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# Synthesis and Evaluation of Macrocyclic Transition State Analogue Inhibitors for α-Chymotrypsin

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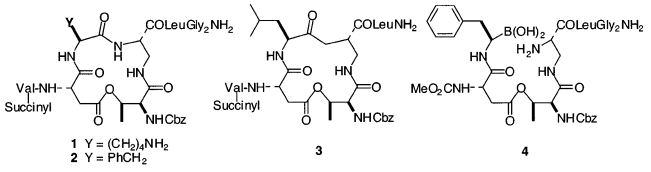
Lactams **1** and **2** are readily formed from acyclic precursors in the presence of trypsin and chymotrypsin, respectively, identifying the macrocyclic ring system as a potential motif for constrained transition state analogue inhibitors of the serine peptidases. Ketone **3** was synthesized and shown to be a modest inhibitor of chymotrypsin ($K_i$ = 220 $\mu$M), albeit 4-fold more potent than the acyclic hydroxy acid **25** ($K_i$ = 1.5 mM as a mixture of epimers). A precursor (**31**) to the amino boronic acid **4** was also prepared; although this derivative was a potent inhibitor of chymotrypsin ($K_i$ = 130 nM) by virtue of the boronic acid moiety, it showed no advantage over the des-amino analogue **32** ($K_i$ = 120 nM), which is not capable of cyclizing.

## Design of Inhibitors

In the preceding paper, we describe the application of an on-bead, enzyme-catalyzed cyclization process as a way to screen for macrocyclic frameworks of potential relevance in the design of conformationally constrained peptidase inhibitors.[1] In this report, we present the synthesis and evaluation of some transition state analogue inhibitors based on a macrocyclic motif identified in this manner. The ring system represented by lactams **1** and **2** is one of the simplest of those whose formation is readily catalyzed by trypsin and α-chymotrypsin. Thus, to assess the validity of this approach for the identification of effective inhibitor scaffolds, ketone **3** and boronic acid **4** were synthesized and evaluated as inhibitors of α-chymotrypsin.




The substrate-like lactam **2** was included among the analogues to be synthesized and evaluated because of the precedents of naturally occurring cyclic depsipeptides as potent inhibitors of serine proteases (see, for example, the cyanopeptolins).[2] Peptidyl boronic acids are among the most potent inhibitors of serine proteases known.[3−6] The ketomethylene derivative **3** was prepared as a stable, macrocyclic transition state analogue.[7] For synthetic expediency, an isobutyl group representing a leucine side chain was incorporated at the P1 position in place of the phenylalanine benzyl group, even though this replacement was expected to reduce the binding affinity approximately 16-fold (based on the rates of deacylation for a series of acyl-α-chymotrypsins).[8,9] Finally, compound **4** was synthesized to explore the possibility that, with a favorable ring conformation, a cyclic boronate ester could be formed in the enzyme active site and lead to even more potent inhibition.[10]

## Synthesis of Inhibitors

**Lactam 2.** PEGA (poly(ethylene glycol)-polyacrylamide) beads bearing a photocleavable linker[11] were subjected to four cycles of standard solid-phase peptide synthesis to afford resin-linked $N^{\alpha}$-Boc-$N^{\beta}$-Fmoc-(*S*)-α,β-diaminopropanoyl-Leu-Gly-Gly, **5** (Scheme 1).[12] Deprotecting the β-amino group followed by acylation with *N*-Cbz-threonine gave alcohol **6**, which was acylated with *N*-Fmoc-L-aspartic acid 1-allyl ester to give ester **7**. After cleavage of the allyl protecting group,[13] the resulting acid

† The Center for New Directions in Organic Synthesis is supported by Bristol-Myers Squibb as a sponsoring member and Novartis Pharma as supporting member.

