JOC Article

Synthesis of Macrocyclic, Potential Protease Inhibitors Using a Generic Scaffold

Estelle Dumez, John S. Snaith, and Richard F. W. Jackson*,†

Department of Chemistry, Bedson Building, The University of Newcastle upon Tyne, Newcastle upon Tyne, NE1 7RU, UK

Andrew B. McElroy, John Overington, and Martin J. Wythes

Pfizer Central Research, Sandwich, Kent, CT13 9NJ, UK

Jane M. Withka and Thomas J. McLellan

Exploratory Medicinal Sciences, Pfizer Global Research and Development, Groton, Connecticut 06340

r.f.w.jackson@shef.ac.uk

Received February 17, 2002

A generic macrocyclic peptide structure **2** was designed as a potential inhibitor of a range of proteinases, by using as a basis for the design the known structures of a series of enzyme−inhibitor complexes. The macrocyclic nature of the target **2** was chosen so as to reduce the entropic advantage in the hydrolytic enzymatic step, and thereby to inhibit the function of the enzyme. The nature of the linking group was identified as a benzoxazole by molecular modeling, so as to preserve the recognized conformation of the peptide chain. The specificity of the potential inhibitor was tuned by variation of the P_1 group (by incorporating phenylalanine, aspartic acid, or lysine), to allow recognition by different enzyme classes. The targets were prepared from the bis-amino acid derivative **5**, itself prepared using the Pd-catalyzed coupling of an organozinc reagent with the iodobenzothiazole **7** and subsequent macrocyclization of the open-chain derivatives **22**−**24** using HATU. None of the macrocylic compounds **25**, **28**−**30**, and **32** inhibited their target enzymes. NMR and MS studies on the interaction of macrocycle **29** and chymotrypsin established that compound **29** was in fact a substrate of the enzyme. This result indicated that while the design had been partially successful in identifying a compound that bound, the reduction in entropic advantage due to its macrocyclic nature was not sufficient to allow **29** to act as an inhibitor.

Introduction

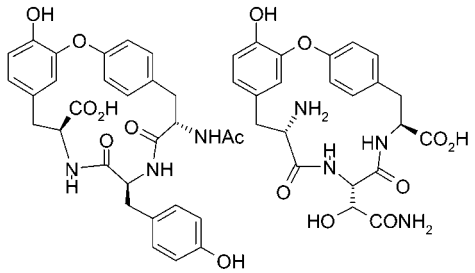
Many therapeutic areas are potentially amenable to intervention by protease inhibition. However, such targets present a major medicinal chemistry challenge to incorporate potency, low molecular size, bioavailability, and both chemical and metabolic stability into the same molecule. Recent progress in the area has been the subject of a perspective,[1] with recent reviews of inhibition of serine proteases[2] and of cysteine proteases.[3] A powerful strategy for forcing potential protease inhibitors into a bioactive conformation is to reduce their flexibility by incorporating the recognition site into a macrocyclic structure.[4] There has been much recent effort in this area,[5−10] and a very recent paper reports the design and synthesis of aspartic protease inhibitors constrained by macrocyclization.[11] This class of inhibitor has also been exemplified in several natural-product protease inhibitors, notably K-13 and OF4949-1. K-13 is a moderately potent inhibitor of ACE (IC_{50} = 350 nM, Captopril IC_{50} = 5 nM)[12] given that its structure is unoptimized for this enzyme, and we have recently reported a total synthesis

† Present address: Department of Chemistry, Dainton Building, University of Sheffield, Brook Hill, Sheffield, S3 7HF, UK.

(1) Leung, D.; Abbenante, G.; Fairlie, D. P. *J. Med. Chem.* **2000**, *43*, 305−341.
(2) Ripka, W. C.; Vlasuk, G. P. In *Annual Reports in Medicinal Chemistry*; Academic Press: San Diego, CA, 1997; Vol. 32, pp 71−89.
(3) Marquis, R. W. In *Annual Reports in Medicinal Chemistry*; Academic Press: San Diego, CA, 2000; Vol. 35, pp 309−320.
(4) Fairlie, D. P.; Abbenante, G.; March, D. R. *Curr. Med. Chem.* **1995**, *2*, 654−686.
(5) Tian, Z. Q.; Brown, B. B.; Mack, D. P.; Hutton, C. A.; Bartlett, P. A. *J. Org. Chem.* **1997**, *62*, 514−522.
(6) Janetka, J. W.; Raman, P.; Satyshur, K.; Flentke, G. R.; Rich, D. H. *J. Am. Chem. Soc.* **1997**, *119*, 441−442.
(7) Tyndall, J. D. A.; Reid, R. C.; Tyssen, D. P.; Jardine, D. K.; Todd, B.; Passmore, M.; March, D. R.; Pattenden, L. K.; Bergman, D. A.; Alewood, D.; Hu, S. H.; Alewood, P. F.; Birch, C. J.; Martin, J. L.; Fairlie, D. P. *J. Med. Chem.* **2000**, *43*, 3495−3504.
(8) Kohn, W. D.; Zhang, L. S.; Weigel, J. A. *Org. Lett.* **2001**, *3*, 971−974.
(9) Belvisi, L.; Bernardi, A.; Checchia, A.; Manzoni, L.; Potenza, D.; Scolastico, C.; Castorina, M.; Cupelli, A.; Giannini, G.; Carminati, P.; Pisano, C. *Org. Lett.* **2001**, *3*, 1001−1004.
(10) Xue, C.-B.; Voss, M. E.; Nelson, D. J.; Duan, J. J.-W.; Cherney, R. J.; Jacobson, I. C.; He, X.; Roderick, J.; Chen, L.; Corbett, R. L.; Wang, L.; Meyer, D. T.; Kennedy, K.; DeGrado, W. F.; Hardman, K. D.; Teleha, C. A.; Jaffee, B. D.; Liu, R.-Q.; Copeland, R. A.; Covington, M. B.; Christ, D. D.; Trzaskos, J. M.; Newton, R. C.; Magolda, R. L.; Wexler, R. R.; Decicco, C. P. *J. Med. Chem.* **2001**, *44*, 2636−2660.
(11) Ripka, A. S.; Satyshur, K. A.; Bohacek, R. S.; Rich, D. H. *Org. Lett.* **2001**, *3*, 2309−2312.
(12) Yasuzawa, T.; Shirahata, K.; Sano, H. *J. Antibiot.* **1987**, *40*, 455−458.

10.1021/jo025615o CCC: $22.00
Published on Web 05/29/2002

of K-13.[13] OF4949-1 has been reported to be a potent inhibitor of aminopeptidase-B with confirmed antitumor activity but lacking cytotoxic side effects.[14]



K-13 **OF4949-I**

Theoretical Rationale. A proteolytic enzyme is designed to (1) bind the substrate, (2) stabilize the tetrahedral proteolytic transition state, (3) eject the amine product, (4) catalyze the formation of a hydrolytic transition state, and (5) eject the acid product. For a metalloproteinase, the proteolytic and hydrolytic steps are one and the same. The third step in the process described above is encouraged by an increase in entropy when the product dissociates. However, if the two halves of the substrate are linked together by a rigid template, this entropic drive can in principle be markedly reduced to the extent that the stabilized hydrolytic transition state has a lower free energy than the cleaved products. Under these circumstances, the product amine will not dissociate from the enzyme and the substrate will remain bound at the active site probably oscillating across the transition structure.

Hence, the challenge in discovering this type of inhibitor is to produce a substrate with both sides of the target amide tethered in such a way that the backbone conformation is constrained to match ideally the enzyme-bound conformation and that all other conformations are energetically unfavorable such that the substrate fit is optimized. Provided that no additional strain is introduced, stabilization of the preferred amide conformation in this way will provide the optimum reduction in the entropy of dissociation and optimum binding affinity.

This strategy for protease inhibition is widely utilized in nature, for example in the serpin, Kunitz, Kazal, Bowman–Burke-type, and Potato types I and II protease inhibitors.[15] In these protein-based inhibitors, the "baited trap" substrate is constrained in the optimal conformation by a rigid protein backbone. Crystal structures are available for several of these inhibitors together with their target–protease complex.[16] Inhibitors from the different classes have markedly different tertiary structures but, for a given target protease, exhibit almost identical conformations in the active-site-directed region.

Target Design. The overriding design strategy was based on a desire to take advantage of the strong similarity of the substrate backbone conformation found for several unrelated classes of proteinases, in particular,

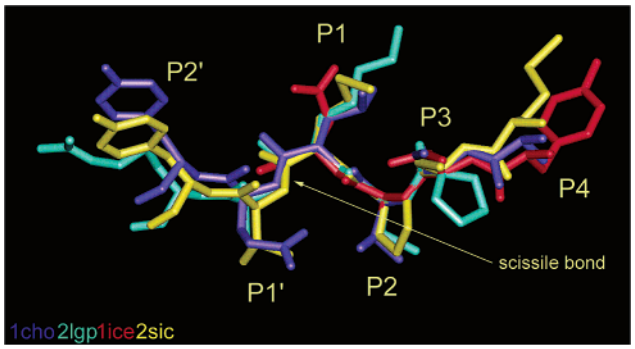


FIGURE 1. Conserved backbone conformations.

the trypsin-like and subtilisin-like serine proteinases and the caspase-like cysteine proteinases. The aim was to design inhibitors with a backbone main chain conformation that would provide a generally applicable (at least to the families of proteinases outlined above), rigid framework for the display of side chains that could be used to tune potency and selectivity for particular serine or cysteine proteinases.

To establish the required backbone conformations, enzyme–inhibitor complex structures (**1**) bovine chymotrypsin with turkey ovomucoid third domain (PDB code 1CHO), (**2**) bovine trypsin with bovine pancreatic trypsin inhibitor (PDB code 2TGP), (**3**) Interleukin converting enzyme-1 complexed with a substrate-based inhibitor (PDB code 1ICE), and (**4**) subtilisin complexed with a subtilisin inhibitor (PDB code 2SIC) were overlapped on the basis of the common regions of protein structure using standard superposition methods (Figure 1). Note that this overlap includes two related but functionally distinct serine proteinases (chymotrypsin and trypsin) and two unrelated protein-based inhibitors (ovomucoid third domain and pancreatic trypsin inhibitor), functional constraints have clearly selected a common substratelike binding conformation for the two inhibitor structures. This overlap establishes the strong structural similarity over the cleaved peptide bond region, running from P3 to P2′ for this pair of inhibitors. We chose to focus on the area that spanned the scissile bond (from P2 to P1′), since this region is known to be important in defining the general features of substrate specificity for this family and also would lead to tripeptide-based inhibitors as a basis for potentially orally active inhibitors.

Examination of the overlaid inhibitor structures uncovered a further feature, exploited in design, namely, the close structural and conformational similarity of an H-bond acceptor, stabilizing the conformation of the peptide bond at P1′ through a hydrogen bond to the main chain NH (the atoms involved in the two inhibitors are OE1 of Glu19 in 1CHO and O of Gly36 in 2TGP). This novel stabilizing interaction was factored into the inhibitor design to maintain, and possibly further enhance, the rigid display of the scissile bond to the enzyme and potentially to act by precluding the attack of the water that cleaves the acyl enzyme intermediate. These observations led to the generic cyclization design principle shown in Figures 2 and 3.

A visual inspection of the structure in Figure 1 suggested a potential cyclization strategy through a heterocyclic ring with a hydrogen bond acceptor at the position

(13) Perez-Gonzalez, M.; Jackson, R. F. W. *Chem. Commun.* **2000**, 2423–2424.
(14) Sano, S.; Ikai, K.; Yoshikawa, Y.; Nakamura, T.; Obayashi, A. *J. Antibiot.* **1987**, *40*, 512–518.
(15) Laskowski, M., Jr.; Kato, I. *Annu. Rev. Biochem.* **1980**, *49*, 593–626.
(16) Bode, W.; Huber, R. *Eur. J. Biochem.* **1992**, *204*, 433–451.

