was obtained as a soft white solid (2.07 g, 96% yield, >98% pure): mp 154−156 °C; $[\alpha]_{D,20}$ −21.1° (*c* 0.1, methanol); FTIR (KBr powder, DR), 3567.9 (broad), 3298.9 (broad), 3063.0, 2938.1, 1718.8, 1710.3, 1689.4, 1672.8, 1654.0, 1650.0, 1636.8, 1543.1, 1499.3, 1433.6, 1420.9, 1399.8, 1358.4, 1341.3, 1331.7, 1263.9, 973.9, 910.0, 757.5, 748.7, 739.8, 716.2 cm^{-1}; ^1H NMR (DMSO-d_6, 270 MHz, 35 °C) δ 1.34 (t, 3H, *J* = 7.1 Hz, Glu-α-OCH$_2$C*H*$_3$), 1.42−1.60 (m, 4H, Lys-H_γ + Lys-H_δ), 1.69−1.77 (m, 2H, Lys-H_β), 2.03 (m, 1H, Glu-$H_{\beta1}$), 2.12 (m, 1H, Glu-$H_{\beta2}$), 2.46 (dd, 2H, $J_{\beta1,\gamma}$ = 5.1 Hz, $J_{\beta2,\gamma}$ = 7.2 Hz, Glu-H_γ), 2.69 (dd, 1H, $J_{\alpha,\beta1}$ = 7.9 Hz, $J_{\beta1,\beta2}$ = 16.4 Hz, Asp-$H_{\beta1}$), 2.91 (dd, 1H, $J_{\alpha,\beta2}$ = 4.9 Hz, $J_{\beta1,\beta2}$ = 16.4 Hz, Asp-$H_{\beta2}$), 3.15 (dt, 2H, $J_{\delta,\epsilon}$ = $J_{\epsilon,NH\zeta}$ = 6.2 Hz, Lys-H_ϵ), 3.83−3.97 (2dd, 2H, Gly-$H_{\alpha1}$ + Gly-$H_{\alpha2}$), 4.14 (m, 1H, Lys-H_α), 4.24 (q, 2H, *J* = 7.1 Hz, Glu-α-OCH_2CH$_3$), 4.39 (m, 1H, Glu-H_α), 4.83 (m, 1H, Asp-H_α), 5.17 (d, 1H, J_{a1a2} = 12.7 Hz, Z_α-CH_{a1}-CH$_{a2}$), 5.18 (s, 2H, Z_ϵ-CH_2), 5.24 (d, 1H, J_{a1a2} = 12.7 Hz, Z_α-CH$_{a1}$CH_{a2}), 7.35 (t-broad, 1H, Lys-NH_ζ-Z_ζ), 7.47−7.58 (m, 10H, Arom-*H*), 7.71 (d, 1H, $J_{\alpha,NH\alpha}$ = 7.0 Hz, Lys-NH_α-Z_α), 8.20 (d, 1H, $J_{\alpha,NH\alpha}$ = 8.2 Hz, Gly-N*H*-Asp), 8.48−8.50 (m, 2H, Lys-N*H*-Gly + Asp-N*H*-Glu) ppm; ^{13}C NMR (DMSO-d_6, 67.8 MHz, 35 °C), δ 13.912 (Glu-α-OCH$_2$C*H*$_3$), 22.645 (Lys-γ), 25.879 (Glu-β), 28.934 (Lys-δ), 30.138 (Glu-γ), 31.216 (Lys-β), 36.266 (Asp-β), 40.416 (Lys-ϵ), 42.034 (Gly-α), 49.131 (Asp-α), 51.737 (Glu-α), 54.900 (Lys-α), 60.344 (Glu-α-OCH$_2$CH$_3$), 64.998 (Z_ζ-CH_2), 65.393 (Z_α-CH_2), 127.585/128.070 (arom), 115.958/156.048/168.500/170.656/171.303/171.519/172.417/173.855 (carbonyl) ppm; FAB-MS (glycerol matrix, 8 keV Xe) low resolution 744 (M + H), 700 (M + H − CO_2), 610 (M + H − CBZ); FAB-MS accurate mass (M + H) calcd for $C_{35}H_{46}N_5O_{13}$ 744.3092, obsd 744.3048. Elemental analysis. Calcd for $C_{35}H_{45}N_5O_{13}$: C, 56.50; H, 6.10; N, 9.42; Found: C, 56.46; H, 6.04; N, 9.47.