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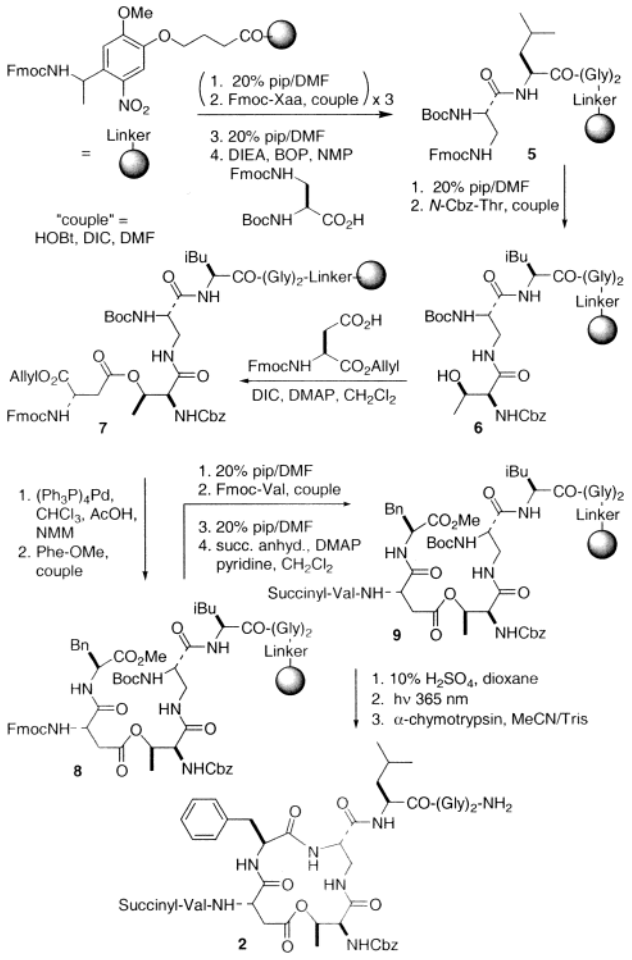
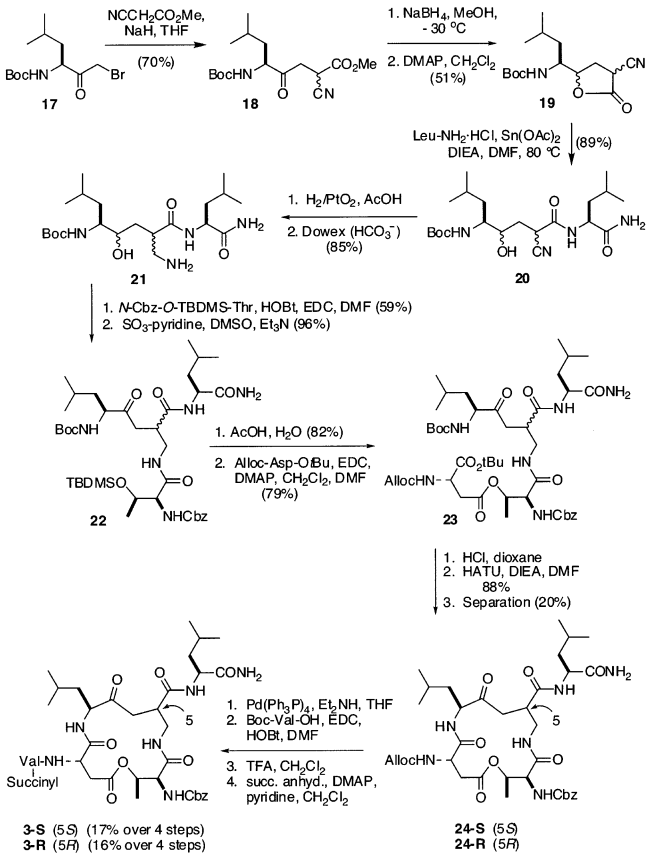
**SCHEME 1**