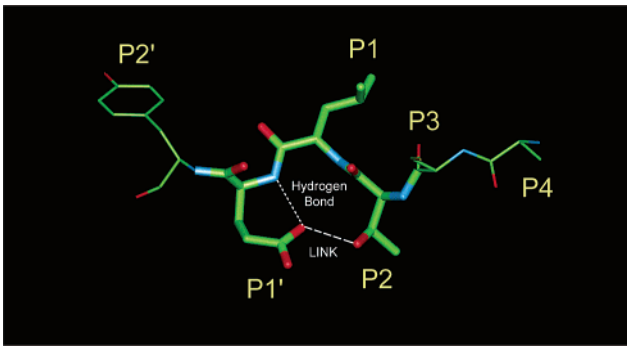


FIGURE 2. Inhibitor framework.

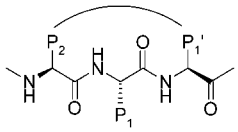
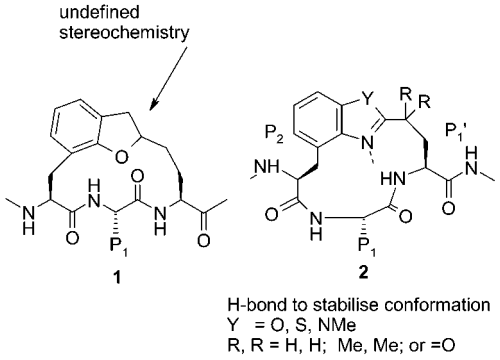


FIGURE 3. General synthetic target.

of the carboxylate oxygen of the P1′ glutamic acid residue in Figure 2. This led to the linking of residues P_2 and P_1' to form the generic cyclic structure shown in Figure 3.

To help select suitable P2 to P1′ linking groups, a search of a Web database of known proteinase inhibitor classes (developed by James Bray and John Overington, http://www.yorvic.york.ac.uk/~proteinase/) was undertaken using the HOOK program within QUANTA. The results of this study indicate that the most promising initial candidates for geometrically reasonable linkers involve connection to Cβ of each of the P_2 and P_1' side chains. Connections to Cα (at least with the database available at the time) tended to result in less synthetically amenable targets. The HOOK program suggested compound **1** using the Cβ linking strategy. Compound **1** provided the inspiration for the subsequent design of a series of potentially synthetically more accessible heterocycles, including **2**. An important criterion for selecting suitable heterocycles was their ability to form the stabilizing intramolecular H-bond between the benzoxazole/benzothiazole/benzimidazole nitrogen and the NH of the P1−P1′ amide bond. Electostastic surface calculations (Figure 4) confirmed that the chosen heterocycle linkers had the potential to form the required intramolecular H-bonds.



In an attempt to investigate the conformational stability of the ring structure for compounds of general structure **2**, we prepared a series of molecular dynamics

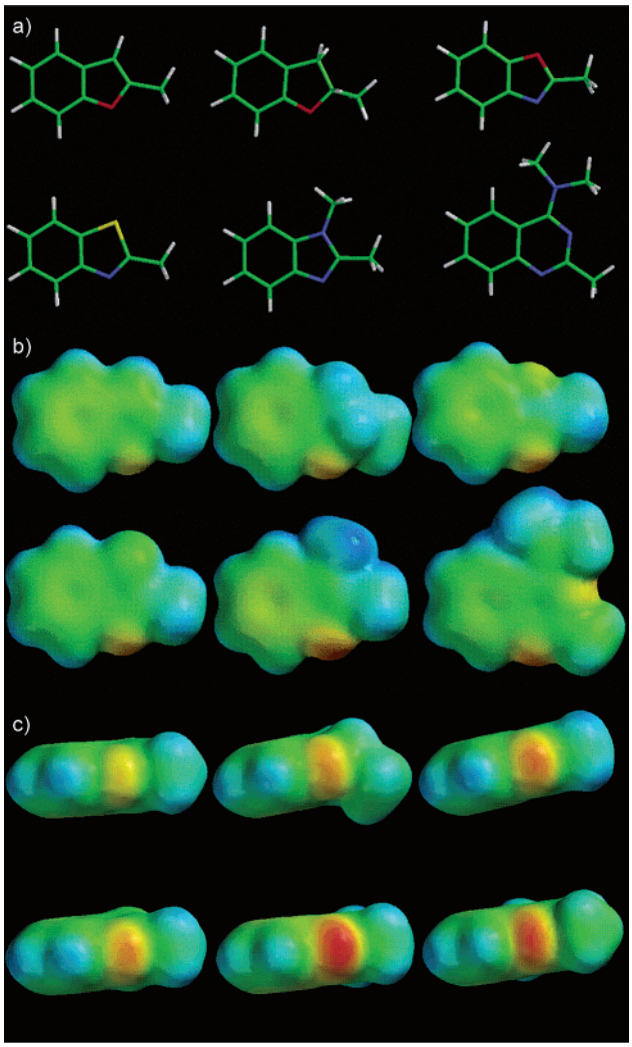
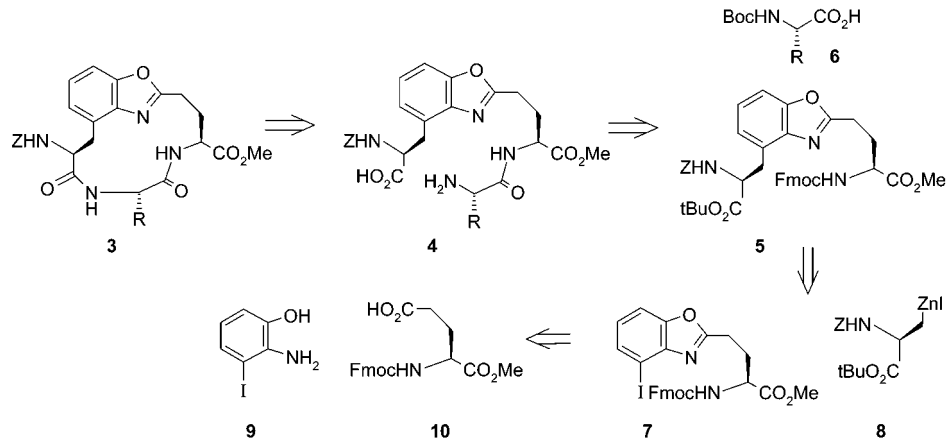


FIGURE 4. Modeled heterocycles (a) and front (b) and side (c) views of electrostatic surfaces (common scale).

simulations. Simulations were performed in a simulated "box of water" with the SYBYL molecular modeling package (Tripos Associates, St. Louis, MI) using default parameters. The ring was conformationally "stable" upon extended simulation, in that no significant conformational changes were observed (for example, flipping of amide bonds or loss of the important intramolecular hydrogen bond). Although such simulation results need necessarily to be treated with some caution, the preliminary study did support the adopted cyclization strategy.

Synthetic Strategy. For reasons of ease of synthesis, we identified the general target structures **3**, in which the side chain R corresponds to the side chain of Lys, Phe, and Asp, the canonical P1 recognition residues for trypsin, chymotrypsin, and ICE-1 proteases, respectively. The retrosynthetic strategy for the macrocycles **3** is indicated in Scheme 1 and proceeds via the open chain derivative **4**, which in turn could be prepared from the key bis(amino acid) **5** and the Boc-protected amino acid **6**, in which variation of the side chain functionality would allow recognition by the specific target enzyme. The design principle behind this class of inhibitor, constraint of the substrate in a rigid, optimized conformation, should ensure the success of the cyclization reaction, which is

SCHEME 1. Retrosynthetic Analysis for Target 3



the only intrinsically challenging step in the synthesis of this class of compound. The synthesis of the dipeptide **5** was expected to proceed from the iodobenzoxazole **7** and the protected serine-derived organozinc reagent **8**,[17] by using our palladium-catalyzed cross-coupling procedure.[18] We envisaged that the key heterocyclic building block **7** for our proposed protease inhibitors **3** could be prepared[19] from the trisubstituted aromatic **9** and protected glutamic acid **10**. In turn, it appeared that the trisubstituted aromatic **9** would be amenable to synthesis by directed ortho metalation.[20,21]

Synthesis. Our intial route to the trisubstituted aromatic **9** involved protection of 2-aminophenol as the *N*-Boc derivative **11** (98%). Treatment of **11** with 3 equiv of *tert*-butyllithium, in an attempt to form the trianion, and subsequent quenching with TMSCl failed to give any of the desired product, and so the acidic phenol function was protected as its TBDMS ether **12**. While ortho lithiation and quenching with TMSCl gave a complex mixture, the quench with either 1,2-diiodoethane or molecular iodine afforded yields of around 30% of **13**. TFA treatment smoothly removed the Boc group, while the TBS group was rapidly cleaved by reaction with TBAF, providing the desired target **9** in an overall yield of 67% from **13** after chromatography. After we had carried out this work, a report indicated that the yields in the lithiation/iodine quench could be substantially increased by the use of the analogous TIPS ether.[22]

While this route was amenable to scale-up, the poor overall yield prompted us to investigate more robust protection for the phenol, and we therefore examined the lithiation of the Boc-protected methyl ether **14**, which had been reported by Schlosser.[21] Lithiation at −25 °C and quenching with 1,2-chloroiodoethane, also at −25 °C, proceeded uneventfully to give the iodide **15** (61%), although careful temperature control in the lithiation step was crucial. Shortly after we had developed the method, a very similar route to the iodide **15** was reported; the only differences were in the conditions used for the quench, namely, the use of iodine at a temperature of −100 °C.[22] Our results suggest that, at least with 1,2-chloroiodoethane as the quenching agent, this low temperature may not be required. Complete deprotection of **15** was readily achieved using boron tribromide, affording 2-amino-3-hydroxy-1-iodobenzene **9** (95%).

With the aromatic nucleus in hand, attention was turned to the amino acid portion. To ensure the appropriate orthogonality of protecting groups, FmocGluOMe **10** was selected. Accordingly, FmocGluOMe **10** was uneventfully prepared from commercially available HGluOMe, and an *iso*-butylchloroformate-mediated coupling with **9**, a process with very good precedent,[19] proceeded smoothly. The product mixture from this reaction was complex, likely reflecting rotationally restricted O- and N-acylated species, and so rather than attempt to characterize it, we subjected the crude product directly to the cyclization conditions. Heating at reflux in propionic acid for 24 h smoothly yielded benzoxazole **7**, in 68% overall yield after chromatography.

Palladium-Catalyzed Couplings. One of the key steps of this approach was the palladium-catalyzed coupling reaction of the iodobenzoxazole **7** with organozinc reagent **8**.[17] Zinc reagent **8** was prepared from the protected iodoalanine derivative **16**, using activated zinc. Use of our normal conditions[18] for the cross-coupling reaction (DMF, with a catalyst prepared from 2.5 mol % $Pd_2(dba)_3$ and 10 mol % P(*o*-tolyl)$_3$) gave the desired cross-coupling product **5** (27−33%), along with the biaryl **17** derived from a homocoupling reaction (15−25%). Efforts to improve the yield of the desired target (including use of a variety of temperatures and different phosphine ligands) were unsuccessful, and only when the reaction was carried out using $PdCl_2(PPh_3)_2$ (10 mol %) as a catalyst did the yield of the coupled product **5** improve to an acceptable and reproducible level of 58−67% (based on iodobenzoxazole **7**), with only 7−12% of the biaryl **17**. Although $PdCl_2(PPh_3)_2$ has been successfully used for the coupling of aryl iodides with organozinc reagents previously,[23] it is in our experience unusual for it to give yields superior to those obtained using P(*o*-tolyl)$_3$-derived catalysts.