$O^{5.1}$,$O^{1.2}$-Diethyl N^1-Cbz-α-L-glutamyl-L-serinate (1b). Ethyl L-serinate hydrochloride (L-Ser(OH)-OEt·HCl, 10.17 g, 60 mmol) was suspended in 150 mL of ethanol containing 1.0 mL of water. A 1.0 M ethanolic solution of sodium hydroxide (58.8 mL) was slowly added with stirring, followed by 0.2 g of potassium carbonate and 0.2 g of potassium bicarbonate. After the mixture was stirred for 15 min, the volume was adjusted to 200 mL with ethanol and the mixture filtered through Celite. This solution was mixed with O^1,O^5-diethyl *N*-Cbz-L-glutamate (*N*-Cbz-L-Glu(OEt)-OEt, 10.11 g, 30 mmol) dissolved in ethanol (40 mL). A solution of chymopapain (0.50 g of purified enzyme in 6.0 mL of 0.25 M CHES buffer (pH 9.0) containing 10 mM EDTA) was slowly added to the substrate solution while stirring vigorously, followed by dithiothreitol (1.25 g) dissolved in ethanol (4.0 mL). After 20 h of stirring at 37 °C, HPLC analysis showed that 86% of the dipeptide had been formed, and the reaction mixture was filtered through Celite and rotary evaporated to dryness. The semisolid obtained was triturated with petroleum ether and washed successively with 4 × 50 mL of 10:1 petroleum ether:ethyl acetate, 1 × 30 mL of 3:1 petroleum ether:ethyl acetate, 3 × 30 mL of ice cold 0.05 M aqueous potassium bicarbonate, 3 × 30 mL of ice cold 0.05 M aqueous citric acid, and finally 3 × 30 mL of water. After drying, the title compound was obtained as a white solid (9.86 g, 78% yield, >99% purity): mp 124−127 °C; $[\alpha]_{D,20}$ −13.9° (*c* 0.1, methanol); FTIR (KBr powder, DR), 3497.4, 3296.6 (broad), 3073.0, 3036.1, 2981.8, 2962.6, 2932.8, 1743.7, 1709.0, 1682.1, 1645.5, 1556.1, 1537.8, 1466.7, 1455.0, 1444.1, 1413.4, 1388.2, 1370.4, 1335.5, 1302.6, 1279.1, 1253.9, 1201.9, 1129.6, 1071.3, 1047.7, 1019.2, 911.7, 860.4, 787.9, 776.9, 754.6, 719.3 cm^{-1}; ^1H NMR (DMSO-d_6, 270 MHz, 35 °C) δ 1.30 (t, 6H, *J* = 7.3 Hz, Glu-γ-OCH_2CH_3 + Ser-OCH_2CH_3), 1.93 (m, 1H, Glu-$H_{\beta1}$), 2.06 (m, 1H, Glu-$H_{\beta2}$), 2.49 (t, 2H, $J_{\beta1,\gamma} = J_{\beta,\gamma}$ = 8.0 Hz, Glu-H_γ), 3.78 (m, 1H, Ser-$H_{\beta1}$), 3.88 (m, 1H, Ser-$H_{\beta2}$), 4.13−4.28 (m, 5H, Glu-H_α + Glu-γ-OCH_2CH_3 + Ser-OCH_2CH_3), 4.44 (ddd, 1H, $J_{\alpha,\beta1} = J_{\alpha,\beta2}$ = 4.4 Hz, $J_{\alpha,NH}$ = 7.6 Hz, Ser-H_α), 5.13 (dd, 1H, $J_{\beta1,OH} = J_{\beta2,OH}$ = 8.8 Hz, Ser-O*H*), 5.15 (s, 2H, Z-CH_2), 7.42−7.53 (m, 5H, arom-*H*), 8.28 (d, 1H, $J_{\alpha,NH}$ = 7.6 Hz, Glu-N*H*-Ser) ppm; ^{13}C NMR (DMSO-d_6, 67.8 MHz, 35 °C) δ 13.840 (Glu-γ-OCH_2CH_3), 13.930 (Ser-OCH_2CH_3), 27.335 (Glu-β), 29.976 (Glu-γ), 53.462 (Glu-α), 54.648 (Ser-α), 59.697 (Glu-γ-OCH_2CH_3), 60.398 (Ser-OCH_2CH_3), 61.027 (Ser-β), 65.340 (Z-CH_2), 127.333/127.531/127.638/128.196 (arom), 155.724/170.225/171.447/172.220 (carbonyl) ppm; FAB-MS (glycerol matrix, 8 keV Xe) (M + H) calcd for $C_{20}H_{29}N_2O_9$ 425.1923, obsd 425.1956. Elemental analysis. Calcd for $C_{20}H_{28}N_2O_9$: C, 56.58; H, 6.65; N, 6.60; Found: C, 56.51 ; H, 6.64 ; N, 6.63.

