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Probing Enzyme Stereospecificity. Evaluation of β-Alkoxy-α-amino Acids with Two Stereocenters as Inhibitors of Serine Proteases.

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Abstract : *L*-N-Acetyl-O-(arylalkyl)-serines incorporating two stereocenters were prepared *via* ring-opening reactions of an aziridine precursor, derived from *L*-serine, with each of *R* and *S* 1-phenyl- and 2-methoxy-2-phenylethanol. These were than evaluated as inhibitors of the serine proteases subtilisin Carlsberg and α–chymotrypsin in order to probe the effects on binding of the different stereocenter configurations. Each *L*-N-Ac-O-(arylalkyl)-serine derivative was a competitive inhibitor for both enzymes, but significant stereocenter-configuration discrimination was observed only in the inhibition of subtilisin Carlsberg by the enantiomeric O-(1-phenylethyl)-serine derivatives.

INTRODUCTION

In recent years, enzymes have been increasingly exploited as chiral catalysts in organic synthesis.[1] However, relatively little is known about the factors determining their stereospecificity preferences towards the unnatural substrate structures that dominate their applications in asymmetric synthesis. Among the interactions generally considered to be among the key determinants of the strength of enzyme-substrate binding are steric effects, electrostatic and hydrogen bonds, hydrophobic effects arising from solvent reorganization, and stacking interactions.[2] In order to take full and rational advantage of the synthetic potential that enzymes offer, it is becoming increasingly important to identify, and to extend our understanding of, the factors controlling substrate binding. The use of substrate analogs that can act as competitive inhibitors of enzymes provides a convenient approach to such studies since the inhibition constants (K_I) reflect the corresponding enzyme-inhibitor (EI) binding efficiencies directly.

Currently, many applications of enzymes in asymmetric synthesis have focussed on the creation of chiral synthons with a single stereocenter. However, the large chiral environments

provided by enzymes have the capacity to discriminate and control many stereocentres concurrently, thereby potentially providing access to any multiple-stereocenter combination desired in a chiral synthon. As first steps towards the delineation of enzyme stereospecificity requirements in this regard, we have begun to examine the abilities of synthetically useful serine proteases to discriminate between diastereomers of two-stereocenter inhibitors. Subtilisin Carlsberg (SC, EC 3.4.21.14) and α-chymotrypsin (CT, EC 3.4.21.1) were chosen as the initial enzymes for this study, being commercially available serine proteases that have been applied in a wide range of synthetic transformations.[3] The active sites of these enzymes possess extended binding regions composed of several subsites, of which the S_1-pocket[4] dominates the structural specificity, as illustrated schematically in Figure 1.[5]

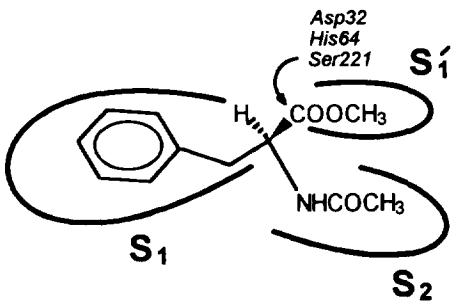
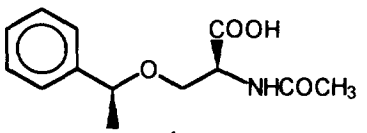


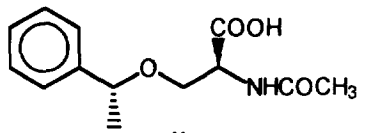
Figure 1. Schematic representation of the active site of subtilisin Carlsberg, with the representative good substrate N-acetyl-*L*-phenylalanine methyl ester (NAPME) bound in the productive ES-complex. The non-polar benzyl group of NAPME fits well in the hydrophobic S_1 pocket, and dominates the binding process. The S_1' region that accommodates the leaving group is not very large, and contains the serine residue of the catalytically vital Ser221-His64-Asp32 serine protease triad. Serine 221 is correctly positioned to attack the substrate carbonyl group, as indicated by the arrow. The S_2 pocket is the next of several adjacent binding sites, and is occupied by the N-acetyl group in this case.

In this paper, the two-stereocenter β-arylalkyloxy-*L*-serine derivatives **1a**,**b** and **2a**,**b** have been employed as inhibitor-probes of the capacity of the S_1-pockets of SC and CT to discriminate between *R*- and *S*-configurations of a stereocenter remote from the α-*L*-center of the serine parent, for which the natural *L*(*S*)-preference of both enzymes is overwhelming. Routine control of the configuration of stereocenters that are remote from the catalytic operators represents an unsolved problem in asymmetric synthesis at the present time, in both chemical[6] and enzymic[1]

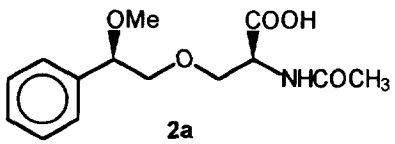
methodologies. Even for the most widely studied enzyme group, that of the hydrolases, very few examples have been reported where the stereocenter configuration can be controlled or discriminated when it is three or more bonds from the reacting carbonyl group.[7] In the current studies, the second, remote, stereocenters of **1a,b** and **2a,b** are four and five bonds respectively removed from the carboxyl group, whose carbonyl group simulates that of an ester substrate's C=O that undergoes attack by the active site serine nucleophile (*cf.* Figure 1).