Elaboration to the Macrocycle. The next step of the synthesis was the regioselective formation of the peptide

(17) Gair, S.; Jackson, R. F. W.; Brown, P. A. *Tetrahedron Lett.* **1997**, *38*, 3059−3062.
(18) Jackson, R. F. W.; Moore, R. J.; Dexter, C. S. *J. Org. Chem.* **1998**, *63*, 7875−7884.
(19) Nestor, J. J.; Horner, B. L.; Ho, T. L.; Jones, G. H.; McRae, G. I.; Vickery, B. H. *J. Med. Chem.* **1984**, *27*, 320−325.
(20) Snieckus, V. *Chem. Rev.* **1990**, *90*, 879−933.
(21) Maggi, R.; Schlosser, M. *J. Org. Chem.* **1996**, *61*, 5430−5434.
(22) Kondo, Y.; Kojima, S.; Sakamoto, T. *J. Org. Chem.* **1997**, *62*, 6507−6511.

TABLE 1. Macrocyclization Reactions[a]

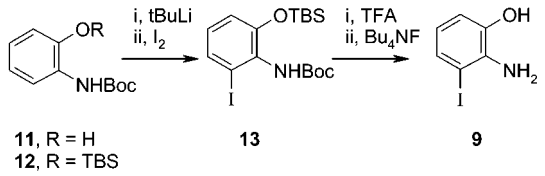
tripeptide	macrocycle	conditions A, yield (%)	conditions B, yield (%)
22	**25**	30−56	48
23	**26**	26	41
24	**27**	8	43

[a] Conditions A: (i) DIPEA (6 equiv) in DMF (C_S = 0.0125 M); (ii) HBTU (3 equiv), rt, 3 h. Conditions B: slow addition of tripeptide and DIPEA (10 equiv) to a solution of HATU (4 equiv) in DMF (C_S = 0.005 M), at 0 °C over 2 h, followed by 24 h at rt.

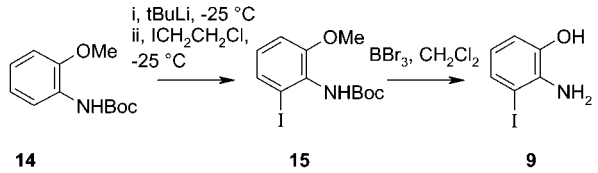
bond; after the removal of the Fmoc-protection in **5** by treatment with 20% piperidine in DMF, the free amino compound **18** was directly coupled in DMF to BocPheOSu, BocAsp(OBn)OSu, or BocLys(Pht)OSu using HOBt to form the tripeptides **19**, **20**, and **21** in 60, 60, and 53% yields (overall for the two steps), respectively. TFA was then used to remove simultaneously the Boc protecting group and cleave the *tert*-butyl ester of the tripeptides **19**, **20**, and **21** to give the TFA salts **22**, **23**, and **24**, respectively. In the first attempt, the macrolactamization was performed by treating the amine−TFA salt **22** with DIPEA (6 equiv) and HBTU (3 equiv) in DMF; this afforded the macrocycle **25** in yields of between 30 and 56% after flash chromatography, although the 30% yield was more representative. Application of these same conditions to the Asp-derivative **23** gave the corresponding macrocycle **26** (26%), while use of the Lys-derivative **24** gave a very modest yield of the macrocycle **27** (8%). In an effort to improve the yield of this cyclization, we decided to modify the experimental procedure and to evaluate a different peptide coupling additive. Better, more consistent, results were obtained when a solution of the amine−salt **22** was first treated with DIPEA and then slowly added to HATU in DMF, which gave the macrocycle **25** in 48% yield. Application of these conditions to the Asp-derivative **23** and the Lys-derivative **24** gave the macrocycles **26** and **27** in 41 and 43% yields, respectively. The results for the cyclizations are summarized in Table 1.

To emulate more closely the natural substrate, it was desirable to convert **25** into an acetamide at the N-terminus and a primary amide at the C-terminus. Removal of the benzyloxycarbonyl group by hydrogenolysis proceeded smoothly in acetic acid and acetic anhydride to trap the rather unstable free amine, affording a yield of 80% of acetamide **28**. Aminolysis of the methyl ester proved to be surprisingly difficult, with a variety of methods proving to be unsuccessful. Under the rather forcing conditions of 50 atm of ammonia at 70 °C in an autoclave, a 30% yield of the target compound **29** was obtained. As an alternative, a two-step procedure for the conversion of **28** involving methyl ester hydrolysis (96%) and subsequent EDCI-mediated amidation (58%) proved to be more efficient. The Asp-macrocycle **26** was converted into the acetamido free acid **30**, using the same procedure as employed for the preparation of **28**. The Lys-macrocycle **27** was first converted into the acetamido derivative **31**, and finally treatment with hydrazine gave the free side-chain amino derivative **32**, albeit in poor yield (20%), Scheme 7. This latter reaction was not

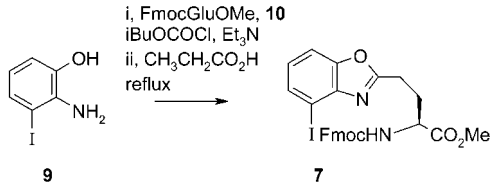
SCHEME 2



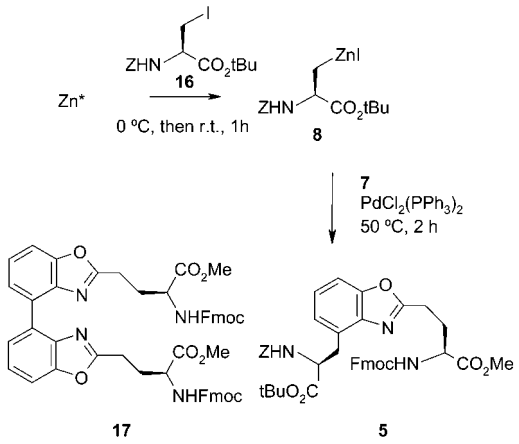
SCHEME 3



SCHEME 4



SCHEME 5



especially clean, and the product had to be purified by preparative HPLC. There was some evidence for hydrazinolysis of the methyl ester functionality.

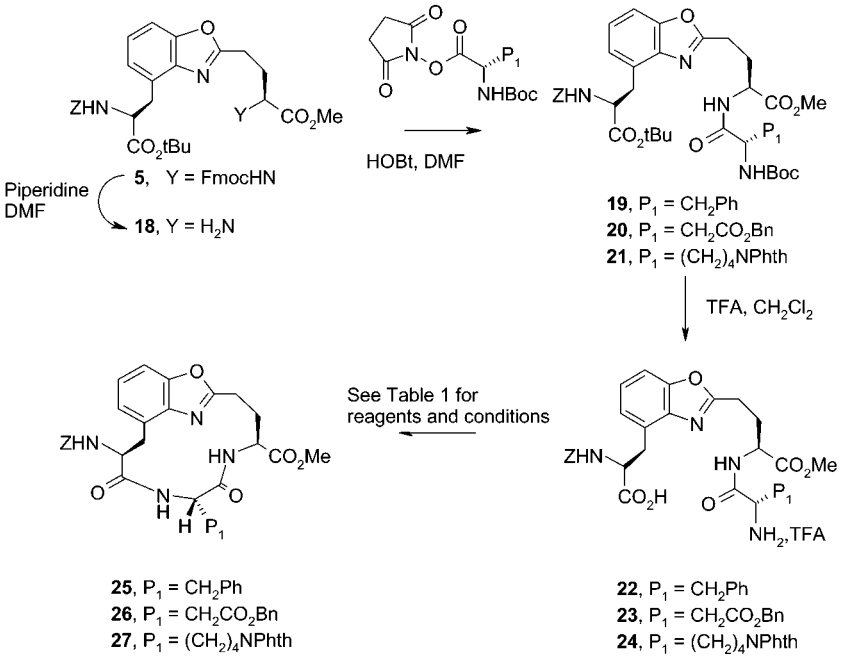
The trans configuration of the two amide bonds in the derivative **25** was established by means of a 2D ROESY spectrum (in deuteriochloroform). However, compound **25** was not soluble in water, and the NMR results in deuteriochloroform need to be treated with some caution since such a solvent would not be expected to "compete" for the intramolecular hydrogen bond in the same way that aqueous solvent conditions would.

Biological Evaluation. Compounds **25**, **28**−**30**, and **32** were tested for inhibition of a range of proteases with recognition sequences corresponding to the incorporated amino acid side chain (Table 2). We were disappointed to find that no significant inhibition of protease activity was observed.

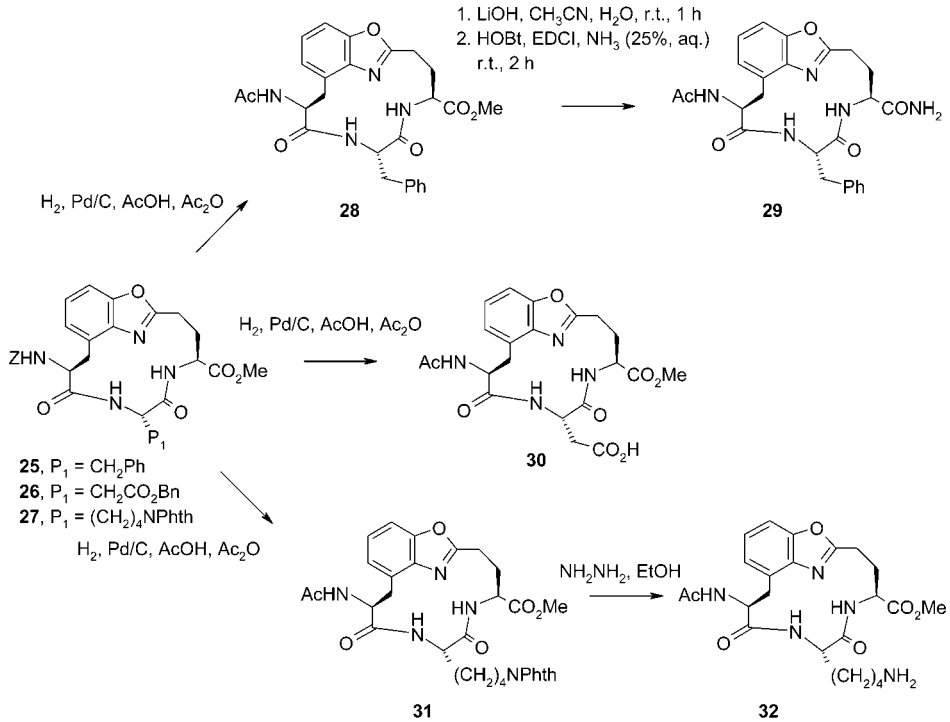
Compound **29**, in contrast to compound **25**, was soluble in water. Its structure was assigned and evaluated by homonuclear NMR methods in D_2O/H_2O to determine if an internal hydrogen bond exists in solution to support

(23) Smyth, M. S.; Burke, T. R., Jr. *Tetrahedron Lett.* **1994**, *35*, 551−554.