$O^{5.1}$,O^3-Diethyl N^1-Cbz-α-L-glutamyl-L-seryl-L-leucinate (2b). $O^{3.1}$,$O^{1.2}$-Diethyl N^1-Cbz-α-L-glutamyl-L-serinate (**1b**, 9.34 g, 22 mmol) and ethyl L-leucinate (L-Leu-OEt, 10.50 g, 66 mmol) were dissolved in 100 mL of 5:1 ethanol:acetonitrile containing 0.1% v/v of water. Immobilized pronase E (7.5 g) was added, and the mixture was incubated in a reciprocal shaker at 37 °C. After 15 h, HPLC analysis showed that 66% of tripeptide had been formed. At this point, a further 50 mL of solvent together with 5.0 g of immobilized enzyme was added, and the reaction mixture was incubated for a further 10 h, by which time the conversion had increased to 87%. The reaction mixture was then filtered, the immobilized enzyme washed thoroughly with ethanol, and the filtrate rotary evaporated to dryness. The semisolid obtained was triturated with petroleum ether, then washed succesively with 4 × 40 mL of petroleum ether, 2 × 15 mL of ice cold 0.05 M aqueous potassium bicarbonate, 3 × 15 mL of ice cold 0.05 M aqueous citric acid, 4 × 15 mL of 10:1 water:ethanol, then finally 3 × 20 mL of water. After drying, the product was obtained as a white solid (9.31 g, 79% yield, >98% pure): mp 134−136 °C; $[\alpha]_{D,20}$ −34.1° (*c* 0.1, methanol); FTIR (KBr powder, DR) 3284.6 (broad), 3079.1, 2961.1, 2937.9, 2876.8, 1729.3, 1693.6, 1644.3, 1544.7 (broad), 1467.4, 1447.0, 1397.8, 1373.8, 1334.2, 1279.9, 1233.6, 1212.8, 1194.8, 1156.8, 1113.3, 1095.7, 1079.8, 1069.3, 1045.9, 1016.2, 847.3, 762.9, 741.8, 734.9, 718.2 cm^{-1}; ^1H NMR (DMSO-d_6, 270 MHz, 35 °C) δ 0.98 (d, 3H, $J_{\gamma,\delta1}$ = 6.6 Hz, Leu-$H_{\delta1}$), 1.03 (d, 3H, $J_{\gamma,\delta2}$ = 6.2 Hz, Leu-$H_{\delta2}$), 1.31 (t, 3H, *J* = 7.2 Hz, Glu-γ-OCH_2CH_3), 1.32 (m, 3H, *J* = 6.9 Hz, Leu-OCH_2CH_3), 1.67 (dd, 2H, $J_{\alpha,\beta}$ = 8.4 Hz, $J_{\beta,\gamma}$ = 5.9 Hz, Leu-H_β), 1.76 (m, 1H, Leu-H_γ), 1.94 (m, 1H, Glu-$H_{\beta1}$), 2.07 (m, 1H, Glu-$H_{\beta2}$), 2.49 (t, 2H, $J_{\beta1,\gamma} = J_{\beta2,\gamma}$ = 7.9 Hz, Glu-H_γ), 3.73 (dd, 2H, $J_{\alpha,\beta}$ = 5.9 Hz, $J_{\beta,OH}$ = 5.1 Hz, Ser-H_β), 4.19 (q, 2H, *J* = 7.2 Hz, Glu-γ-OCH_2CH_3), 4.23 (q, 2H, *J* = 6.9 Hz, Leu-OCH_2CH_3), 4.29 (m, 1H, Glu-H_α), 4.39−4.51 (m, 2H, Ser-H_α + Leu-H_α), 4.97 (t, 1H, $J_{\beta,OH}$ = 5.1 Hz, Ser-O*H*), 5.18 (s, 2H, Z-CH_2), 7.49−7.51 (m, 5H, arom-*H*), 7.58 (d, 1H, $J_{\alpha,NH}$ = 7.9 Hz, Glu-N*H*-Z), 8.05 (d, 1H, $J_{\alpha,NH}$ = 7.7 Hz, Ser-N*H*-Leu), 8.28 (d, 1H, $J_{\alpha,NH}$ = 7.9 Hz, Glu-N*H*-Ser) ppm; ^{13}C NMR (DMSO-d_6, 67.8 MHz, 35 °C) δ 14.541 (Glu-γ-OCH_2CH_3), 14.631 (Ser-OCH_2CH_3), 21.962 (Leu-δ_1), 23.238 (Leu-δ_2), 24.729 (Leu-γ), 27.910 (Glu-β), 30.677 (Glu-γ), 40.416 (Leu-β), 41.045 (Leu-α), 54.450 (Glu-α), 55.546 (Ser-α), 60.398 (Glu-γ-OCH_2CH_3), 61.009 (Leu-OCH_2CH_3), 62.105 (Ser-β), 66.094 (Z-CH_2), 127.980/128.196/128.357/128.914 (arom), 156.533/170.495/171.789/172.759/172.957 (carbonyl) ppm; FAB-MS (glycerol matrix, 8 keV Xe) (M + H) calcd for $C_{26}H_{40}N_3O_9$ 538.2764, obsd 538.2790. Elemental analysis. Calcd for $C_{26}H_{39}N_3O_9$: C, 58.07; H, 7.32; N, 7.82. Found: C, 58.01; H, 7.33; N, 7.85.