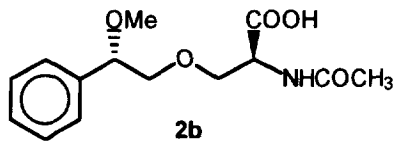
1a



1b



2a



2b

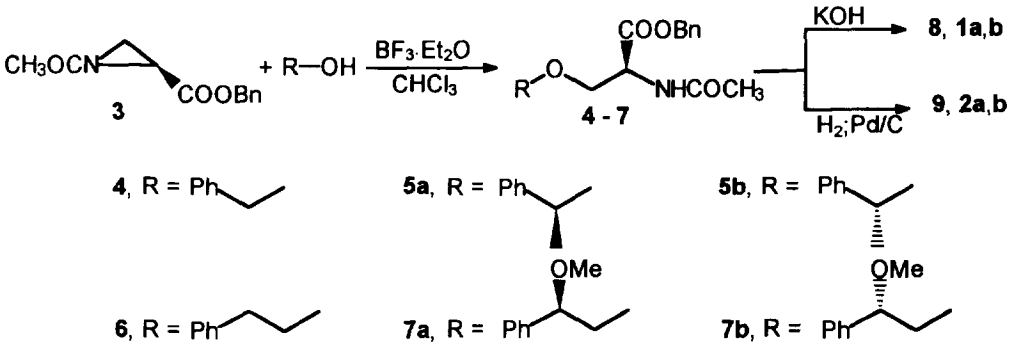
The selection of the inhibitor structures was based on their basic resemblance of good substrates such N-acetyl-*L*-phenylalanine methyl ester (NAPME). Carboxylic acid inhibitors of the **1** and **2** type were selected since aromatic carboxylic acids are known to be competitive inhibitors for CT,[8] and because the carboxylate function confers sufficient water-solubility for the kinetic studies to be performed in aqueous solutions, thereby avoiding the need to add organic co-solvents that can themselves act as inhibitors or denaturing agents.[9] Furthermore, connecting the chiral arylalkyl alcohol moieties to the serine-OH *via* ether linkages was not only a synthetically convenient method of introducing a remote second stereocenter, but also contributed further to the water solubility of the inhibitors.

For the target inhibitors **1a**,**b**, and **2a**,**b**, and their one-stereocenter reference compound analogs **8** and **9**, it was concluded on the basis of graphics analyses, and the penchant of SC and CT for accommodating large groups in S_1,[5,10] that binding of structures such as **1a**,**b**, **2a**,**b** to the active sites of SC and CT would position the second stereocenter of the largely non-polar

arylalkyloxy side chains in the hydrophobic S_1 pockets, thereby permitting the capacity of the S_1 site for stereoselective discrimination on such structures to be evaluated and compared for both enzymes.

RESULTS AND DISCUSSION

Simple O-alkyl-serine or S-alkyl-cysteine compounds have been prepared from aziridine-2-carboxylate derivatives *via* opening of the aziridine rings with the alcohols or thiols.[11-13] Accordingly, this approach was adopted in this work (Scheme 1). The required aziridine precursor, Ac-*L*-Azy-OBn (**3**) was prepared from *L*-serine according to the literature methods.[14]



Scheme 1

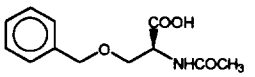
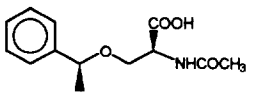
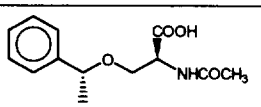
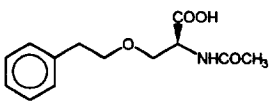
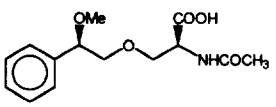
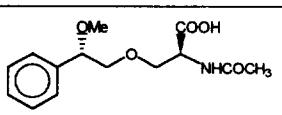
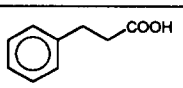
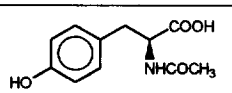
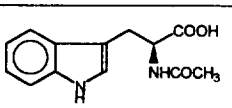
Although in the literature precedents[11-13] the alcohols or thiol nucleophiles were used in large excess or as co-solvents, in the present study, good yields, typically ~70%, of **4**, **5a,b** and **6** were achieved on BF_3-catalyzed ring-opening of Ac-*L*-Azy-OBn (**3**) with only three-fold excesses of the alcohol nucleophiles. While the yields could be increased somewhat by higher excesses of the alcohols, variations in the amounts of BF_3-etherate did not affect the efficiencies of the reactions. The unreacted alcohols were easily separable from the reaction products by chromatography and could be reused. The *L*-N-Ac-O-(arylalkyl)-serine targets **2a,b**, and **9** were then readily obtained by hydrogenolysis of **7a,b** and **6** respectively. Several Pd catalysts were tested for the