SCHEME 6



SCHEME 7. Modification of Macrocycles



the design of a rigid protease inhibitor scaffold. However, deuterium exchange experiments showed no indication of slowed amide exchange and, therefore, no evidence of hydrogen bond formation. Since delayed deuterium exchange could be difficult to observe under these conditions due to the potential flexibility of the small molecule, binding studies were also carried out to determine if this scaffold could bind weakly to chymotrypsin. Protein−ligand binding experiments were carried out by transferred NOE methods, which are optimal for μM−mM affinities.[24] No evidence of ligand binding was observed; however, the chemical shifts of the ligand in solution were shown to change dramatically as a function of time suggesting chemical modification in the presence of the enzyme. These results suggested that **29** was acting as a substrate rather than an inhibitor. To confirm this behavior, **29** was added to chymotrypsin in the presence of a cocktail of standard protease inhibitors. Under these

(24) Meyer, B.; Weimar, T.; Peters, T. *Eur. J. Biochem.* **1997**, *246*, 705−709.

TABLE 2. Biological Evaluation of Potential Inhibitors

macrocycle	protease	residue of natural substrate	test concn (μM)	% inhibition
30	Interleukin converting enzyme	Asp	50	0, 2, 4
32	Factor VIIa	Lys	200	30, 19
32	Factor Xa	Lys	200	14, 34
32	Trypsin	Lys	200	17, 19
32	Thrombin	Lys	200	11, 13
25	Chymotrypsin	Phe	10	11, 0
28	Chymotrypsin	Phe	100	2, 0
29	Chymotrypsin	Phe	100	0, 0

conditions, no chemical modification of **29** was observed, which further indicated that **29** was a substrate. These observations, both in the absence and in the presence of the cocktail of standard protease inhibitors, were reproducible.

At this point, the number and location of cleavage sites was unknown. One major product was evident using both MS and NMR methods, and the TOCSY spectrum indicated that only one amide bond had been disrupted. Mass spectroscopy confirmed that the cleavage site was in the cyclic portion of the molecule; however, the exact product of the cleavage could not be umambiguously determined by MS/MS methods. Acetylation of the product was carried out, and samples were re-evaluated by MS/MS methods. In the accurate mass MS/MS spectrum, only the fragment ion corresponding to the cleavage product **33** was observed (*m*/*z* 373), thus unequivocally confirming it as the product (Figure 5). These data showed that cleavage occurred at the amide bond, which was initially thought to be involved in the internal hydrogen bond, to give the acyclic tripeptide **33**, rather than the isomeric derivative **34**, in a way that is entirely consistent with the known selectivity of chymotrypsin.

Our conclusion, therefore, is that while the design of the generic target has been successful in producing a compound that fits the active site of chymotrypsin, the hypothesis that the macrocyclic nature of the compounds can overcome the entropic drive for hydrolysis was not borne out.

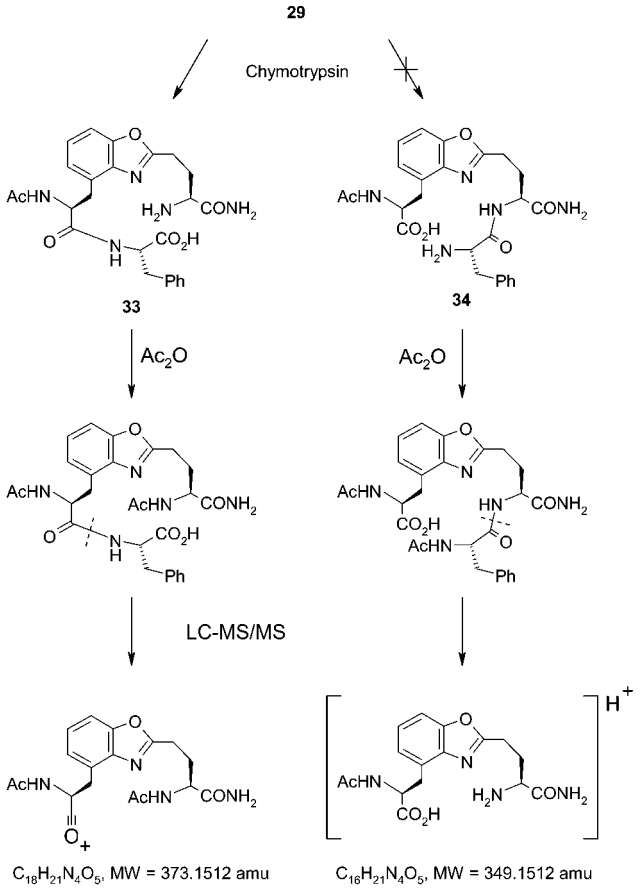


FIGURE 5. Schematic of the proposed major fragmentation pathway based on the MS/MS data for the cleavage product from compound **29**.

Experimental Section

***tert*-Butyl 2-(*R*)-(Benzyloxycarbonyl)amino-3-iodopropionate (16).** Methyltriphenoxyphosphonium iodide (7.23 g, 16 mmol) was added in four portions over 10 min to a stirred solution of *tert*-butyl 2-(*S*)-(benzyloxycarbonyl)amino-3-hydroxypropionate[25] (2.36 g, 8 mmol) in anhydrous DMF (30 mL), under nitrogen and at room temperature. The flask was covered with foil and the mixture stirred for 3 h until no starting material remained as judged by TLC using 3/1 petrol/ethyl acetate as an eluant. Methanol (20 mL) was added and the mixture stirred for 15 min. The reaction mixture was poured into water (150 mL) and the product extracted with diethyl ether (2 × 100 mL). The combined organic fractions were washed with water (3 × 150 mL), 5% sodium hydroxide solution (2 × 50 mL), water (3 × 100 mL), sodium thiosulfate solution (100 mL), and water (100 mL). The organic layer was dried over magnesium sulfate and concentrated under reduced pressure to give a pale yellow oil. Flash chromatography using 5/1 petrol/ethyl acetate yielded the iodide **16** a white solid (2.88 g, 89%): mp 47−49 °C; $[\alpha]_D^{22}$ −5.6 (*c* 1.05, EtOH). MS (*m*/*z* EI$^+$, %) 349 ($MH^+ - t$-Bu, 87%), 304 (50), 178 (72), 151 (13), 91 (100), 57 (96); IR ν_{max} (film) 3360, 2978, 2930, 1724, 471 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.50 (9H, s), 3.59 (2H, d, J = 3.5), 4.41 (1H, dt, J = 7, 3.5), 5.13 and 5.14 (2H, AB, J = 12), 5.60 (1H, d, J 7), 7.28−7.37 (5H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 8.38, 28.0, 54.0, 67.1, 83.6, 128.1, 128.2, 128.6, 136.1, 155.4, 168.1. Anal. Calcd for $C_{15}H_{20}NO_6I$: C, 44.5; H, 5.0; N, 3.5. Found: C, 44.8; H, 5.1; N, 3.45.

2-Amino-3-hydroxy-1-iodobenzene (9). 2-*N*-*t*-Butoxycarbonylamino-1-iodo-3-methoxybenzene **15** (5 g, 14.3 mmol) in dry CH_2Cl_2 (50 mL) was cooled to −78 °C under nitrogen, and boron tribromide (6.44 g, 2.43 mL, 25.7 mmol) was added dropwise via syringe over 5 min. The mixture was stirred for 16 h, warming to room temperature. Water (20 mL) was added cautiously and the mixture stirred for 30 min before partitioning between CH_2Cl_2 (3 × 50 mL) and water (50 mL). The pH of the aqueous layer was adjusted to 7 with sodium bicarbonate solution and extracted with ethyl acetate (3 × 50 mL), washed with brine (20 mL), and dried over sodium sulfate. Evaporation afforded the compound **9** as a yellow solid, which was sufficiently pure for further work (3.2 g, 95%): 3/1 petrol/ethyl acetate (R_f 0.4); mp 138 °C dec (lit.[26] 137 °C dec); calcd for C_6H_6INO [M$^+$] 234.9494, found 234.9493; MS (*m*/*z* EI$^+$, %) 235 (M$^+$, 100%), 108 (23), 80 (22); IR ν_{max} (KBr)/cm^{-1} 3372, 3297, 3037 (br); ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.10 (2H, br s), 4.95 (1H, br s), 6.41 (1H, t, J = 8), 6.68 (1H, dd, J = 8, 1), 7.25 (1H, dd, J = 8, 1); ^{13}C NMR (125 MHz, $CDCl_3$) δ 83.1, 114.0, 118.7, 128.8, 137.3, 144.0.

(25) Chevallet, P.; Garrouste, P.; Malawska, B.; Martinez, J. *Tetrahedron Lett.* **1993**, *34*, 7409−7412.

(26) Hodgson, H. H.; Kershaw, A. *J. Chem. Soc.* **1928**, 2703−2705.

***N*-9-Fluorenylmethyloxycarbonylglutamic Acid α-Methyl Ester (10).** To a solution of sodium bicarbonate (1.98 g, 23.58 mmol) and glutamic acid α-methyl ester (3.80 g, 23.58 mmol) in water (80 mL) was added *N*-fluoren-9-yl methoxycarbonyloxy succinimide (FmocOSu) (7.95 g, 23.58 mmol) dissolved in THF (150 mL) and the resulting mixture stirred for 16 h at room temperature. After dilution with water (150 mL), the pH was adjusted to 8 using saturated sodium bicarbonate solution and any unreacted FmocOSu removed by extraction with ether (2 × 200 mL). The pH was lowered to 1 with 1 M HCl and the milky white suspension extracted with ethyl acetate (2 × 150 mL). The combined ethyl acetate layers were washed with water (2 × 150 mL) and brine (20 mL) and dried over sodium sulfate. Evaporation afforded FmocGluOMe **10** as a white solid (8.31 g, 92%): mp 139−140 °C (lit.[27] 130 °C); $[\alpha]_D^{22}$ 6.6 (*c* 1.4, $CHCl_3$); calcd for $C_{21}H_{21}NO_6$ [M^+] 383.1369, found 383.1355; MS (*m*/*z* EI$^+$, %) 383 (M^+, <1%), 365 (2), 324 (1), 196 (26), 178 (100), 165 (85); IR ν_{max} (KBr) 3349, 3034, 2953, 1750, 1686 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.00 (1H, m), 2.23 (1H, m), 3.78 (3H, s), 4.20 (1H, t, J = 7), 4.40 (3H, m), 5.66 (1H, d, J = 8.5), 7.31 (2H, m), 7.40 (2H, *J*, 7.5), 7.60 (2H, m), 7.75 (2H, *d*, J = 7.5); ^{13}C NMR (125 MHz, $CDCl_3$) δ 27.2, 29.8, 47.0, 52.5, 53.1, 67.0, 119.9, 125.0, 127.0, 127.6, 141.2, 143.6, 143.7, 156.0, 172.4, 177.7. Anal. Calcd for $C_{21}H_{21}NO_6$: C, 65.79; H, 5.52; N, 3.65. Found: C, 65.7; H, 5.5; N, 3.7.