$O^{5.1}$-Ethyl N^1-Cbz-α-L-glutamyl-L-seryl-L-leucyl-L-alaninamide (3b). $O^{3.1}$,O^3-Diethyl N^1-Cbz-α-L-glutamyl-L-seryl-L-leucinate (**2b**, 4.6 g, 8.6 mmol) and L-alaninamide (L-Ala-NH_2, 1.51 g, 17.2 mmol) were dissolved in 75 mL of 7:3 ethanol:acetonitrile containing 0.4% v/v water. Immobilized proteinase K (7.5 g) was added, and the mixture was incubated in a reciprocal shaker at 37 °C. After 15 h, HPLC analysis showed that 81% of the tetrapeptide had been formed. At this point, a further 75 mL of 7:3 ethanol:acetonitrile containing 0.4% v/v of water was added, followed by 7.5 g of immobilized enzyme, and the reaction mixture was incubated for a further 10 h, by which time complete conversion had been achieved. The reaction mixture was then filtered, the immobilized enzyme washed thoroughly with warm 1:1 methanol:ethanol, and the filtrate rotary evaporated to dryness. The solid obtained was triturated with ice cold brine then washed succesively with 1 × 20 mL of ice cold 0.05 M aqueous potassium bicarbonate containing 0.3 M sodium chloride, 2 × 20 mL of ice cold 0.05 M aqueous citric acid containing 0.3 M sodium chloride, 2 × 15 mL of ice cold 0.2 M aqueous sodium chloride, and finally 2 × 10 mL of ice cold water. After drying, the title compound was obtained as a white solid (4.54 g, 92% yield, >98% pure): mp 207−209 °C (dec); $[\alpha]_{D,20}$ −10.1° (*c* 0.1, 1:1 methanol:DMSO); FTIR (KBr powder, DR), 3290.0, 3072.1 (broad), 2981.4, 2952.1 (broad), 2918.2, 2871.1, 1724.3, 1673.5, 1659.4, 1633.1, 1538.1, 1455.1, 1416.7, 1338.6, 1280.3, 1258.7, 1223.9, 1196.1, 1180.9, 1154.5, 1113.5, 1048.1, 1027.4, 912.1, 851.6, 778.1, 727.1, 718.2 cm^{-1}; ^1H NMR (DMSO-d_6, 270 MHz, 35 °C) δ 0.98 (d, 3H, $J_{\gamma,\delta1}$ = 6.6 Hz, Leu-$H_{\delta1}$), 1.03 (d, 3H, $J_{\gamma,\delta2}$ = 6.3 Hz, Leu-$H_{\delta2}$), 1.32 (t, 3H, *J* = 7.2 Hz, Glu-γ-OCH_2CH_3), 1.35 (d, 3H, $J_{\alpha,\beta}$ = 6.6 Hz, Ala-H_β), 1.66 (m, 2H, Leu-H_β), 1.79 (m, 1H, Leu-H_γ), 1.95 (m, 1H, Glu-$H_{\beta1}$), 2.07 (m, 1H, Glu-$H_{\beta2}$), 2.49 (t, 2H, $J_{\beta1,\gamma} = J_{\beta2,\gamma}$ = 7.8 Hz, Glu-H_γ), 3.67−3.73 (m, 2H, Ser-$H_{\beta1}$ + Ser-$H_{\beta2}$), 4.19 (q, 2H, *J* = 7.2 Hz, Glu-γ-OCH_2CH_3), 4.25−4.32 (m, 2H, Glu-H_α + Ala-H_α), 4.40 (m, 1H, Leu-H_α), 4.48 (m, 1H, Ser-H_α), 5.19 (s, 2H, Z-CH_2), 5.26 (t, 1H, $J_{\beta1,OH} = J_{\beta2,OH}$ = 5.3 Hz, Ser-O*H*), 7.12 (s-broad, 1H, Ala-NH_aH_b), 7.20 (s-broad, 1H, Ala-NH_aH_b), 7.46−7.52 (m, 5H, arom-*H*), 7.62 (d, 1H, $J_{\alpha,NH}$ = 7.6 Hz, Glu-N*H*-Z), 7.91 (d, 1H, $J_{\alpha,NH}$ = 7.6 Hz, Leu-N*H*-Ala), 8.09 (d, 1H, $J_{\alpha,NH}$ = 7.3 Hz, Glu-N*H*-Ser), 8.22 (d, 1H, $J_{\alpha,NH}$ = 7.6 Hz, Ser-N*H*-Leu) ppm; ^{13}C NMR (DMSO-d_6, 67.8 MHz, 35 °C) δ 14.343 (Glu-γ-OCH_2CH_3), 18.261 (Ala-β), 21.585 (Leu-δ_1), 23.346 (Leu-δ_2), 24.370 (Leu-γ), 27.479 (Glu-β), 30.354 (Glu-γ), 40.722 (Leu-β), 48.359 (Ala-α), 51.719 (Leu-α), 54.163 (Glu-α), 55.097 (Ser-α), 60.039 (Glu-γ-OCH_2CH_3), 61.854 (Ser-β), 65.375 (Z-CH_2), 127.836/127.980/128.555 (arom), 156.227/170.567/171.627/172.382/172.543/174.340 (carbonyl) ppm; FAB-MS (glycerol matrix, 8 keV Xe) (M + H) calcd for $C_{27}H_{42}N_5O_9$ 580.2982, obsd 580.2975. Elemental analysis. Calcd for $C_{27}H_{41}N_5O_9$: C, 55.93; H, 7.13; N, 12.09. Found: C, 55.87; H, 7.16; N, 12.11.