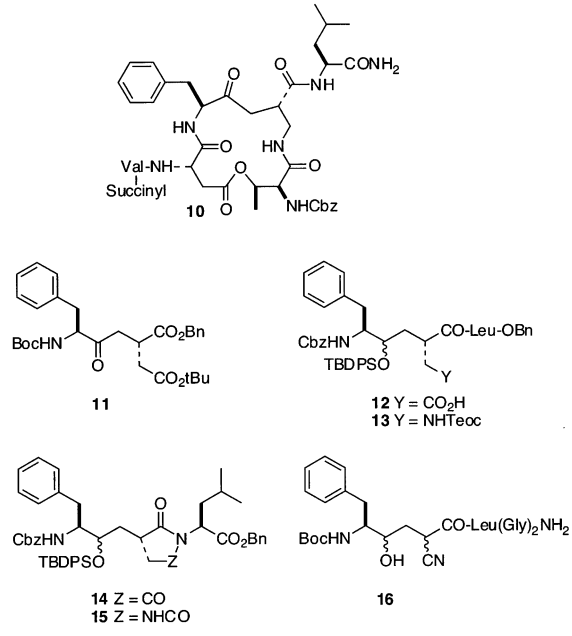

was activated and coupled with L-phenylalanine methyl ester to provide compound **8**. The succinyl-valyl moiety was then introduced in a stepwise fashion, and the Boc protecting group was removed from the α-position group of the diaminopropanoyl (Dap) unit prior to photolytic cleavage from the resin. Cyclization of the amino ester was accomplished in high yield in acetonitrile/water with α-chymotrypsin.

**Macrocyclic Ketone 3.** Prior to carrying out the synthesis of macrocycle **3**, with a leucine side chain at the P1 position, we undertook to synthesize the phenylalanine analogue **10**. Two routes were explored, but the difficulties encountered in each led us to abandon this target. In the first route, we prepared the ketone **11** described by Déziel et al. as a 5:1 mixture of diastereomers.[14] After several steps involving protecting group manipulation, we obtained the carboxylic acid **12**. Unfortunately, attempts to introduce the side chain amino group by Curtius rearrangement and trapping of the isocyanate intermediate with a suitable alcohol to give carbamate **13** were sidetracked by formation of the lactam **14** or dihydrouracil **15** instead. An alternative, non-stereoselective route was then pursued, analogous

**SCHEME 2**




to the sequence outlined below for the leucine analogue (see Scheme 2). However, at the stage of intermediate **16**, we were unable to effect hydrogenation of the nitrile group without concomitant reduction of the phenyl ring. Although in hindsight we should have carried the resulting cyclohexylalanine derivative forward,[15] we elected to prepare the leucine analogue **3** instead.




The key component required for synthesis of ketone **3** was a suitably protected ketomethylene analogue of

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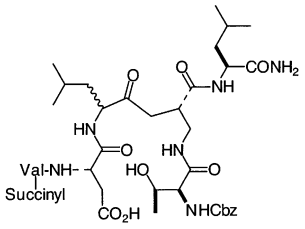
*N*-(*N*$^{\alpha}$-leucyl-α,*β*-diaminopropanoyl)leucinamide (Leu-(*S*)-Dap-Leu-$NH_2$). This moiety was constructed as the reduced amino alcohol **21**, as shown in Scheme 2. Alkylation of the Boc-leucine bromomethyl ketone **17**[16] with methyl cyanoacetate afforded cyano ketone **18**, which possesses the carbon skeleton of the Leu-Dap dipeptide analogue. To avoid problems anticipated on generation of a primary amine in proximity to the ketone and the methyl ester, **18** was reduced with $NaBH_4$ in MeOH and the resulting mixture of hydroxy ester and lactone **19** was converted entirely to the latter with DMAP in 52% overall yield.[17] Although four diastereomers of lactone **19** were present, separation was not attempted at this stage since one of the stereocenters was to be removed later and because separation and assignment of the two final isomers were more readily accomplished when the macrocyclic materials were in hand.