Methyl (2*S*)-2-{[(9*H*-Fluoren-9-ylmethoxy)carbonyl]amino}-4-(4-iodo-1,3-benzoxazol-2-yl)butanoate (7). To a solution of Fmoc glutamic acid α-methyl ester (4.08 g, 10.64 mmol) and triethylamine (1.075 g, 1.48 mL, 10.64 mmol) in CH_2Cl_2 (60 mL) at −10 °C was added isobutyl chloroformate (1.45 g, 1.38 mL, 10.64 mmol). The solution was stirred under nitrogen at this temperature for 30 min before 2-amino-3-hydroxy-1-iodobenzene **9** (2.5 g, 10.64 mmol) was added as a suspension in CH_2Cl_2 (50 mL). The mixture was stirred for 16 h, warming to room temperature before being washed with water (2 × 100 mL) and brine (10 mL) and dried over sodium sulfate. After evaporation, the residue was taken up in propionic acid (25 mL) and heated under reflux for 3 h protected by a calcium chloride guard tube. The propionic acid was evaporated and the residue taken up in ether (100 mL), washed with sodium bicarbonate solution (3 × 50 mL) and brine (50 mL), and dried over sodium sulfate. Evaporation afforded a tan foam that was subjected to chromatography over silica, eluting with 1/2 ethyl acetate/petrol (R_f 0.3) to afford the iodobenzoxazole **7** as a cream solid (4.21 g, 68%): mp 135−136 °C; $[\alpha]_D^{22}$ 18.7 (*c* 0.53, CH_2Cl_2); calcd for $C_{27}H_{23}N_2IO_5$ [M^+] 582.0653, found 582.0682; MS (*m*/*z* EI$^+$, %) 583 (MH^+, 9%), 582 (M^+, <1%), 523 (8), 454 (23), 387 (24), 360 (55), 301 (60), 272 (65), 178 (100); IR ν_{max} (film) 3333, 3065, 2952, 1721 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.31 (1H, m), 2.51 (1H, m), 3.02 (2H, m), 3.73 (3H, s), 4.18 (1H, t, J = 7.0), 4.38 (2H, d, J = 7.0), 5.71 (1H, d, J = 8), 7.05 (1H, t, J = 8), 7.28 (2H, dt, J = 7.5, 1), 7.36 (2H, t, J = 7.5), 7.42 (1H, dd, J = 8, 1), 7.57 (2H, m), 7.67 (1H, dd, J = 8, 1), 7.73 (2H, m, ArH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 24.9, 29.3, 47.1, 52.7, 53.4, 67.1, 84.5, 110.3, 112.0, 125.0, 126.2, 127.0, 127.7, 133.5, 141.3, 143.7, 143.9, 149.3, 155.9, 165.8, 172.0. Anal. Calcd for $C_{27}H_{23}N_2IO_5$: C, 55.68; H, 3.98; N, 4.81. Found: C, 55.85; H, 3.9; N, 4.7.

Methyl (2*S*)-4-{4-[(2*S*)-2-{[(Benzyloxy)carbonyl]amino}-3-(*tert*-butoxy)-3-oxopropyl]-1,3-benzoxazol-2-yl}-2-{[(9*H*-fluoren-9-ylmethoxy)carbonyl]amino}butanoate (5). Trimethylsilyl chloride (62 mg, 72 μL, 0.57 mmol) was added to a suspension of zinc dust (325 mesh, 371 mg, 5.7 mmol) in dry DMF (1 mL) under nitrogen, and the resulting mixture was stirred for 30 min at room temperature. A solution of *tert*-butyl 2-(*R*)-(benzyloxycarbonyl)amino-3-iodopropionate **16** (1.53 g, 3.78 mmol) in DMF (1 mL) was added dropwise to the activated zinc over 5 min with ice cooling before being stirred at room temperature for 1 h to afford the serine-derived organozinc reagent. A solution of the iodobenzoxazole **7** (1 g, 1.72 mmol) in DMF (2 mL) was added to this mixture, followed by bis(triphenylphosphine) palladium(II) chloride (122 mg, 0.172 mmol), and the resultant mixture was stirred at 25 °C for 15 min before being warmed to 50 °C for 2 h. The reaction mixture was filtered through a Celite pad to remove the inorganic materials, and then poured into water (100 mL) and extracted with ethyl acetate (3 × 50 mL). The organic extracts were washed with water (30 mL) and brine (30 mL), dried over magnesium sulfate, and concentrated under reduced pressure to give a crude product as an oil. Flash chromatography over silica eluting with 2/1 petrol/ethyl acetate (R_f 0.26) yielded the pure bis-amino acid **5** as a pale yellow foam (845 mg, 67%). Also eluted was 63 mg (8%) of the bis-benzoxazole side product **17** (R_f 0.1). Data for bis(amino acid) **5**: $[\alpha]_D^{22}$ 5.9 (*c* 1.7, CH_2Cl_2); calcd for $C_{42}H_{43}N_3O_9$ [M^+] 733.2999, found 733.2983; MS (*m*/*z* EI$^+$, %) 733 (M^+, <1%), 632 (<1), 588 (1), 511 (12), 410 (8), 259 (8), 178 (100); IR ν_{max} (film) 3331, 3064, 2977, 2954, 1722 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.34 (9H, s), 2.25 (1H, m), 2.48 (1H, m), 2.96 (2H, m), 3.42 (2H, m), 4.21 (1H, t, J = 7), 4.42 (2H, d, J = 7), 4.65 (1H, m), 5.06 (2H, s), 5.62 (1H, d, J = 8), 6.61 (1H, d, J = 8), 7.15 (1H, d, J = 8.0), 7.23 (1H, t, J = 8), 7.30 (2H, m), 7.35 (5H, m), 7.38 (1H, d, J = 8), 7.40 (2H, m), 7.55 (2H, m), 7.75 (2H, d, J = 7.5); ^{13}C NMR (125 MHz, $CDCl_3$) δ 24.7, 27.8, 29.0, 33.9, 47.1, 52.5, 53.3, 55.2, 66.5, 67.0, 81.7, 109.2, 119.9, 124.6, 125.0, 125.6, 127.0, 127.6, 127.8, 127.9, 128.4, 128.6, 136.6, 140.3, 141.2, 143.6, 143.8, 150.5, 156.0, 165.4, 170.5, 172.0. Selected data for the side product (**17**): MS *m*/*z* (electrospray) 933 ([M + Na]$^+$, 23%), 737 (100), 711 (84); ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.13 (1H, m), 2.32 (1H, m), 2.87 (2H, m), 3.53 (3H, s), 3.94 (1H, *t*, J = 7), 4.16 (2H, d, J = 7), 5.65 (1H, d, J = 8), 7.06 (2H, m), 7.18 (2H, m), 7.24 (1H, m), 7.31 (1H, m), 7.33 (2H, m), 7.54 (2H, d, J = 7.5), 7.78 (1H, d, J = 7.5, ArH); ^{13}C NMR (125 MHz, $CDCl_3$) δ 25.0, 29.2, 47.1, 52.6, 53.6, 67.0, 109.7, 119.9, 124.7, 125.1, 125.8, 127.0, 127.6, 129.0, 139.2, 141.2, 143.7, 151.2, 156.0, 165.7, 172.2.

Methyl (2*S*)-4-{4-[(2*S*)-2-{[(Benzyloxy)carbonyl]amino}-3-(*tert*-butoxy)-3-oxopropyl]-1,3-benzoxazol-2-yl}-2-({(2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-phenylpropanoyl}-amino)butanoate (19). Coupled product **5** (440 mg, 0.6 mmol) was dissolved in DMF (5 mL), and piperidine (1 mL) was added at 0 °C. The solution was stirred under nitrogen at room temperature for 1 h before the solvents were removed by distillation in a Kugelrohr apparatus at a pressure of 1 mmHg and an oven temperature of 50 °C, affording the free amine as a beige solid. The crude free amine was taken up in DMF (10 mL) and *N*-hydroxybenzotriazole (92 mg, 0.6 mmol) added, followed by N^{α}-*t*-butoxycarbonylphenylalanine *N*-hydroxysuccinimide ester (217 mg, 0.6 mmol). After stirring under nitrogen for 48 h the solution was poured into water, producing a white precipitate, and extracted with ethyl acetate (3 × 100 mL). The organic extracts were washed with brine (30 mL) before being dried over magnesium sulfate and evaporated to afford an oil that was subjected to chromatography over silica eluting with 4/1 ether/petrol (R_f 0.24) to afford the tripeptide **19** as a colorless solid (259 mg, 57%): mp 56−58 °C; $[\alpha]_D^{22}$ −10.7 (*c* 0.14, CH_2Cl_2); calcd for $C_{41}H_{50}N_4O_{10}$ [M^+] 758.3527, found 758.3548; MS (*m*/*z* EI$^+$, %) 758 (M^+, <1%), 629 (5), 539 (8), 511 (14), 367 (40), 146 (54), 120 (62), 91 (100); IR ν_{max} (film) 3314, 3063, 2979, 1717, 1684 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.34 (9H, s), 1.41 (9H, s), 2.23, (1H, m), 2.48 (1H, m), 2.80−3.40 (6H, m), 3.67 (3H, s), 4.40−4.65 (3H, m), 5.00 (3H, m), 6.65 (1H, s), 6.89 (1H, d, J = 7.5), 7.2−7.4 (13H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 24.5, 28.0, 28.4, 28.8, 34.1, 38.0, 51.7, 52.7, 55.3, 55.8, 66.6, 82.0, 109.4, 125.0, 126.0, 127.0, 128.0, 128.1, 128.5, 128.7, 135.4, 136.2, 136.5, 136.7, 140.0, 150.4, 155.2, 156.0, 156.7, 165.6, 170.5, 171.4.

Methyl (2*S*)-4-{4-[(2*S*)-2-{[(Benzyloxy)carbonyl]amino}-3-(*tert*-butoxy)-3-oxopropyl]-1,3-benzoxazol-2-yl}-2-({(2*S*)-2-[(*tert*-butoxycarbonyl)amino]-3-(benzyloxycarbonyl)-

(27) Webster, K. L.; Rutherford, T. J.; Gani, D. *Tetrahedron Lett.* **1997**, *38*, 5713−5716.

propanoyl}amino)butanoate (20). Coupled product **5** (440 mg, 0.6 mmol) was deprotected as for the preparation of **19** using DMF (5 mL) and piperidine (1 mL). The crude free amine was taken up in DMF (10 mL) and *N*-hydroxybenzotriazole (92 mg, 0.6 mmol) added, followed by N^{α}-*t*-butoxycarbonylaspartic acid α-*N*-hydroxysuccinimide ester *β*-benzyl ester (252 mg, 0.6 mmol). After stirring under nitrogen for 24 h, the solution was poured into water, producing a white precipitate, and extracted with ethyl acetate (3 × 100 mL). The organic extracts were washed with brine (30 mL) before being dried over magnesium sulfate and evaporated to afford an oil that was subjected to chromatography over silica eluting with 4/1 ether/petrol (R_f 0.3) to afford the tripeptide **20** as a colorless oil (299 mg, 61%): $[\alpha]_D^{22}$ 12.1 (*c* 0.38, CH_2Cl_2); calcd for $C_{43}H_{52}N_4O_{12}$ [M^+] 816.3582, found 816.3553; MS (*m*/*z* EI$^+$, %) 816 (M^+, <1%), 615 (2), 539 (2), 466 (2), 146 (54), 91 (100); IR ν_{max} (film) 3314, 3062, 3032, 3004, 2979, 2955, 2932, 2872, 1717, 1684 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.35 (9H, s), 1.47 (9H, s), 2.24, (1H, m), 2.51 (1H, m), 2.74 and 2.91 (4H, m), 3.35 (2H, m), 3.70 (3H, s), 4.6−4.8 (3H, m), 5.1 (4H, m), 5.75 (1H, d, *J* = 7.5), 6.63 (1H, d, *J* = 8), 7.15 (1H, d, *J* = 7.5), 7.2−7.4 (12H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 24.5, 27.8, 28.2, 28.8, 33.8, 36.1, 50.7, 51.7, 52.5, 55.2, 66.5, 66.8, 81.7, 109.2, 124.5, 125.4, 128.1, 128.2, 128.2, 128.3, 128.4, 128.5, 128.5, 135.3, 136.2, 136.7, 140.4, 150.5, 155.4, 156.0, 156.6, 165.4, 170.6, 170.8, 171.5.