$O^{5.1}$-Ethyl α-L-Glutamyl-L-seryl-L-leucyl-L-alaninamide Formate Salt (4b). Tetrapeptide (**3b**) was *N*-deprotected by transfer hydrogenation as follows: $O^{5.1}$-Ethyl N^1-Cbz-α-L-glutamyl-L-seryl-L-leucyl-L-alaninamide (**3b**, 4.0 g, 6.9 mmol) was dissolved in 200 mL of methanol containing 5% v/v of water and 5% v/v of formic acid. Palladium on alumina (5% w/w loading, 4.0 g) was added, and the mixture was stirred at 20 °C for 30 min, by which time deprotection was complete. The reaction mixture was diluted with ethanol (50 mL) then filtered through Celite, and the filtrate was concentrated by rotary evaporation at 30 °C to approximately 25 mL. The concentrate was then diluted with 200 mL of water and freeze-dried. The solid obtained was thoroughly washed with diethyl ether (4 × 40 mL) to give the title compound as a hygroscopic white solid (3.31 g, 97% yield, >98% purity): mp 170−173 °C (dec); $[\alpha]_{D,20}$ −14.5° (*c* 0.1, methanol); FTIR (KBr powder, DR), 3264.2 (broad), 3073.8 (broad), 2955.4 (very broad), 2803.4, 1737.8, 1731.7, 1667.3, 1650.3, 1633.1, 1614.1, 1567.3, 1555.8, 1537.8,

1504.1, 1469.9, 1453.9, 1416.2, 1381.6, 1346.0, 1281.8, 1214.8, 11064.3, 951.9, 921.8, 897.4, 854.3, 781.8, 765.4, 709.9 cm^{-1}; ^1H NMR (DMSO-d_6, 270 MHz, 35 °C) δ 0.95 (d, 3H, $J_{\gamma,\delta1}$ = 6.3 Hz, Leu-H$_{\delta1}$), 0.99 (d, 3H, $J_{\gamma,\delta2}$ = 6.6 Hz, Leu-H$_{\delta2}$), 1.29 (t, 3H, J = 6.9 Hz, Glu-γ-OCH$_2$C*H*$_3$), 1.31 (d, 3H, $J_{\alpha,\beta}$ = 7.2 Hz, Ala-H$_\beta$), 1.63 (m, 2H, Leu-H$_\beta$), 1.74 (m, 1H, Leu-H$_\gamma$), 1.89 (m, 1H, Glu-H$_{\beta1}$), 1.99 (m, 1H, Glu-H$_{\beta2}$), 2.49 (t, 2H, $J_{\beta1,\gamma}$ = $J_{\beta2,\gamma}$ = 7.9 Hz, Glu-H$_\gamma$), 3.58 (m, 1H, Glu-H$_\alpha$), 3.68 (m, 1H, Ser-H$_{\beta1}$), 3.76 (m, 1H, Ser-H$_{\beta2}$), 4.16 (q, 2H, J = 6.9 Hz, Glu-γ-OC*H*$_2$CH$_3$), 4.26 (m, 1H, Ala-H$_\alpha$), 4.38 (dt, 1H, $J_{\alpha,\beta}$ = 6.6 Hz, $J_{\alpha,NH}$ = 7.2 Hz, Leu-H$_\alpha$), 4.47 (m, 1H, Ser-H$_\alpha$), 7.07 (s-broad, 1H, Ala-N*H*$_a$H$_b$), 7.18 (s-broad, 1H, Ala-NH$_a$*H*$_b$), 7.92 (d, 1H, $J_{\alpha,NH}$ = 7.6 Hz, Leu-N*H*-Ala), 8.33−8.46 (m, 3H, Glu-N*H*-Ser + Ser-N*H*-Leu + *H*CO$_2^-$) ppm; ^{13}C NMR (DMSO-d_6, 67.8 MHz, 35 °C) δ 14.002 (Glu-γ-OCH$_2$*C*H$_3$), 17.901 (Ala-β), 21.225 (Leu-δ_1), 23.004 (Leu-δ_2), 24.083 (Leu-γ), 28.737 (Glu-β), 29.689 (Glu-γ), 40.416 (Leu-β), 48.035 (Ala-α), 51.396 (Leu-α), 52.905 (Glu-α), 54.594 (Ser-α), 59.733 (Glu-γ-O*C*H$_2$CH$_3$), 61.710 (Ser-β), 164.260 (*H*CO$_2^-$), 170.207/171.339/171.986/172.435/174.035 (carbonyl) ppm; FAB-MS (glycerol matrix, 8 keV Xe) (M + H) calcd for $C_{19}H_{36}N_5O_7$ 446.2614, obsd 446.2611. Elemental analysis. Calcd for $C_{20}H_{37}N_5O_9$ (formate salt): C, 48.85; H, 7.59; N, 14.25. Found: C, 48.79; H, 7.55; N, 14.20.