The lactone of **19** served as an activated form of the carboxyl group, coupling with leucinamide in the presence of stannous acetate in DMF to give amide **20** in good yield. Hydrogenation of the nitrile moiety over $PtO_2$ in acetic acid, followed by neutralization with an anion-exchange resin, provided the key intermediate **21** in 85% yield.

The remaining steps in elaboration of macrocyclic ketone **3** began with acylation of the primary amine in **21** with an N,O-diprotected threonine derivative.[18] The yield in this straightforward reaction was reduced by competing relactonization, which led to loss of the leucinamide moiety. $SO_3$/pyridine oxidation[19] of the free hydroxyl group in the acylated product gave keto amide **22**, and removal of the silyl protecting group[20] and esterification with the *β*-carboxyl group of *N*-Alloc-L-aspartate α-*tert*-butyl ester[21] then provided compound **23** in good yield. Cleavage of the *tert*-butyl ester and N-Boc protecting group followed by cyclization of the resulting amino acid with *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU)[22] and DIEA in DMF provided macrocycle **24** as a mixture of diastereomers in 88% yield.[23] The diastereomeric products were separated with difficulty by silica gel chromatography, providing small amounts (∼8%) of each epimer in ca. 80% purity. Distinctive NOESY NMR spectra were obtained for each diastereomer, but the interactions observed were not conclusive in assigning the stereochemistry of each compound. The remaining synthetic steps were carried out on each diastereomer separately, and the *S*- and *R*-configurations at C-5 were assigned at a later stage based on their activity as inhibitors of chymotrypsin.

The Alloc protecting group was removed from macrocycles **24-S** and **24-R** with tetrakis(triphenylphosphine)-palladium(0) and diethylamine as the allyl scavenger. Boc-valine was coupled to each amino terminus and deprotected with TFA in $CH_2Cl_2$. Finally, succinic anhydride, DMAP, and pyridine in $CH_2Cl_2$ provided the succinylated compounds **3-S** and **3-R** in 16−17% yield over the four steps.

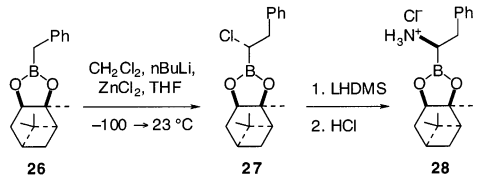
**Acyclic Ketone 25.** To provide a comparison compound for the macrocyclic ketone **3-S**, the lactone linkage was cleaved with aq $K_2CO_3$[24] to give the hydroxy acid **25**. These conditions also caused epimerization at the stereocenter adjacent to the ketone to give a 60/40 mixture of diastereomers.




**25**

**Boronic Acid 4.** Synthesis of the boronic acid analogue **4**, in the form of precursor **31**, began with preparation of the (+)-pinanediol boronate analogue of L-phenylalanine, **28**. This material was prepared in enantiomerically pure form using the procedure of Matteson and Sadhu (Scheme 3).[25,26] Benzylboronic acid was formed

**SCHEME 3**




**26** **27** **28**

from the Grignard reagent and trimethyl borate and esterified with (+)-pinanediol in the presence of $MgSO_4$. The product **26** was homologated with dichloromethyllithium (generated at −100 °C) in the presence of zinc chloride to give the (*S*)-1-chloro-2-phenylethyl boronate diastereomer **27**. We found the stereoselectivity of this transformation to be very sensitive to moisture; with strictly anhydrous $ZnCl_2$, diastereomerically pure material was obtained, but without rigorous care to exclude water, 1:1 diastereomeric mixtures resulted. The chloride was displaced with inversion with lithium hexamethyldisilazide in THF at −78 °C, and the crude product as a solution in hexane was converted to the hydrochloride **28** with HCl in dioxane, again at −78 °C. Although we did not optimize the procedures, and thus obtained the final product in only a modest overall yield of 45%, we include the experimental details in the Supporting Information, since we do not believe they have been reported previously for this analogue.