Methyl (2*S*)-4-{4-[(2*S*)-2-{[(Benzyloxy)carbonyl]amino}-3-(*tert*-butoxy)-3-oxopropyl]-1,3-benzoxazol-2-yl}-2-({(2*S*)-2-[(*tert*-butoxycarbonyl)amino]-6-phthalimidohexanoyl}amino)butanoate (21). Coupled product **5** (440 mg, 0.6 mmol) was deprotected as for the preparation of **19** using DMF (5 mL) and piperidine (1 mL). The crude free amine was taken up in DMF (10 mL) and *N*-hydroxybenzotriazole (92 mg, 0.6 mmol) added, followed by *N*-$^{\alpha}$*t*-butoxycarbonyl-N^{ϵ}-phthalimidolysine *N*-hydroxysuccinimide ester (284 mg, 0.6 mmol). After stirring under nitrogen for 24 h, the solution was poured into water, producing a white precipitate, and extracted with ethyl acetate (3 × 100 mL). The organic extracts were washed with brine (30 mL) before being dried over magnesium sulfate and evaporated to afford an oil that was subjected to chromatography over silica eluting with 4/1 ether/petrol (R_f 0.2) to afford the tripeptide **21** as a colorless oil (287 mg, 55%): $[\alpha]_D^{22}$ 3.2 (*c* 0.56, CH_2Cl_2); calcd for $C_{34}H_{44}N_4O_{10}$ [$M - C_{12}H_{11}NO_2$] 668.3057, found 668.3025; MS (*m*/*z* EI$^+$, %) 869 (M^+, <1%), 668 (3), 539 (7), 423 (15), 367 (15), 231 (25), 108 (60), 57 (100); IR ν_{max} (film) 3320, 2978, 2939, 1771, 1713, 1630, 1575 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.41−1.45 (2H, m), 1.32 (9H, s), 1.41 (9H, s), 1.59−1.66 (2H, m), 1.79−1.87 (1H, m), 2.23−2.27 (1H, m), 2.29−2.36 (1H, m), 2.46−2.54 (1H, m), 3.36 (1H, dd, *J* = 14, 7.5), 3.42 (1H, dd, *J* = 14, 4.5), 3.62−3.68 (2H, m), 3.65 (3H, s), 4.07−4.12 (1H, m), 4.61−4.65 (1H, m), 4.67−4.75 (1H, m), 5.03 and 5.12 (2H, AB, *J* = 12), 5.30 (1H, d, *J* = 7.5), 6.75 (1H, d, *J* = 6.5), 7.09 (1H, d, *J* = 8), 7.12 (1H, d, *J* = 7.5), 7.20 (1H, t, *J* = 7.5), 7.27−7.36 (5H, m), 7.34 (1H, d, *J* = 8.5), 7.65−7.69 (2H, m), 7.80−7.83 (2H, m); ^{13}C NMR (125 MHz, $CDCl_3$) δ 22.5, 24.7, 27.8, 28.2, 28.5, 28.6, 31.5, 33.8, 37.2, 51.6, 52.4, 54.4, 55.1, 66.4, 79.8, 81.7, 109.2, 123.1, 124.5, 125.5, 127.8, 127.8, 128.3, 128.5, 132.0, 133.8, 136.7, 140.3, 150.4, 155.0, 156.3, 165.6, 168.3, 171.0, 171.6, 172.2.

***N*$^{\alpha}$-*t*-butoxycarbonyl-*N*$^{\epsilon}$-phthalimidolysine *N*-Hydroxysuccinimide Ester.** To a suspension of N^{α}-*t*-butoxycarbonyl-N^{ϵ}-phthalimidolysine (0.8 g, 2.13 mmol) in CH_2Cl_2 (25 mL) was added EDCI (490 mg, 2.56 mmol), giving a solution. *N*-Hydroxysuccinimide (294 mg, 2.56 mmol) was added and the solution stirred under nitrogen for 48 h before being washed with brine, 1 M citric acid, and brine and dried over magnesium sulfate to afford the title compound as a fluffy white solid that was used without further purification (806 mg, 80%): calcd for $C_{19}H_{18}N_3O_7$ [$M - t$-BuO]$^+$ 400.1145, found 400.1151; MS (*m*/*z* EI$^+$, %) M^+ not found, 400 ($M - {}^t$BuO, 30%), 303 (32), 160 (56), 57 (100); IR ν_{max} (KBr) 3372, 2977, 2943, 1818, 1773, 1743, 1710 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.42 (9H, s), 1.54 (2H, m), 1.73 (2H, m), 1.84 (1H, m), 1.93 (1H, m, CH*H*), 2.82 (4H, s), 3.72 (2H, t, *J* = 7), 4.65 (1H, m), 5.06 (1H, d, *J* = 7.5), 7.72 (2H, dd, *J* = 5, 3), 7.84 (2H, dd, *J* = 5, 3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 22.2, 25.5, 28.0, 28.2, 32.2, 37.3, 51.8, 80.5, 123.2, 132.2, 133.8, 154.2, 168.4 (3 × C=O coincident).

Methyl (7*S*,10*S*,13*S*)-10-Benzyl-7-{[(benzyloxy)carbonyl]amino)-8,11-dioxo-19-oxa-9,12,17-triazatricyclo-[14.2.1.0^{6,18}]nonadeca-1(18),2,4,16-tetraene-13-carboxylate (25). The cyclization precursor **19** (190 mg, 0.25 mmol) was stirred in trifluoroacetic acid (4 mL) for 1 h, protected by a calcium chloride guard tube. The trifluoroacetic acid was evaporated under reduced pressure and the residue triturated with ether, concentrated under reduced pressure, and thoroughly dried under high vacuum for 6 h to afford the trifluoroacetate salt **22** as a white solid. This residue was dissolved in DMF (20 mL) and then treated with diisopropylethylamine (323 mg, 431 *μ*L, 2.5 mmol), and the mixture was stirred at room temperature for 15 min. This solution was slowly added, over 2 h, to HATU (380 mg, 1 mmol) in DMF (30 mL) at 0 °C, and then the resulting mixture was stirred for 16 h at room temperature under nitrogen before being poured into water (50 mL) and extracted with ether (3 × 30 mL). The organic extracts were washed with brine (20 mL) and then dried over magnesium sulfate to give a solid that was purified by silica chromatography eluting with 90% CH_2Cl_2/10% MeOH to yield the macrocylic tripeptide **25** (R_f 0.64) as a crystalline white solid (70 mg, 48%): mp 221−222 °C; $[\alpha]_D^{22}$ −6.7 (*c* 0.24, CH_2Cl_2); calcd for $C_{32}H_{32}N_4O_7$ [M^+] 584.2271, found 584.2263; MS (*m*/*z* EI$^+$, %) 584 (M^+, 37%), 476 (32), 302 (25), 91 (100); IR ν_{max} (film) 3303, 3063, 3030, 2951, 2930, 2855, 1722, 1645 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.29−2.33 (1H, m), 2.47 (1H, ddt, *J* = 15.5, 9.5, 3), 2.85 (1H, dd, *J* = 13.5, 6), 2.88 (1H, ddd, *J* = 18.5, 9.5, 2), 2.94 (1H, ddd, *J* = 18.5, 7.5, 2.5), 3.18 (1H, dd, *J* = 13.5, 6), 3.34 (1H, dd, *J* = 14, 6.5), 3.42 (1H, dd, *J* = 14, 2.5), 3.68 (3H, s), 4.40 (1H, td, *J* = 6.5, 2.5), 4.50 (1H, td, *J* = 6.5, 3.5), 4.65 (1H, dt, *J* = 9, 6), 5.09 and 5.12 (2H, AB, *J* = 12.5), 5.59 (1H, d, *J* = 9), 5.75 (1H, d, *J* = 6.5), 6.98 (1H, dd, *J* = 8, 2), 7.01 (1H, d, *J* = 7.5), 7.10−7.14 (4H, m), 7.24 (1H, dd, *J* = 8, 1), 7.30−7.34 (6H, m), 8.49 (1H, d, *J* = 6); ^{13}C NMR (125 MHz, $CDCl_3$) δ 25.0, 25.3, 33.3, 37.4, 52.6, 53.7, 54.3, 55.2, 67.0, 109.6, 125.0, 126.3, 127.2, 128.2, 128.3, 128.7, 128.9, 129.4, 136.1, 136.6, 139.7, 150.7, 155.6, 166.9, 170.1, 170.6, 171.3.

Methyl (7*S*,10*S*,13*S*)-10-(Benzylethanoate)-7-{[(benzyloxy)carbonyl]amino)-8,11-dioxo-19-oxa-9,12,17-triazatricyclo[14.2.1.0^{6,18}]nonadeca-1(18),2,4,16-tetraene-13-carboxylate (26). Employing the macrocyclization procedure described for the preparation of **25** and using the cyclization precursor **20** (204 mg, 0.25 mmol) afforded macrocylic tripeptide **26** (52.3 mg, 41%) as a crystalline white solid: mp 172−173 °C; $[\alpha]_D^{22}$ 4.1 (*c* 0.22, CH_2Cl_2); calcd for $C_{34}H_{34}N_4O_9$ [M^+] 642.2326, found 642.2338; MS (*m*/*z* EI$^+$, %) 642 (M^+, 13%), 491 (13), 270 (10), 242 (10), 146 (80), 91 (100); IR ν_{max} (KBr) 3419, 3387, 3347, 3065, 3030, 2996, 2957, 2900, 2885, 1747, 1725, 1698, 1684, 1645 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.40−2.44 (1H, m), 2.50 (1H, dd, *J* = 17.5, 4), 2.63−2.68 (1H, m), 2.99 (1H, dd, *J* = 19, 10.5), 3.10 (1H, dd, *J* = 19, 6.5), 3.34 (1H, dd, *J* = 17.5, 3.5), 3.42 (1H, dd, *J* = 14, 6), 3.67 (1H, dd, *J* = 14, 2.5), 3.75 (3H, s), 4.54 (1H, dd, *J* = 8.5, 4.5), 4.62−4.65 (1H, m), 4.89−4.93 (1H, m), 4.99 and 5.08 (2H, AB, *J* = 12.0), 5.09 and 5.13 (2H, AB, *J* = 12), 5.75 (1H, d, *J* = 7.5), 6.97 (1H, d, *J* = 9.5), 7.06 (1H, d, *J* = 7.5), 7.22 (1H, t, *J* = 8), 7.33−7.40 (11H, m), 9.74 (1H, d, *J* = 4.5); ^{13}C NMR (125 MHz, $CDCl_3$) δ 24.2, 24.4, 32.8, 35.2, 49.2, 52.2, 53.7, 54.4, 67.0, 67.1, 109.4, 124.8, 126.4, 126.6, 128.1, 128.2, 128.4, 128.5, 128.6, 135.1, 136.3, 139.4, 150.7, 155.4, 167.4, 170.1, 170.5, 171.2, 172.5.