$O^{5.5}$-Ethyl $N^{2.1}$,$N^{6.1}$-Di-Cbz-L-lysylglycyl-α-L-aspartyl-α-L-glutamyl-α-L-glutamyl-L-seryl-L-leucyl-L-alaninamide (5). $O^{1.4}$-Ethyl dihydrogen $N^{2.1}$,$N^{6.1}$-di-Cbz-L-lysylglycyl-α-L-aspartyl-L-glutamate (**4a**, 1.0 g, 1.34 mmol), $O^{5.1}$-ethyl α-L-glutamyl-L-seryl-L-leucyl-L-alaninamide formate salt (**4b**, 0.66 g, 1.34 mmol), and *N,N*-diisopropylethylamine (0.24 mL, 1.40 mmol, 5% molar excess) were dissolved in 40 mL of 8:2 ethanol:acetonitrile. A solution of V8 protease (15.0 mg dissolved in 1.6 mL of 150 mM ammonium bicarbonate buffer, pH 7.8) was then added, and the mixture was stirred for 8 h at 37 °C, when HPLC analysis showed that 91% of the octapeptide had been formed. The reaction mixture was diluted with 80 mL of 1:1 methanol:ethanol containing 0.1% v/v of acetic acid, then filtered through Celite, and the filtrate was rotary evaporated to dryness at 30 °C. The solid obtained was triturated with 0.1 M aqueous citric acid containing 0.2 M sodium chloride, then filtered off and washed successively with 2 × 10 mL of 0.05 M aqueous citric acid, 2 × 10 mL of 7:3 water:ethanol, 2 × 10 mL of 7:3 ether:ethanol, 2 × 10 mL of 2:1 ether:dichloromethane, then finally 2 × 10 mL of ether. After drying, the product was obtained as a white solid (1.31 g, 84% yield, > 98% pure): decomposes without melting above 190 °C; $[\alpha]_{D,20}$ −8.6° (*c* 0.1, 1:1 methanol:DMSO); FTIR (KBr powder, DR), 3585.9 (broad), 3286.1 (broad), 3131.7, 3069.2, 3016.3, 2938.1, 2881.2, 2871.4, 1722.3, 1702.2, 1644.0, 1590.8, 1538.2 (broad), 1483.3, 1453.8, 1436.2, 1399.0, 1384.0, 1370.1, 1351.9, 1341.6, 1325.1, 1262.2, 1193.9, 1181.7, 1105.6, 1050.3, 1044.0, 1024.7, 974.7, 910.9, 889.9, 858.7, 804.0, 800.0, 776.7, 756.7, 750.4 cm^{-1}; ^1H NMR (DMSO-d_6, 270 MHz, 35 °C) δ 0.92 (d, 3H, $J_{\gamma,\delta1}$ = 6.6 Hz, Leu-H$_{\delta1}$), 0.96 (d, 3H, $J_{\gamma,\delta2}$ = 6.6 Hz, Leu-H$_{\delta2}$), 1.26 (t, 3H, J = 6.9 Hz, Glu$_5$-γ-OCH$_2$CH$_3$), 1.29 (d, 3H, $J_{\alpha\beta}$ = 6.7 Hz, Ala-H$_\beta$), 1.32−1.51 (m, 4H, Lys-H$_\gamma$ + Lys-H$_\delta$), 1.53−1.65 (m, 3H, Lys-H$_{\beta1}$ + Leu-H$_\beta$), 1.68−1.80 (m, 2H, Lys-H$_{\beta2}$ + Leu-H$_\gamma$), 1.88 (m, 1H, Glu$_4$-H$_{\beta1}$), 1.94−2.08 (m, 3H, Glu$_4$-H$_{\beta2}$ + Glu$_5$-H$_{\beta1}$ + Glu$_5$-H$_{\beta2}$), 2.31−2.46 (m, 4H, Glu$_4$-H$_\gamma$ + Glu$_5$-H$_\gamma$), 2.61 (dd, 1H, $J_{\alpha,\beta1}$ = 7.9 Hz, $J_{\beta1,\beta2}$ = 16.0 Hz, Asp-H$_{\beta1}$), 2.78 (dd, 1H, $J_{\alpha,\beta2}$ = 5.3 Hz, $J_{\beta1,\beta2}$ = 16.0 Hz, Asp-H$_{\beta2}$), 3.06 (dt, 2H, $J_{\delta,\epsilon}$ = 6.0 Hz, $J_{\epsilon,NH\zeta}$ = 6.2 Hz, Lys-H$_\epsilon$), 3.63−3.78 (m, 2H, Ser-H$_{\beta1}$ + Ser-H$_{\beta2}$), 3.82 (d-broad, 2H, Gly-H$_\alpha$), 4.08 (m, 1H, Lys-H$_\alpha$), 4.12 (q, 2H, J = 76.9 Hz, Glu$_5$-γ-OC*H*$_2$CH$_3$), 4.18 (m, 1H, + Ala-H$_\alpha$), 4.22−4.41 (m, 4H, Glu$_4$-H$_\alpha$ + Glu$_5$-H$_\alpha$ + Ser-H$_\alpha$ + Leu-H$_\alpha$), 4.69 (m, 1H, Asp-H$_\alpha$), 5.08 (d, 1H, J_{a1a2} = 13.0 Hz, Z$_\alpha$-C*H*$_{a1}$CH$_{a2}$), 5.09 (s, 