hydrogenolysis over a range of reaction times, with 5%Pd/C and a 20 minute reaction time giving the best results. When Pd black or 10%Pd/C were employed as catalysts in removing the benzyl ester functions, cleavage of the methoxyl group from **7a,b** also occurred to some degree. The hydrogenolysis approach was used since it had worked successfully in the literature precedent.[11a, 12] However, it could not be applied to conversion of **4** and **5a,b** to **8** and **1a,b** respectively, because of the sensitivity of the benzyl ether functionality to the hydrogenolysis conditions. Accordingly, for these compounds, the benzyl esters were cleaved instead by hydrolysis. The diastereomeric purities of **1a,b**, **2a,b**, **5a,b** and **7a,b** were determined by ^{1}H NMR analyses of the the amide N*H*-peaks, since this proton exhibited a different chemical shift for each diastereomer. After recrystallization, the target inhibitors **1a,b** and **2a,b** were > 98% *de*. The Scheme 1 method thus represents a very convenient access to O-substituted serines, with both primary and secondary alcohols reacting smoothly and without any significant racemization.

The individual inhibition constants for each *L*-N-Ac-O-(arylalkyl)-serine **1a,b**, **2a,b**, **8**, and **9** for SC and CT was determined by the method of Waley.[15] Each *L*-N-Ac-O-(arylalkyl)-serine was found to be a good competitive inhibitor of both enzymes, with the K_I's being within the fairly narrow range of 4.7 to 8.3 mM for both enzymes. The results are recorded in Table 1. For comparison purposes, the literature K_I-values for competitive inhibition of SC and CT by hydrocinnamic acid (**10**),[8b] N-acetyl-*L*-tyrosine (**11**),[8c] and N-acetyl-*L*-tryptophan (**12**)[8d] are also included.

The Table 1 data show that each of the *L*-N-Ac-O-(arylalkyl)-serines is a superior inhibitor to the achiral, phenylalanine-length, acid **10** and to N-acetyl-*L*-tyrosine (**11**) and -tryptophan (**12**). Of the reference standards **10-12**, the lowest K_I (10 mM) is that of N-acetyl-*L*-tryptophan (**12**) for CT, which reflects the superior binding of its large, hydrophobic, indolyl residue with the S_1-site. The fact that each of the arylalkyloxyserine inhibitors has a lower K_I than **12** for both enzymes is in accord with the initial graphics analyses conclusions that the arylalkyl side chains interact well with S_1, as designed.

Table 1. Inhibition of Subtilisin Carlsberg and α-Chymotrypsin by *L*-N-Ac-O-(arylalkyl)-serines,[a] Hydrocinnamic Acid and Aromatic *L*-N-Ac-Amino Acid Derivatives.

Inhibitor		Subtilisin Carlsberg K_I (mM)	α–Chymotrypsin K_I (mM)
	8	6.9 ± 0.6	4.7 ± 0.9
	1a	4.0 ± 0.3	7.4 ± 1.5
	1b	6.5 ± 0.5	7.0 ± 1.4
	9	7.2 ± 0.6	5.1 ± 1.0
	2a	8.1 ± 0.6	7.1 ± 1.4
	2b	8.4 ± 0.7	7.3 ± 1.5
	10	140[b, 8b]	25[c]
	11	N.A	80[d, 8c]
	12	N.A	10[e, 8d]

[a] K_I values for both enzymes were determined in duplication at pH 7.5 at 25 °C in 0.1 M NaH_2PO_4, 0.5 M NaCl, 0.25 mM of substrate, 5% DMSO in volume, inhibitor concentrations in the range of 5.9 mM-9.1mM, enzyme concentrations 4.5 nM (SC) and 16 nM (CT). K_M and k_{cat} values for the standard substrate Suc-Ala-Ala-Pro-Phe-PNA are 3.62×10^{-4} M, 411 S^{-1} for SC and 3.42×10^{-5} M, 31.2 S^{-1} for CT.

[b] Data obtained at 37 °C at pH 8.0 in 0.1 M KCl containing 8% dioxane by volume with N-acetyl-*L*-tyrosine ethyl ester as the substrate.

[c] Data obtained at 25 °C at pH 7.8.

[d] Data obtained at 25 °C at pH 7.7 in 0.5 M Tris-HCl.

[e] Data obtained at 25 °C at pH 7.9 to 8.0 in 0.1 M NaCl.

N.A indicates not available.