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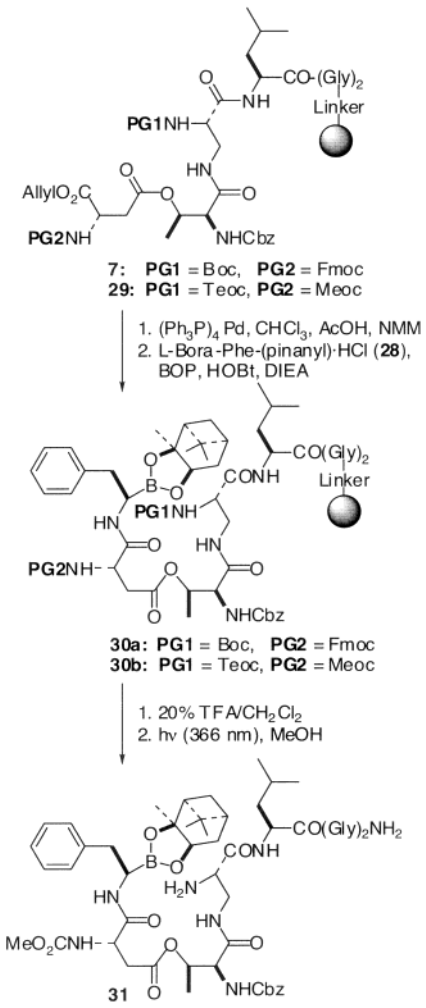
**SCHEME 4**




Subsequent steps in the synthesis of boronate **31** followed closely the route described for the lactam precursor above (Scheme 4). After deallylation of depsipeptide intermediate **7**, considerable effort was required to optimize the coupling reaction between the resin-bound acid and the boro-Phe derivative. Conventional coupling conditions, such as DIC/HOBt, PyBOP, or BOP and DIEA, with or without HOBt, produced significant amounts of the deboronated 2-(phenylethyl)amide as a contaminant. This side reaction appeared to be a function of the DIEA; indeed, when the boro-Phe component was treated with the base prior to addition of the other reagents, deboronated material was the major product. Conversely, when boro-Phe was added 10 min *after* all the other components were combined and the resin-bound acid had presumably been activated, the desired product **30a** was formed in acceptable yield (ca. 65%) with only a trace of the contaminant.

Conditions required for removal of either the Boc or the Fmoc protecting groups of **30a** proved to be incompatible with the boronate moiety, at least in the context of these complex analogues. A number of acid reagents were explored for cleavage of the Boc group, including TFA/$CH_2Cl_2$ mixtures, HCl or $H_2SO_4$ in ether or dioxane, or TMSCl/phenol, but loss of the boronate ester function and formation of a plethora of products resulted in every case. The inclusion of scavengers (anisole, thioanisole, thiophenol, or triisopropylsilane/water) did not suppress these side reactions. The sensitivity of **30a** was not a function of the resin-depsipeptide component, since removal of the Boc group from the precursor **7** could be effected cleanly under a variety of conditions. Nor was the sensitivity due to the α-acyl boro-Phe unit alone; the *N*-acetyl derivative of boro-Phe pinanyl ester was perfectly stable to all of the Boc-deprotection conditions evaluated. While the Achilles heel of **30a** was not identified with certainty, the need to replace the Boc group with a protecting group that could be removed under different conditions was clearly indicated.

The instability of boro-Phe derivative **28** to DIEA led us to worry that the conditions for removal of the Fmoc protecting group from the aspartic acid unit might also be incompatible with the boronate ester. Unfortunately, these concerns were justified, as deboronated compounds were the major products resulting from treatment of **30a** with 20% piperidine in DMF for 20 min. Replacement of the Fmoc group of **7** with an acetyl moiety prior to coupling with boro-Phe proceeded without complication, but the coupled product (**28a**, with **PG2** = Ac) was formed as a mixture of diastereomers, presumably having epimerized at the Asp α-position during activation.