2H, Z$_\epsilon$-C*H*$_2$), 5.15 (d, 1H, J_{a1a2} = 13.0 Hz, Z$_\alpha$-CH$_{a1}$C*H*$_{a2}$), 7.03 (s-broad, 1H, Ala-N*H*$_a$H$_b$), 7.10 (s-broad, 1H, Ala-NH$_a$*H*$_b$), 7.24 (t-broad, 1H, Lys-N*H*$_\zeta$-Z$_\zeta$), 7.38−7.46 (m, 10H, arom-*H*), 7.51 (d-broad, 1H, Lys-NH$_\alpha$-Z$_\alpha$), 7.80 (d, 1H, $J_{\alpha,NH}$ = 7.6 Hz, Leu-N*H*-Ala), 8.05−8.19 (m, 5H, Gly-N*H*-Asp + Asp-N*H*-Glu$_4$ + Glu$_4$-N*H*-Glu$_5$ + Glu$_5$-N*H*-Ser + Ser-N*H*-Leu), 8.22 (t-broad, 1H, Lys-N*H*-Gly) ppm; ^{13}C NMR (DMSO-d_6, 67.8 MHz, 35 °C) δ 14.002 (Glu$_5$-γ-OCH$_2$*C*H$_3$), 17.865 (Ala-β), 21.261 (Leu-δ_1), 22.663 (Lys-γ), 23.022 (Leu-δ_2), 24.047 (Leu-γ), 26.868/27.083 (Glu$_4$-β + Glu$_5$-β), 28.970 (Lys-δ), 29.922 / 30.102 (Glu$_4$-γ + Glu$_5$-γ), 31.252 (Lys-β), 36.463 (Asp-β), 40.434 (Lys-ϵ), 40.650 (Leu-β), 41.944 (Gly-α), 48.107 (Ala-α), 49.527 (Asp-α), 51.467 (Leu-α), 52.006/52.312 (Glu$_4$-α + Glu$_5$-α), 54.810 (Lys-α), 55.187 (Ser-α), 59.715 (Glu$_5$-γ-O*C*H$_2$CH$_3$), 61.368 (Ser-β), 65.034 (Z$_\zeta$-C*H*$_2$), 65.447 (Z$_\alpha$-C*H*$_2$), 127.603/128.231 (arom), 155.994/156.048/170.279/170.692/171.016/171.106/171.339/171.932/172.256/172.346/173.963/174.035 (carbonyl) ppm; FAB-MS (glycerol matrix, 8 keV Xe) low resolution 1143 (M + H), 1126 (M + H − OH), 1009 (M + H − Cbz); FAB-MS accurate mass (M + H) calcd for $C_{52}H_{75}N_{10}O_{19}$ 1143.5210, obsd 1143.5285. Elemental analysis. Calcd for $C_{52}H_{74}N_{10}O_{19}$: C, 54.62; H, 6.53; N, 12.26. Found: C, 54.69; H, 6.57; N, 12.19.

L-Lysylglycyl-α-L-aspartyl-α-L-glutamyl-α-L-glutamyl-L-seryl-L-leucyl-L-alaninamide Bis(trifluoroacetate) Salt (Delicious Peptide Amide Bis(trifluoroacetate) Salt, 6). Octapeptide **5** was deprotected by transfer hydrogenation followed by alkaline hydrolysis. $O^{5.5}$-Ethyl $N^{2.1}$,$N^{6.1}$-di-Cbz-L-lysylglycyl-α-L-aspartyl-α-L-glutamyl-α-L-glutamyl-L-seryl-L-leucyl-L-alaninamide (**5**) (0.57 g, 0.5 mmol) was dissolved in 5 mL of cold 1:1 trifluoroacetic acid:acetic acid containing 4% v/v of formic acid, and the solution was cooled to 0 °C in an ice bath. Palladium on alumina (5% w/w loading, 1.0 g) was added, the mixture was stirred at 0 °C for 30 min, then allowed to warm up to 20 °C and stirring continued for a further 30 min, by which time deprotection was complete. The reaction mixture was diluted with ice cold water (50 mL) and filtered, and the filtrate was freeze-dried. The solid trifluoroacetate salt obtained was dissolved in 10 mL of 3:1 methanol:water and cooled to 0 °C in an ice bath. Sodium hydroxide (5.2 mL of a 0.5 M solution in 3:1 ethanol:water, 2.6 mmol) was then added to the stirred solution over a period of 0.5 h, and stirring continued at 0 °C for a further 4 h. The solution was then neutralized with trifluoroacetic acid (2.6 mL of a 1.0 M solution in 3:1 methanol:water), diluted with 20 mL of water, and freeze-dried. The solid obtained was extracted with 6:1 acetonitrile:ethanol and filtered, and the filtrate was rotary evaporated to dryness at 20 °C. The title product was thus obtained as an extremely hygroscopic