However, while each of **1a,b** and **2a,b** is a good competitive inhibitor of both SC and CT, with K_I's of magnitudes approximating the K_M of 7.9 mM[5c] and 2.0 mM[5d] of the excellent substrate NAPME with SC and CT respectively, neither enzyme is able to discriminate significantly between *R*- or *S*-configurations of a second stereocenter of the side chain. Nor is the nature of the side chain substituent distinguished, with the K_I's of the unsubstituted homologs **8** and **9**, and methyl- (**1a,b**) and methoxy-substituted (**2a,b**) inhibitors, being comparable. This relative lack of structural or configurational recognition indicates that the side chains of the *L*-N-Ac-O-(arylalkyl)-serines **1a,b** and **2a,b** may be too long to fit completely into the S_1-pockets of SC and CT, and that, in the respective EI-complexes, the second stereocenter regions become tilted out of the sphere of S_1's binding influence. Thus in order for the chiral environment of S_1 to be exploitable for discriminating remote stereocenters, either substrates or inhibitors whose P_1-groups[4] can be fully accommodated by the natural S_1 dimensions must be used, or the size and structural characteristics of S_1 must be expanded to provide a more extended binding environment. Further studies are planned to explore these aspects in more detail, using remote stereocenter inhibitor structures that are more closely compatible with the dimensions of the S_1-binding region to probe the first aspect, and applying site-directed mutagenesis to explore the second possibility.

EXPERIMENTAL

General Methods. Anhydrous reagents were prepared according to literature procedures.[16] Melting points were determined on an Electrothermal IA9000 Series digital melting point apparatus and are uncorrected. Analytical TLC was performed on precoated plates (silica gel 60F-254). Purifications by radial TLC were performed on a model 7924T Chromatotron from Harrison Research. Plates of 4mm and 2mm thickness were coated with E. Merck Silica Gel 60 PF_{254} containing gypsum. Absorption measurements were performed on a Perkin Elmer Lambda 2 UV/VIS spectrometer. IR spectra were determined on KBr pellets (for solids) and films (for liquids) on a Nicolet 5DX FTIR spectrophotometer. NMR (^1H, ^{13}C) spectra were recorded on a Gemini 200 (200 MHz, 50 MHz respectively) spectrometer. ^1H NMR chemical shifts are reported

in ppm relative to the TMS peak (δ = 0.0), and ^{13}C NMR chemical shifts relative to the $CDCl_3$ peak (δ = 77.0), both with $CDCl_3$ as solvent. High-resolution mass spectrum was measured on an AEIMS 3074. Optical rotations were measured on a Perkin-Elmer 141 polarimeter. Elemental analyses were performed by Galbraith Laboratories (TX).

Reagent grade chemicals, 2-phenethanol, *R*-(-)- and *S*-(+)-2-methoxy-2-phenylethanol (98% *ee*), were purchased from Aldrich and *R*-(+)- and *S*-(-)-α–phenylethanol from Norse Laboratories Inc.. SC (EC 3.4.21.14), CT (EC 3.4.21.1) and succinyl-*L*-Ala-*L*-Ala-*L*-Pro-*L*-Phe-p-nitroanilide (Suc-Ala-Ala-Pro-Phe-PNA) were purchased from the Sigma Chemical Company. The concentrations of SC and CT were assayed from their catalysis of Suc-Ala-Ala-Pro-Phe-PNA[17] and p-nitrophenyl-ethyl acetate (PNA)[18] hydrolyses, respectively. Benzyl (*S*)-1-acetyl-2-aziridinecarboxylate (**3**, Ac-*L*-Azy-OBn) was prepared according to the literature procedure[14]: $[\alpha]^{22}_D$ -39.3° (c 1.3, MeOH); (lit.[14] $[\alpha]^{23}_D$ -39.6° (c 1.0, MeOH)); ^1H NMR δ 2.13 (3H, s), 2.50 (1H, d of d, *J* = 5.4, 1.7 Hz), 2.61 (1H, d, *J* = 1.7 Hz), 3.18 (1H, d of d, *J* = 5.4, 2.9 Hz), 5.22 (2H, s), 7.38 (5H, s).

General Procedure A for Preparation of 1 a.b and 8.