The difficulties encountered with the Boc and Fmoc protecting groups were avoided by repeating the synthesis of the depsipeptide precursor, incorporating 2-(trimethylsilyl)ethoxycarbonyl (Teoc) and methoxycarbonyl (Meoc) groups, respectively, in intermediate **9**. We anticipated that the Teoc group could be removed from the Dap unit with fluoride under alkaline or mildly acidic conditions, and the Meoc group was to be retained in the final product.[27] With the optimized coupling conditions described above, the boro-Phe pinanyl ester was coupled to the Asp carboxyl group after deallylation to give **30b** in 55−63% yield and without any deboronated product.

Ultimately, Teoc proved to be the right protecting group but for the wrong reason. Attempts to effect cleavage with fluoride ion also resulted in significant deboronation, whether under basic (TBAF), slightly acidic (HF/pyridine), or neutral conditions (tris(dimethylamino)-sulfonium difluorotrimethyl silicate, TAS-F).[28,29] Fortunately, however, the Teoc group was sufficiently acid labile that it could be removed cleanly with 20% TFA in dichloromethane, with no significant amount of deboronation. We do not have a good explanation for why the Teoc group, but not the Boc group, could be removed cleanly under essentially the same conditions.

After the conditions had been optimized for the solid-phase synthesis of the boronate ester **31**, a preparative-scale synthesis was carried out and material was isolated and purified after photolytic detachment from the resin at 366 nm. Photolysis was carried out with three cycles of irradiation for 4 h, at which point no further material was isolated from the resin. The crude product was purified by reverse-phase HPLC to give the final product in 16% isolated yield over the 16 steps of the synthesis.

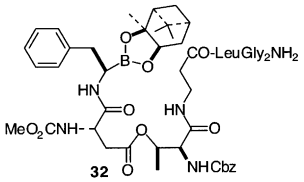
To assess whether any potential cyclic boronate-enzyme adducts would result in enhanced binding affinity, the comparison compound **32** lacking the nucleophilic amino group was also synthesized. The route followed that outlined in Scheme 4, except for the simplification

**TABLE 1. Inhibition of α-Chymotrypsin by Macrocyclic Transition State Analogues**[a]

| compound | $K_i$ ($\mu$M) | comments |
|---|---|---|
| lactam **2** | 1100 | slow substrate |
| ketone **3-S** | 220 | configuration assigned on |
| ketone **3-R** | 1700 | basis of affinity |
| acyclic ketone **25** | 1500 | as mixture of epimers |
| boronate **31** | 0.13 | pinanediol esters hydrolyze to |
| boronate **32** | 0.12 | boronic acids in buffer |

[a] Inhibition was determined at 25 °C in pH 7.8 Tris, 0.10 M $CaCl_2$ buffer or pH 7.0 HEPES buffer (for boronates **3** and **16**) with succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide as substrate.

afforded by replacement of the protected α,*β*-Dap moiety with 3-(Fmoc-amino)propanoic acid.