Methyl (7*S*,10*S*,13*S*)-10-(4′-Phthalimidobutyl)-7-{[(benzyloxy)carbonyl]amino)-8,11-dioxo-19-oxa-9,12,17-triazatricyclo[14.2.1.0^{6,18}]nonadeca-1(18),2,4,16-tetraene-13-carboxylate (27). Employing the macrocyclization procedure described for the preparation of **25** and using the cyclization precursor **24** (217 mg, 0.25 mmol) afforded macrocylic tripep-

tide **27** (75 mg, 43%) as a crystalline white solid: mp 189−190 °C; $[\alpha]_D^{22}$ 16.7 (*c* 0.12, CH_2Cl_2); calcd for $C_{37}H_{37}N_5O_9$ [M^+] 695.2591, found 695.2586; MS (*m*/*z* EI$^+$, %) 695 (M^+, 12%), 587 (36), 219 (70), 131 (50), 91 (92), 69 (100); IR ν_{max} (KBr) 3313, 3064, 3032, 2942, 2860, 1772, 1739, 1712, 1646 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.24−1.32 (2H, m), 1.58−1.64 (1H, m), 1.67−1.73 (1H, m), 1.76−1.78 (1H, m), 1.98−2.01 (1H, m), 2.38−2.44 (1H, m), 2.60−2.65 (1H, m), 2.96 (1H, dd, *J* = 18.5, 11.5), 3.09 (1H, dd, *J* = 18.5, 7.5, H), 3.46−3.50 (1H, m), 3.65 (1H, dd, *J* = 13.5, 6.5), 3.66 (2H, t, *J* = 5.5), 3.75 (3H, s), 4.33−4.37 (1H, m), 4.65−4.68 (1H, m), 4.73−4.75 (1H, m), 5.10 and 5.18 (2H, AB, *J* = 12.5), 5.76 (1H, d, *J* = 7), 6.58 (1H, d, *J* = 7.5), 7.04 (1H, d, *J* = 7.5), 7.17 (1H, t, *J* = 8), 7.31−7.34 (6H, m), 7.70 (2H, dd, *J* = 4.5, 3), 7.86 (2H, dd, *J* = 4.5, 3), 8.84 (1H, d, *J* = 6); ^{13}C NMR (125 MHz, $CDCl_3$) δ 22.2, 25.2, 28.1, 30.7, 33.2, 36.6, 52.5, 53.4, 53.8, 54.5, 66.8, 109.3, 123.5, 124.7, 126.4, 127.2, 128.1, 128.6, 132.0, 134.2, 136.6, 139.6, 150.7, 155.4, 166.7, 169.0, 170.8, 171.5, 171.6.

Methyl (7*S*,10*S*,13*S*)-7-(Acetylamino)-10-benzyl-8,11-dioxo-19-oxa-9,12,17-triazatricyclo[14.2.1.0^{6,18}]nonadeca-1(18),2,4,16-tetraene-13-carboxylate (28). A solution of the macrocycle **25** (44 mg, 0.08 mmol) in acetic acid (5 mL) and acetic anhydride (0.5 mL) was hydrogenated at room temperature (H_2 = 1 atm) over a catalytic amount (10 mol %) of 5% palladium on carbon. After 48 h, the mixture was filtered through a Celite pad and the solvents evaporated. The crude residue was taken up in CH_2Cl_2 (30 mL), washed with a potassium bicarbonate solution (2 × 30 mL) and brine (30 mL), dried over magnesium sulfate, and evaporated to afford the acetamido derivative **28** as a white solid (33 mg, 85%), which was used without further purification: 90% CH_2Cl_2/10% MeOH (R_f 0.5); $[\alpha]_D^{22}$ −12.5 (*c* 0.2, CH_2Cl_2); calcd for $C_{26}H_{28}N_4O_6$ [M^+] 492.2009, found 492.2021; MS (*m*/*z* EI$^+$, %) 492 (M^+, 100%), 449 (15), 433 (18), 174 (56), 120 (73), 91 (76); IR ν_{max} (film) 3286, 3063, 3031, 2952, 2928, 1741, 1634 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.28−2.34 (1H, m), 2.46−2.50 (1H, m), 2.86−2.98 (3H, m), 3.10 (1H, dd, *J* = 14.0, 6.5), 3.36 (1H, dd, *J* = 13.5, 6.5), 3.42 (1H, dd, *J* = 13.5, 2.5), 3.68 (3H, s), 4.51−4.55 (1H, m), 4.62−4.68 (2H, m), 5.98 (1H, d, *J* = 8.5), 6.49 (1H, d, *J* = 6.5), 6.99 (1H, dd, *J* = 7.5, 2.5), 7.03 (1H, d, *J* = 7.5), 7.08−7.17 (4H, m), 7.16 (1H, t, *J* = 8), 7.25 (1H, d, *J* = 8), 8.63 (1H, d, *J* = 5.5); ^{13}C NMR (125 MHz, $CDCl_3$) δ 23.3, 24.8, 25.1, 32.8, 37.4, 52.5, 53.4, 53.6, 54.3, 109.4, 124.7, 126.0, 127.0, 127.4, 128.7, 129.2, 136.1, 139.6, 150.6, 165.8, 169.6, 170.3, 170.6, 171.2.

Methyl (7*S*,10*S*,13*S*)-7-(Acetylamino)-10-(4′-phthalimidobutyl)-8,11-dioxo-19-oxa-9,12,17-triazatricyclo[14.2.1.0^{6,18}]-nonadeca-1(18),2,4,16-tetraene-13-carboxylate (31). Macrocycle **27** (52 mg, 0.08 mmol) was reacted under an atmosphere of hydrogen for 24 h, according to the procedure above, to give the acetamide **31** as a white solid (36 mg, 75%), which was used without further purification: 90% CH_2Cl_2/10% MeOH (R_f 0.65); mp 168−170 °C; $[\alpha]_D^{22}$ −14.0 (*c* 0.2, CH_2Cl_2); calcd for $C_{31}H_{33}N_5O_3$ [M^+] 603.2334; found 603.2329; MS (*m*/*z* EI$^+$, %) 603 (M^+, 100), 560 (21), 559 (23), 546 (25), 257 (37), 244 (61), 231 (76), 187 (45), 174 (63), 160 (54), 146 (43), 84 (75); IR ν_{max} (KBr) 3327, 2925, 2853, 1772, 1741, 1711, 1684, 1640 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.19−1.25 (2H, m), 1.61−1.63 (1H, m), 1.70−1.74 (1H, m), 1.99 (3H, s), 1.81−1.84 (1H, m), 2.02−2.13 (1H, m), 2.45−2.49 (2H, m), 2.57−2.63 (1H, m), 2.94−2.99 (1H, m), 3.14−3.16 (1H, m), 3.65−3.71 (1H, m), 3.73−3.75 (1H, m), 3.67 (2H, t, *J* = 7.0), 3.76 (3H, s), 4.33−4.37 (1H, m), 4.66 (1H, td, *J* = 6, 3), 4.93 (1H, td, *J* = 6.5, 2), 6.41 (1H, d, *J* = 6.5), 6.56 (1H, d, *J* = 7.5), 7.10 (1H, d, *J* = 7.0), 7.23 (1H, t, *J* = 8), 7.36 (1H, d, *J* = 7.5), 7.76 (2H, dd, *J* = 5.5, 3), 7.92 (2H, dd, *J* = 5.5, 3), 9.11 (1H, d, *J* = 6); ^{13}C NMR (125 MHz, $CDCl_3$) δ 22.1, 23.3, 24.9, 25.0, 28.0, 29.7, 32.8, 36.3, 52.4, 53.2, 53.3, 54.0, 109.3, 123.5, 124.6, 126.4, 127.4, 131.8, 134.3, 139.8, 150.7, 166.7, 169.1, 169.4, 171.3, 171.4, 171.5. Anal. Calcd for $C_{31}H_{33}N_5O_3$: C, 61.68; H, 5.51; N, 11.60. Found: C, 61.6; H, 5.4; N, 11.7.

7-Acetylamino-10-carboxymethyl-8,11-dioxo-19-oxa-9,12,17-triazatricyclo[14.2.1.0^{5,18}]nonadeca-1(18),2,4,16-tetraene-13-carboxylic Acid Methyl Ester (30). A mixture of the macrocycle **26** (48 mg, 0.08 mmol) and a catalytic amount (10 mol %) of 5% palladium on carbon in acetic acid (5 mL) and acetic anhydride (0.5 mL) was hydrogenated at room temperature for 24 h before being filtered through a Celite pad, and the solvents were removed. The crude residue was taken up in CH_2Cl_2 (30 mL), washed with water (10 mL) and brine (30 mL), dried over magnesium sulfate, and concentrated under reduced pressure to afford the acetamide free acid **30** as a white solid (35 mg, 95%), which was used without further purification: 90% CH_2Cl_2/10% MeOH (R_f 0.08); mp 135−137 °C; $[\alpha]_D^{22}$ −17.6 (*c* 0.2, CH_2Cl_2); calcd for $C_{21}H_{24}N_4O_8$ [M^+] 460.1586, found 460.1594; MS (*m*/*z* EI$^+$, %) 460 (M^+, 61), 401 (31), 279 (53), 205 (28), 167 (32), 149 (100), 57 (42); MS (*m*/*z* CI$^+$, %) 461 (MH^+, 100), 149 (26), 279 (18), 167 (28), 149 (58), 83 (37); IR ν_{max} (KBr) 3500−3308, 2955, 2926, 2854, 1741, 1651 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.01 (3H, s), 2.34−2.39 (1H, m), 2.58−2.62 (1H, m), 2.61 (1H, dd, *J* = 17.5, 4.5), 2.94 (1H, dd, *J* = 19, 11.5), 3.02 (1H, dd, *J* = 17.5, 5), 3.04 (1H, dd, *J* = 19.0, 8), 3.28 (1H, dd, *J* = 14, 6), 3.64−3.66 (1H, m), 3.68 (3H, s), 4.51−4.54 (1H, m), 4.83−4.87 (1H, m), 5.01−5.04 (1H, m), 6.63 (1H, s), 6.99 (1H, d, *J* = 7.5), 7.20 (1H, t, *J* = 8), 7.32 (1H, d, *J* = 8.5), 7.52 (1H, d, *J* = 8.5), 9.64 (1H, d, *J* = 4.5); ^{13}C NMR (125 MHz, $CDCl_3$) δ 23.3, 24.4, 24.7, 33.0, 35.3, 49.6, 52.5, 53.0, 53.8, 109.6, 124.9, 126.3, 127.2, 139.6, 150.9, 167.5, 170.4, 171.0, 171.2, 171.4, 174.8.