N-Acetyl-O-benzyl-*L*-serine (8). To a solution of Ac-*L*-Azy-OBn (**3,** 307 mg, 1.4 mmol, 1.0 mol equiv.) in dry $CHCl_3$ (4 mL) at 20 °C under N_2 was added benzyl alcohol (454 mg, 4.2 mmol, 3.0 mol equiv.), followed by $BF_3.Et_2O$ (3 drops). The reaction mixture was then stirred at 20 °C under N_2 for 24h and concentrated under reduced pressure. The residue was dissolved in EtOAc and washed with 5% aqueous $NaHCO_3$ and then with H_2O. The organic solution was dried ($MgSO_4$), filtered, and concentrated under reduced pressure. The residue was chromatographed by using the Chromatotron (hexanes/EtOAc, 70:30), to give N-acetyl-O-benzyl-*L*-serine benzyl ester (**4**, 280 mg, 61%) as a colorless oil: $[\alpha]^{23}_D$ +8.3° (c 0.5, CH_2Cl_2); IR 3307, 3073, 2953, 1741, 1644 cm^{-1}; ^1H NMR δ 2.02 (3H, s), 3.68 (1H, d of d, *J* = 9.5, 3.1 Hz), 3.92 (1H, d of d, *J* = 9.5, 2.9 Hz), 4.45 (2H, d, *J* = 4.4 Hz), 4.81 (1H, d of t, *J* = 8.4, 3.1 Hz), 5.18 (2H, d, *J* = 3.3 Hz), 6.41 (1H, d, *J* = 6.9 Hz), 7.17-7.32 (10H, m); ^{13}C NMR δ 23.1, 52.7, 67.2, 69.8, 73.3, 127.6, 127.8, 128.1, 128.3, 128.4, 128.5, 135.3, 137.4, 169.9, 170.2.

To a solution of N-acetyl-O-benzyl-*L*-serine benzyl ester (**4**, 205 mg, 0.63 mmol) in THF (3 mL), was added aqueous 1M KOH (1.5 mL). The reaction mixture was stirred at 20 °C for 2h. When TLC showed the reaction was over, distilled H_2O (15 mL) was added to the reaction mixture and the benzyl alcohol removed by extraction with Et_2O (3 x 20 mL). The aqueous solution was then acidified with 1M HCl and extracted with $CHCl_3$. The organic solution was dried ($MgSO_4$), filtered, and concentrated under reduced pressure to give N-acetyl-O-benzyl-*L*-serine (**8**, 140 mg, 95%) as a white solid. The product was recrystallized from $CHCl_3$/hexanes to give white crystals (136 mg, 91%): mp. 105.9-106.7 °C; $[\alpha]^{24}_D$ +34.6° (c 0.5, CH_2Cl_2); IR 3337, 3050, 2922, 1720, 1610 cm^{-1}; ^1H NMR δ 2.02 (3H, s), 3.70 (1H, d of d, *J* = 9.6, 3.2 Hz), 3.93 (1H, d of d, *J* = 9.5, 3.1 Hz) 4.50 (2H, s), 4.70-4.80 (1H, m), 6.52 (1H, d, *J* = 8.1 Hz), 7.20-7.40 (5H, m), 10.10-10.30 (1H, br); ^{13}C NMR δ 22.6, 52.7, 69.4, 73.3, 127.6, 127.8, 128.3, 137.2, 171.5, 172.8; high-resolution mass spectrum, calcd for MH^+ $C_{12}H_{16}NO_4$ *m/e* 238.1079, found *m/e* 238.1082.

The ring-opening reactions of *Ac-L*-Azy-OBn (**3**) with other alcohols, and hydrolyses of **3a,b**, were carried out in the same manner, as follows:

N-Acetyl-O-((1*S*)-1-phenylethyl)-*L*-serine (1a). From Ac-*L*-Azy-OBn (**3**, 262 mg, 1.19 mmol, 1.0 mol equiv.), *S*-(-)-α-phenylethanol (438 mg, 3.59 mmol, 3.0 mol equiv.), $BF_3{\cdot}Et_2O$ (3 drops), and $CHCl_3$ (3.5 mL). After work-up and purification using the Chromatotron (hexanes/EtOAc 70:30), N-acetyl-O-((1*S*)-1-phenylethyl)-*L*-serine benzyl ester (**5a**, 290 mg, 71%, > 98% *de*) was obtained as a colorless oil: $[\alpha]^{25}_D$ -14.1° (c 0.87, CH_2Cl_2); IR 3306, 3043, 2983, 1745, 1662 cm^{-1}; ^1H NMR δ 1.39 (3H, d, *J* = 6.5 Hz), 2.08 (3H, s), 3.53 (1H, d of d, *J* = 9.5, 3.1 Hz), 3.80 (1H, d of d, *J* = 9.5, 3.0 Hz), 4.34 (1H, q, *J* = 6.4Hz), 4.75 (1H, d of t, *J* = 8.4, 3.0 Hz), 5.14 (2H, d, *J* = 3.5 Hz), 6.47 (1H, d, *J* = 8.4 Hz), 7.0-7.40 (10H, m); ^{13}C NMR δ 23.1, 23.7, 52.7, 67.1, 68.3, 78.6, 126.0, 127.6, 128.2, 128.3, 128.4, 128.5, 134.8, 142.3, 169.9, 170.2.