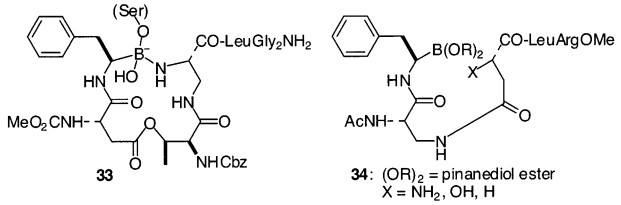



## Inhibition of α-Chymotrypsin

Lactam **2**, the two diastereomers of macrocyclic ketone **3**, and boronate **31** were evaluated as competitive inhibitors of α-chymotrypsin, along with acyclic ketone **25** (mixture of epimers) and boronate **32** as comparison compounds; the results are listed in Table 1. The relative affinities of the two diastereomers of ketone **3** were used to assign their configurations. These isomers differ at the center equivalent to the α-carbon of the P1′ residue; the "natural" S-configuration was therefore assigned to the higher affinity isomer ($K_i$ = 220 $\mu$M). Comparison to the acyclic hydroxy acid **25** indicates that the macrocyclic structure of **3-S** enhances the affinity by about a factor of 4, assuming that the inhibition observed for **25** emanates from only one of the two epimers present. The binding enhancement due to macrocyclization in this case is less than what we have observed for other macrocyclic transition state analogues.[30] Either the macrocyclic ketone **3-S** retains considerable flexibility which must be lost on binding, or the conformation it adopts in the enzyme active site is significantly higher in energy than the low-energy solution conformation(s).

Comparison of **3-S** to lactam **2** suggests that the ketone moiety in **3-S** provides an important enhancement in affinity over the amide unit of the substrate analogue. Lactam **2** is weakly bound to the enzyme ($K_i$ = 1.1 mM) and slowly turned over as a substrate. The ketone **3-S** differs from the lactam **2** in having a leucine side chain at the P1 position, instead of the phenylalanine residue of the lactam. This substitution typically reduces the ground state affinity of α-chymotrypsin substrates (as gauged by $K_m$ values) by about 5-fold[31,32] and their transition state affinity (as gauged by $k_{cat}/K_m$) by 40-fold.[33] Thus, even though the ketone in macrocycle **4-S** is not particularly electron deficient, it nonetheless enhances the binding affinity significantly over what would be expected for an analogue of lactam **2** with leucine at the P1 position.

**CHART 1**




Boronic acid peptide analogues readily form enzyme adducts that mimic the tetrahedral intermediate of the deacylation step in the mechanism of serine proteases and, as a consequence, are among the most potent reversible inhibitors of these enzymes. High affinity was therefore expected for the boronic acids from hydrolysis of **31** and **32**. Although these inhibitors are bound more weakly than analogues that occupy extended binding sites,[4] they are comparable to other analogues limited to the S1 and S2 sites (Chart 1).[10] However, the similar affinity of the amino boronate **31** and the des-amino analogue **32** suggests that the former does not form a macrocyclic adduct with the enzyme (e.g., **33**) that would mimic the tetrahedral intermediate of the *acylation* step of the enzyme mechanism. These results are reminiscent of our earlier attempts to find evidence for such cyclic adducts with the simpler analogues, **34**; these derivatives also had similar inhibition constants (70−130 nM), irrespective of the presence or absence of a nucleophilic group on the P1′ residue.[10]

## Conclusion

In summary, we have synthesized substrate and transition state analogues corresponding to a macrocyclic motif identified from the screening strategy discussed in the preceding paper. The macrocyclic lactam proved to be a weak substrate of α-chymotrypsin, while the macrocyclic ketone was a modest inhibitor. Comparison of the cyclic ketone with an acyclic analogue showed that the macrocyclic structure was advantageous, but not to the extent seen in other cases. Although greater affinity would be anticipated on incorporation of a phenylalanine residue at P1 or a more electrophilic difluoroketone moiety, the potential advantage of the macrocyclic framework did not appear to translate to the boronic acid class of inhibitors.

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(27) Procedures for the preparation of $N^{\alpha}$-Teoc-$N^{\beta}$-Fmoc-α,*β*-diaminopropanoic acid and *N*-methoxycarbonyl-L-aspartic acid 1-allyl ester are provided in the Supporting Information.

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förderung (to B.G.) and the Ligue Nationale Contre le Cancer (to S.G.-P.).

**Supporting Information Available:** Experimental details for the synthesis, characterization, and enzymatic analysis of the inhibitors and intermediates. This material is available free of charge via the Internet at http://pubs.acs.org.

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