(7*S*,10*S*,13*S*)-7-(Acetylamino)-10-benzyl-8,11-dioxo-19-oxa-9,12,17-triazatricyclo[14.2.1.0^{6,18}]nonadeca-1(18),2,4,16-tetraene-13-carboxamide (29). Lithium hydroxide monohydrate (5 mg, 0.12 mmol) was added to a suspension of macrocycle **28** (39.4 mg, 0.08 mmol) in 2 mL of 1/1 acetonitrile/water, and the resulting yellow solution was stirred for 90 min at room temperature. The solvents were evaporated, and the residue was partitioned between ethyl acetate (3 × 10 mL) and water (10 mL). The pH of the aqueous layer was adjusted to 2 with 1 M HCl, extracted with ethyl acetate (4 × 3 mL), and washed with brine (10 mL). The combined organic phases were dried over magnesium sulfate and concentrated to give 37 mg (96%) of the corresponding acid as a white powder that was used directly. To a solution of the acid (5 mg, 0.12 mmol) in THF (13 mL) were added *N*-hydroxybenzotriazole (16 mg, 0.105 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) (13.5 mg, 0.07 mmol). This mixture was stirred at room temperature for 1 h, 25% aqueous ammonia (24.5 mg, 27 μL, 0.07 mmol) was added slowly and the reaction stirred for an additional 2 h at room temperature. After evaporation, the residue was taken up in CH_2Cl_2 (10 mL) and washed with saturated sodium bicarbonate solution (3 × 30 mL). The organic layer was washed with brine (10 mL) and dried over magnesium sulfate. The solvent was evaporated to give a white solid, which was subjected to chromatography over silica (95% CH_2Cl_2/5% MeOH) to afford the acetamido primary amide **29** as a white solid (19.4 mg, 58%): 90% CH_2Cl_2/10% MeOH (R_f 0.4); mp 152−154 °C; $[\alpha]_D^{22}$ −9.1 (*c* 0.2, CH_2Cl_2); calcd for $C_{25}H_{27}N_5O_5$ [M^+] 477.1999, found 477.2012; MS (*m*/*z* EI$^+$, %) 477 (M^+, 81), 433 (100), 341 (51), 299 (69), 286 (82), 258 (55), 216 (91), 174 (63), 120 (68), 91 (84); MS (*m*/*z* CI$^+$, %) 478 (MH^+, 76), 461 (74), 433 (100), 286 (34), 258 (39), 216 (30), 187 (29), 120 (64), 91 (80); IR ν_{max} (KBr) 3410, 3297, 3062, 3031, 2924, 2852, 1665, 1651 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.82 (3H, s), 2.03−2.06 (1H, m), 2.40−2.46 (1H, m), 2.73 (1H, ddd, *J* = 18.5, 7, 5), 2.97−3.01 (1H, m), 2.93 (1H, dd, *J* = 14.5, 5), 3.01 (1H, dd, *J* = 14.5, 7), 3.19 (1H, dd, *J* = 13.5, 2), 3.34 (1H, dd, *J* = 13.5, 9), 4.25 (1H, dt, *J* = 6.5, 5), 4.51−4.54 (2H, m), 5.42 (1H, s), 5.60 (1H, d, *J* = 6), 6.40−6.42 (2H, m), 6.85−6.87 (1H, m), 6.98 (1H, d, *J* = 7.5), 7.08−7.10 (5H, m), 7.67 (1H, d, *J* = 8), 8.75 (1H, d, *J* = 7.5); ^{13}C NMR (125 MHz, $CDCl_3$) δ 23.3, 24.9, 25.1, 32.8, 37.2, 53.1, 53.7, 56.3, 109.8, 125.1, 126.7, 127.1, 127.7, 128.8, 129.2, 135.6, 138.9, 150.7,

167.6, 170.0, 170.2, 172.6, 173.4. Anal. Calcd for $C_{25}H_{27}N_5O_5$: C, 62.88; H, 5.70; N, 14.67. Found: C, 63.2; H, 5.8; N, 14.6.

7-Acetylamino-10-(4-amino-butyl)-8,11-dioxo-19-oxa-9,12,17-triazatricyclo[14.2.1.0^{5,18}]nonadeca-1(18),2,4,16-tetraene-13-carboxylic Acid Methyl Ester (32). Hydrazine monohydrate (44 mg, 43 μL, 0.88 mmol) was added to a solution of macrocycle **31** (24 mg, 0.04 mmol) in ethanol (1 mL). The solution was stirred for 4 h at room temperature during which time a precipitate formed. The reaction mixture was filtered, and the solvent was removed under reduced pressure to afford the crude free amino amine as a white solid (22 mg), which was purified by HPLC using a Phenomonex Luna C18 (1) column (150 × 21.2 mm, 5 um particle size), with mobile phase A of MeCN/water/TFA (5:95:0.1 v/v/v) from JT Baker and mobile phase B of MeCN (Riedel-de-Haan, Chromasolv, far UV grade), a flow rate of 17 mL/min, and UV detection at 225 and 254 nm, to give the free amino derivative **32** (4 mg, 20%): calcd for $C_{23}H_{31}N_5O_6$ [M$^+$] 473.2274; found 473.2259; MS (*m*/*z* EI$^+$, %) 474 (MH$^+$, 41), 473 (M$^+$, 135), 415 (11), 319 (13), 281 (11), 265 (14), 248 (10), 231 (24), 220 (12), 163 (48), 149 (13), 132 (12), 104 (100), 76 (39), 65 (54); MS (*m*/*z* electrospray, %) 496 [M + Na, 11%], 474 (MH$^+$, 100), 163 (17); ^{1}H NMR (400 MHz, CD_3OD) δ 1.34−1.47 (2H, m), 1.51−1.65 (2H, m), 1.91−2.00 (1H, m), 2.05 (3H, s), 2.40 (2H, dquint, J = 8, 2.5), 2.58−2.65 (1H, m), 2.88−2.94 (1H, m), 2.88 (2H, brt, J = 7.5), 3.02 (1H, ddd, J = 18.5, 10.5, 2), 3.17 (1H, ddd, J = 18.5, 8, 2), 3.37 (1H, dd, J = 13.5, 6.5), 3.60 (1H, dd, J = 13.5, 3), 3.74 (3H, s), 4.32−4.38 (1H, m), 4.57−4.61 (1H, m), 4.67 (1H, dd, J = 6.5, 3), 7.22 (1H, d, J = 8), 7.32 (1H, t, J = 8), 7.44 (1H, d, J = 8.5), 9.33 (1H, d, J = 6.5).

Protease Inhibition Testing. Compounds were evaluated for their ability to inhibit the serine proteases, Factor Xa, Tissue Factor/Factor VIIa, thrombin, and trypsin. In vitro kinetic assays were conducted at 37 °C using a kinetic spectrophotometric plate reader. An optimized concentration of human enzyme in buffer (0.5 nM final concentration for thrombin and trypsin, 1 nM for Factor Xa, and 10 nM for Factor VIIa, all from Enzyme Research Laboratories) was combined with 2 μL of inhibitor dilutions (creating a final concentration range from 1 nM to 100 μM) and preincubated for 1 h at room temperature. The assay was initiated by the addition of an appropriate synthetic substrate [S-2765 (Pharmacia Hepar) for Factor Xa, CHROMOZYM TH (Boehringer Mannheim) for thrombin, Spectrozyme VIIa (American Diagnostica) for Tissue Factor/Factor VIIa, and S-2222 (Pharmacia Hepar) for trypsin], at predetermined $2K_m$. The absorbance at 405 nm was determined over 10 min, and the percent inhibition was calculated from the slope of the progress curves during the linear part of the time course at each concentration. Since the test compounds exhibited poor inhibitory activity and failed to reach an IC_{50}, they were instead reported as % inhibition at the highest concentration tested.[28] The above experiment was repeated to give either two or three measurements of inhibition for each test.

Caspase-9 was supplied by Biomol (UK distributor Affiniti, cat no. SE-173), stored at −80 °C, and used at 30 units/sample (activity = 12 units/μg; 1 unit = 1 pmol/minute at 25 °C using Ac-LEHD-pNA substrate). The LEHD-AFC substrate used was from a Caspase-9 Fluorometric assay kit from Immunokontact (UK distributor AMS UK, Cat. No. 310-BV-6F9C). The substrate was stored in the dark at −20 °C at a 1 mM concentration and used at 15 μL per well and a 150 μM final concentration. Half-log dilutions of the test compound were made in buffer consisting of 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, and 10% glycerol. Just prior to use, this buffer was supplemented with DTT to a final concentration of 10 mM, within the range from 100 μM to 10 nM. The resulting dilutions were added (5 μL) to wells in duplicate. In addition, the following controls were included in the assay design: duplicates for no-caspase to find the background fluorescence, add 5 μL of lysis buffer with DMSO in proportion to that of the top compound concentration; duplicates for no-inhibitor to find the highest rate of activity, add 5 μL of buffer as above; the standard inhibitor Z-LEHD-FMK (Alexis biochemicals cat. no. 260−076-M001) at its estimated K_m value (2.23 nM) included as a positive control to inhibit the activity by 50%. Caspase-9 (2 μL per sample diluted with 48 μL of the above buffer) was preincubated with inhibitor (50 μL volume) at 20 °C for 30 min. Substrate (15 μL diluted to 50 μL with above buffer) was then added, and the samples were read in a Fluostar fluorometer with 390 nm excitation and 520 nm emission filters running Biolise software on a kinetics program, every 45 s for 2 h.[28] The mean V values (the slopes of the kinetic curves) were derived from the linear part of the curve, determined for this assay as all readings from 0 to 50. The percent inhibition at that concentration is calculated by the formula (V sample mean)/(V no-inhibitor mean) × 100.

The chymotrypsin assay was conducted using α-chymotrypsin from bovine pancreas (Sigma C-3142) diluted to 1 mg/mL in 0.001 N HCl, diluted 1/100 to 1 μg/mL, and then diluted 1/12.5 to give an 800 ng/mL solution. 3-Carbomethoxypropionyl-L-argininyl-L-prolyl-L-tyrosine-*p*-nitroaniline hydrochloride (Kabi, S-2586) was used as a substrate, stored as a 0.01 M frozen stock solution and diluted 1/5 to give a 0.002 M solution for testing. The test inhibitor was dissolved in water to give a 0.01 M solution and tested in the range from 10^{-4} to 3×10^{-8} M in the assay. Soybean Trypsin inhibitor (Sigma T-9003, 1000 U/mg) was used as a standard inhibitor, diluted to give a 10^{-4} M stock solution, and tested over the range from 10^{-5} to 3×10^{-9} M. The assay was conducted in a Fluostar fluorometer utilizing 20 μL of inhibitor solution, 140 μL of buffer (equal mixture of 100 mM Tris, 940 mM NaCl, and 10 μM $CaCl_2$ at pH 8.3), 20 μL of enzyme solution, and 20 μL of substrate solution.

NMR experiments to identify the chemical modification of **29** were carried out by standard homonuclear 1D proton, 2D COSY, and 2D TOCSY methods. These experiments were carried out for solutions containing α-chymotrypsin (Sigma C-3142) (50 μM) and **29** (500 μM) in 50 mM phosphate buffer pH 7.0 with 50 mM NaCl at 20 °C after 18 h of incubation at room temperature. Control 1D proton and 2D COSY experiments were run for chymotrypsin (50 μM) and **29** (500 μM) in the presence of a cocktail of protease inhibitors (Roche Complete, 40 μL of stock solution prepared by dissolving 1 tablet in 2 mL of phosphate buffer and adding the solution to NMR sample) in 50 mM phosphate buffer pH 7.0 with 50 mM NaCl at 20 °C after 18 h of incubation at room temperature.

Acknowledgment. We thank S. Rapundalo (Biochemistry Department, Pfizer Global Research & Development, Ann Arbor, MI) and R. Ward and E. Hawkeswood (Discovery Biology, Pfizer Global Research & Development, Ramsgate Road, Sandwich, Kent, UK) for carrying out the biological assays and D. Bull (Pfizer Global Research & Development, Ramsgate Road, Sandwich, Kent, UK) for the HPLC purification of compound **32**. We also thank Professor W. McFarlane (University of Newcastle) and T. Peakman (Pfizer Global Research & Development, Ramsgate Road, Sandwich, Kent, UK) for NMR studies.

Supporting Information Available: Experimental procedures for the preparation of compounds **11**−**13** and **15**; ^{1}H and ^{13}C NMR spectra for compounds **5**, **7**, **9**, **10**, **12**, **13**, **15**−**17**, **19**−**21**, and **25**−**31** and ^{1}H spectrum for **32**; and a summary of ROESY data for **25**. This material is available free of charge via the Internet at http://pubs.acs.org.

JO025615O

(28) Dudley, D. A.; Bunker, A. M.; Chi, L. G.; Cody, W. L.; Holland, D. R.; Ignasiak, D. P.; Janiczek-Dolphin, N.; McClanahan, T. B.; Mertz, T. E.; Narasimhan, L. S.; Rapundalo, S. T.; Trautschold, J. A.; Van Huis, C. A.; Edmunds, J. J. *J. Med. Chem.* **2000**, *43*, 4063−4070.