From N-acetyl-O-((1*S*)-1-phenylethyl)-*L*-serine benzyl ester (**5a**, 170 mg, 0.5 mmol), THF (10 mL) and 1M KOH (1.2 mL). After work-up, N-acetyl-O-((1*S*)-phenylethyl)-*L*-serine (**1a**, 122 mg, 98%) was obtained as a white solid. The product was recrystallized from $CHCl_3$/hexanes to

give white crystals (111 mg, 89%, >98% *de*) : mp. 112.4-113.5 °C; $[\alpha]^{25}_D$ -32.0°(c 0.5, MeOH); IR 3352, 3005, 2884, 1707, 1617 cm^{-1}; ^1H NMR δ 1.40 (3H, d, *J* = 6.6 Hz), 2.00 (3H, s), 3.56 (1H, d of d, *J* = 9.4, 3.1 Hz), 3.78 (1H, d of d, *J* = 9.5, 3.3 Hz), 4.40 (1H, q, *J* = 6.6 Hz), 4.65-4.80 (1H, m), 6.83 (1H, d, *J* = 8.1 Hz), 7.18-7.38 (5H, m), 10.87 (1H, br); ^{13}C NMR δ 22.7, 23.6, 52.7, 67.8, 78.5, 126.0, 127.6, 128.4, 142.6, 171.4, 172.9; Anal. Calcd. for $C_{13}H_{17}NO_4$: C 62.14, H 6.82. Found: 61.82, 6.88%.

N-Acetyl-O-((1*R*)-1-phenylethyl)-*L*-serine (1b). From Ac-*L*-Azy-OBn (**3**, 290 mg, 1.32 mmol, 1.0 mol equiv.), *R*-(+)-α-phenylethanol (485 mg, 3.96 mmol, 3.0 mol equiv.), $BF_3{\cdot}Et_2O$ (3 drops), and $CHCl_3$ (4 mL). After work-up and purification using the Chromatotron (hexanes/EtOAc 70:30), N-acetyl-O-((1*R*)-1-phenylethyl)-*L*-serine benzyl ester (**5b**, 310 mg, 69%, > 98% *de*) was obtained as a colorless oil: $[\alpha]^{25}_D$ +43.3° (c 0.93, CH_2Cl_2); IR 3307, 3043, 2990, 1749, 1662 cm^{-1}; ^1H NMR δ 1.31 (3H, d. *J* = 6.4 Hz), 1.98 (3H, s), 3.50 (1H, d of d. *J* = 9.6, 3.2 Hz), 3.80 (1H, d of d, *J* = 9.6, 2.9 Hz), 4.26 (1H, q, *J* = 6.4 Hz), 4.74 (1H, d of t, *J* = 8.4, 3.0 Hz), 5.24 (2H, d of d, *J* = 33.9, 12.3 Hz), 6.35 (1H, d, *J* = 8.3 Hz), 7.1-7.40 (10H, m); ^{13}C NMR δ 23.0, 23.4, 52.7, 67.0, 68.4, 78.8, 126.0, 127.7, 128.2, 128.3, 128.4, 128.5, 135.5, 142.9, 169.7, 170.3.

From N-acetyl-O-((1*R*)-1-phenylethyl)-*L*-serine benzyl ester (**5b**, 230 mg, 0.67 mmol), THF (13.5 mL) and 1M KOH (1.5 mL). After work-up, N-acetyl-O-((1*R*)-1-phenylethyl)-*L*-serine (**1b**, 143 mg, 85%) was obtained as a white solid. The product was recrystallized from $CHCl_3$/hexanes mixture to give white crystals (126 mg, 75%, >98% *de*): mp. 122.6-123.8 °C; $[\alpha]^{25}_D$ +96.4° (c 0.5, MeOH); IR 3427, 3367, 2982, 1723, 1628 cm^{-1}; ^1H NMR δ 1.45 (3H, d, *J* = 6.4 Hz), 2.03 (3H, s), 3.49 (1H, d of d, *J* = 9.7, 4.2 Hz), 3.87 (1H, d of d, *J* = 9.7, 3.5 Hz), 4.45 (1H, q, *J* = 6.5 Hz), 4.65-4.80 (1H, m), 6.61 (1H, d, *J* = 8.1 Hz), 7.20-7.38 (5H, m), 11.18 (1H, br); ^{13}C NMR δ 22.8, 23.5, 52.7, 68.0, 79.0, 126.1, 127.8, 128.5, 142.8, 171.1, 173.3; Anal. Calcd. for $C_{13}H_{17}NO_4$: C 62.14, H 6.82. Found: 62.28, 6.74%.

General Procedure B for Preparation of 9 and 2a,b.