white solid (0.49 g, 93% yield, >97% pure): mp not measurable due to the highly hygroscopic nature of salt; $[\alpha]_{D,20}$ −9.6° (*c* 0.1, 1:1 methanol:DMSO); FTIR (KBr powder, DR), 3668.5, 3586.0, 3564.0, 3304.4 (broad), 3066.6 (broad), 2956.7 (broad), 1682.1, 1651.9, 1557.5, 1539.4, 1520.2, 1506.3, 1472.0, 1455.9, 1435.9, 1418.0, 1206.1, 1139.4, 1026.2, 1004.8, 841.5, 803.5, 724.1 cm^{-1}; ^1H NMR (DMSO-d_6, 270 MHz, 35 °C) δ 0.98 (d, 3H, $J_{\gamma,\delta1}$ = 6.2 Hz, Leu-H$_{\delta1}$), 1.02 (d, 3H, $J_{\gamma,\delta2}$ = 6.2 Hz, Leu-H$_{\delta2}$), 1.36 (d, 3H, $J_{\alpha\beta}$ = 7.3 Hz, Ala-H$_\beta$), 1.57 (m, 2H, Lys-H$_\gamma$), 1.61−1.82 (m, 5H, Lys-H$_\delta$ + Leu-H$_\beta$ + Leu-H$_\gamma$), 1.86−1.98 (m, 4H, Lys-H$_\beta$ + Glu$_4$-H$_{\beta1}$ + Glu$_5$-H$_{\beta1}$), 2.01−2.18 (m, 2H, Glu$_4$-H$_{\beta2}$ + Glu$_5$-H$_{\beta2}$), 2.41 (dd, 2H, $J_{\beta1,\gamma}$ = $J_{\beta2,\gamma}$ = 7.9 Hz, Glu$_5$-H$_\gamma$), 2.49 (dd, 2H, $J_{\beta1,\gamma}$ = $J_{\beta2,\gamma}$ = 7.5 Hz, Glu$_4$-H$_\gamma$), 2.64 (dd, 1H, $J_{\alpha,\beta1}$ = 7.9 Hz, $J_{\beta1,\beta2}$ = 16.0 Hz, Asp-H$_{\beta1}$), 2.89 (dd, 1H, $J_{\alpha,\beta2}$ = 5.0 Hz, $J_{\beta1,\beta2}$ = 16.0 Hz, Asp-H$_{\beta2}$), 2.92 (m, 2H, Lys-H$_\epsilon$), 3.68−3.73 (m, 2H, Ser-H$_{\beta1}$ + Ser-H$_{\beta2}$), 3.95−4.03 (m, 3H, Lys-H$_\alpha$ + Gly-H$_\alpha$), 4.29 (m, 1H, + Ala-H$_\alpha$), 4.33−4.50 (m, 4H, Glu$_4$-H$_\alpha$ + Glu$_5$-H$_\alpha$ + Ser-H$_\alpha$ + Leu-H$_\alpha$), 4.77 (m, 1H, Asp-H$_\alpha$), 7.13 (s-broad, 1H, Ala-N*H*$_a$H$_b$), 7.27 (s-broad, 1H, Ala-NH$_a$*H*$_b$), 7.97 (d, 1H, $J_{\alpha,NH}$ = 6.7 Hz, Leu-N*H*-Ala), 8.09−8.20 (m, 6H, Glu$_4$-N*H*-Glu$_5$ + Ser-N*H*-Leu), 8.24−8.36 (m, 2H, Asp-N*H*-Glu$_4$ + Glu$_5$-N*H*-Ser), 8.50 (d, 1H, $J_{\alpha,NH}$ = 8.2 Hz, Gly-N*H*-Asp), 8.92 (t, 1H, $J_{\alpha,NH}$ = 5.8 Hz, Lys-N*H*-Gly) ppm; ^{13}C NMR (DMSO-d_6, 67.8 MHz, 35 °C) δ 17.847 (Ala-β), 21.118 (Lys-γ), 21.279 (Leu-δ_1), 23.040 (Leu-δ_2), 24.190 (Leu-γ), 26.454 (Lys-δ), 27.101 (Glu$_5$-β), 27.209 (Glu$_4$-β), 29.779 (Glu$_5$-γ), 30.192 (Glu$_4$-γ), 30.426 (Lys-β), 30.605 (Asp-β), 38.566 (Lys-ϵ), 40.434 (Leu-β), 41.926 (Gly-α), 48.305 (Ala-α), 49.617 (Asp-α), 51.629 (Glu$_5$-α), 51.683 (Leu-α), 52.078 (Lys-α), 52.348 (Glu$_4$-α), 55.582 (Ser-α), 61.602 (Ser-β), 115.006/119.409 (*C*F$_3$CO), 158.491/158.959 (CF$_3$*C*O) 168.410/168.967/170.531/170.728/171.196/171.591/171.825/172.903/174.107/174.448/177.682 (carbonyl) ppm; FAB-MS (glycerol matrix, 8 keV Xe) low resolution sample + NaOAc 847 (M + H), 869 (M + Na), 891 (M + 2Na), 913 (M + 3Na). FAB-MS accurate mass (M + H) calcd for $C_{34}H_{59}N_{10}O_{15}$ 847.4161, obsd 847.4172. Elemental analysis. Calcd for $C_{38}H_{60}N_{10}O_{19}F_6$: C, 42.44; H, 5.63; N, 13.03. Found: C, 42.30; H, 5.59; N, 12.91.

Acknowledgment. We would like to thank Dr. X. Jorba, Dr. R. Valivety, and Dr. A. Richards for helpful discussions. In addition, we gratefully acknowledge the AFRC for financial support and the EC for providing a postdoctoral fellowship (ECLAIR) to R.L.-F.

JA942658G