N-Acetyl-O-(2-phenylethyl)-*L*-serine (9). The general procedure A was followed for the ring opening reaction by using Ac-*L*-Azy-OBn (**3**, 310 mg, 1.41 mmol, 1.0 mol equiv.), 2-phenylethanol (521 mg, 4.24 mmol, 3.0 mol equiv.), $BF_3{\cdot}Et_2O$ (3 drops), and $CHCl_3$ (4 mL). After work-up and purification using the Chromatotron (hexanes/EtOAc 70:30), N-acetyl-O-(2-phenylethyl)-*L*-serine benzyl ester (**6**, 320 mg, 0.94 mmol, 67%) was obtained as a colorless oil: $[\alpha]^{23}_D$ +3.9° (c 0.9, CH_2Cl_2); IR 3298, 3033, 2945, 1746, 1663 cm^{-1}; ^1H NMR δ 1.96 (3H, s), 2.78 (2H, t, *J* = 6.7 Hz), 3.55-3.67 (3H, m), 3.89 (1H, d of d, *J* = 9.5, 3.0 Hz), 4.75 (1H, d of t, *J* = 8.3, 3.0 Hz), 5.14 (2H, s), 6.26 (1H, d, *J* = 8.1 Hz), 7.1-7.40 (10H, m); ^{13}C NMR δ 22.9, 35.8, 52.6, 67.1, 70.4, 72.1, 126.2, 128.0, 128.2, 128.4, 128.8, 128.9, 135.3, 138.6, 142.3, 169.8, 170.1.

To a solution of N-acetyl-O-(2-phenylethyl)-*L*-serine benzyl ester (**6**, 200 mg, 0.59 mmol) in MeOH (6.5 mL), was added 5% Pd/C (70 mg). The reaction mixture was stirred at 20 °C under hydrogen for 20 min, filtered and concentrated under reduced pressure. The residue was dissolved in 5% $NaHCO_3$ aqueous solution and washed with Et_2O. The aqueous solution was then acidified with 1M HCl and extracted with $CHCl_3$. The organic solution was dried ($MgSO_4$), filtered and concentrated under reduced pressure to give N-acetyl-O-(2-phenylethyl)-*L*-serine (**9**, 140 mg, 0.56 mmol, 95%) as a white solid. The product was recrystallized from $CHCl_3$/hexanes to give white crystals (127 mg, 0.51 mmol, 86%): mp. 92.3-93.3 °C; $[\alpha]^{25}_D$ +25.4° (c 0.5, MeOH); IR 3343, 3035, 2937, 1734, 1662 cm^{-1}; ^1H NMR δ 1.97 (3H, s), 2.83 (2H, t, *J* = 6.5 Hz), 3.55-3.75 (3H, m), 3.93 (1H, d of d, *J* = 9.4, 3.1 Hz), 4.65-4.75 (1H, m), 6.57 (1H, d, *J* = 8.0 Hz), 7.15-7.35 (5H, m), 8.06 (1H, br); ^{13}C NMR δ 22.6, 35.8, 52.7, 70.0, 72,2, 126.3, 128.3, 128.9, 138.6, 171.4, 172.7; Anal. Calcd. for $C_{13}H_{17}NO_4$: C 62.14, H 6.81. Found: 61.90, 6.78%.

The hydrogenolysis reactions of **7a,b** were carried out in the above manner, as follows:

N-Acetyl-O-((2*R*)-2-methoxy-2-phenylethyl)-*L*-serine (2a). From Ac-*L*-Azy-OBn (**3**, 218 mg, 1.0 mmol, 1.0 mol equiv.), *R*-(-)-2-methoxy-2-phenylethanol (150 mg, 1.0 mmol, 1.0 mol equiv.), $BF_3{\cdot}Et_2O$ (3 drops), and $CHCl_3$ (3 mL). After work-up and purification using the Chromatotron

(hexanes/EtOAc, 50:50), N-acetyl-O-((2*R*)-2-methoxy-2-phenylethyl)-*L*-serine benzyl ester (**7a**, 165 mg, 45%, 85% *de*) was obtained as a colorless oil: IR 3304, 3070, 2938, 1749, 1662 cm^{-1}; ^1H NMR δ 2.04 (3H, s), 3.26 (3H, s), 3.50-3.60 (2H, m). 3.72 (1H, d of d, *J* = 10.0, 3.2 Hz), 4.00 (1H, d of d, *J* = 10.0, 3.0 Hz), 4.25 (1H, m), 4.77 (1H, m), 5.16 (2H, s), 6.59 (1H, d, *J* = 12.0 Hz), 7.20-7.40 (10H, m); ^{13}C NMR δ 22.7, 52.7, 56.7, 66.9, 71.3, 75.9, 82.9, 126.7, 127.9, 128.2, 128.3, 128.4, 128.5, 135.4, 138.3, 170.0, 170.1.

From N-acetyl-O-((2*R*)-2-methoxy-2-phenylethyl)-*L*-serine benzyl ester (**7a**, 47 mg, 0.13 mmol), 5% Pd/C (16 mg), and MeOH (1.4 mL). After work-up, N-acetyl-O-((2*R*)-2-methoxy-2-phenylethyl)-*L*-serine (**2a**, 36 mg, 98%) was obtained as a white solid. The product was recrystallized from a CH_2Cl_2/*i*-Pr_2O mixture to give white crystals (26 mg, 70%, >98% *de*): mp 107-108 °C; $[\alpha]^{25}_D$ -64.5° (c 0.64, CH_2Cl_2); IR 3327, 2894, 1748, 1614 cm^{-1}; ^1H NMR δ 2.10 (3H, s), 3.30 (3H, s), 3.60-3.75 (3H, m), 4.08 (1H, d of d, *J* = 10.0, 3.5 Hz), 4.43 (1H, d of d, *J* = 7.1, 4.0 Hz), 4.65-4.75 (1H, m), 6.68 (1H, d, *J* = 7.4 Hz), 7.25-7.40 (5H, m); ^{13}C NMR δ 22.9, 52.7, 56.8, 70.9, 76.0, 82.9, 126.9, 128.3, 128.6, 137.9, 171.1, 172.5; Anal. Calcd. for $C_{14}H_{19}NO_5$: C 59.76, H 6.81. Found: 59.75, 6.96%.

N-Acetyl-O-((2*S*)-2-methoxy-2-phenylethyl)-*L*-serine (2b). From Ac-*L*-Azy-OBn (**3**, 249 mg, 1.13 mmol, 1.0 mol equiv.), *S*-(+)-2-methoxy-2-phenylethanol (177 mg, 1.16 mmol, 1.0 mol equiv.), $BF_3{\cdot}Et_2O$ (3 drops), and $CHCl_3$ (4.0 mL). After work-up and purification using the Chromatotron (hexanes/EtOAc 50:50), N-acetyl-O-((2*S*)-2-methoxy-2-phenylethyl)-*L*-serine benzyl ester (**7b**, 230 mg, 55%, > 98% *de*) was obtained as a colorless oil: IR 3307, 3070, 2938, 1752, 1660 cm^{-1}; ^1H NMR δ 1.99 (3H, s), 3.25 (3H, s), 3.43-3.70 (3H, m), 4.00 (1H, d of d, *J* = 9.7, 3.2 Hz), 4.28 (1H, d of d, *J* = 7.0, 4.4 Hz), 4.70-4.80 (1H, m), 5.17 (2H, s), 6.29 (1H, d, *J* = 7.7 Hz), 7.20-7.35 (10H, m); ^{13}C NMR δ 22.9, 52.7, 56.8, 67.1, 71.7, 75.5, 82.6, 126.9, 128.0, 128.1, 128.3, 128.4, 128.5, 135.4, 138.8, 170.0, 170.1.

From N-acetyl-O-((2*S*)-2-methoxy-2-phenylethyl)-*L*-serine benzyl ester (**7b**, 63 mg, 0.17 mmol), 5% Pd/C (23 mg), and MeOH (2.0 mL). After work-up, *L*-N-acetyl-O-((2*S*)-2-methoxy-2-phenylethyl)-serine (**2b**, 46 mg, 96%) was obtained as a white solid. The product was

recrystallized from *n*-BuOH/hexanes to give white crystals (30 mg, 63%, >98% *de*): mp. 109-110 °C; $[\alpha]^{25}_{D}$ +77.0° (c 0.51, CH_2Cl_2); IR 3329, 2914, 1734, 1614 cm^{-1}; ^1H NMR δ 2.04 (3H, s), 3.30 (3H, s), 3.63-3.75 (3H, m), 3.95 (1H, d of d, *J* = 5.7, 4.0 Hz), 4.38-4.47 (1H, m), 4.65-4.75 (1H, m), 6.45 (1H, d, *J* = 7.2 Hz), 7.25-7.40 (5H, m); ^{13}C NMR δ 23.0, 52.3, 56.7, 70.4, 76.3, 82.7, 127.0, 128.3, 128.6, 137.9, 171.0, 172.4; Anal. Calcd. for $C_{14}H_{19}NO_5$: C 59.76, H 6.81. Found: 60.02, 6.97%.

Kinetic Measurements.

Kinetic determinations for both SC and CT were performed at 25 °C by measuring absorbance of PNA (ε = 8800 at 410 nm) released during the hydrolysis of Suc-Ala-Ala-Pro-Phe-PNA. All kinetic runs were carried out in duplicate.

For SC, the K_M and k_{cat} values for the standard substrate Suc-Ala-Ala-Pro-Phe-PNA were determined by measuring initial rates of hydrolysis at different substrate concentrations (0.031, 0.042, 0.067, 0.125, 0.250, 0.500, 1.000, 2.000 mM), and fitting the obtained data to the Michaelis-Menten equation using the Grafit program (version 3.0, Erithacus Software Ltd., UK). Assays were run in 0.1 M NaH_2PO_4 buffer at pH 7.5, containing 0.5 M NaCl, with 5% (v/v) DMSO, and with the enzyme concentration 0.47 nM. The K_I for each inhibitor for SC was determined by Waley's method.[15] The progress curve without inhibitor was obtained from the reaction mixture of pH 7.5 buffer containing 0.2 M NaH_2PO_4 and 1.0 M NaCl (1.47 mL), pH 7.5 buffer with 0.1 M NaH_2PO_4 (50 μL), 1.0 M NaCl (30 μL), 25 mM substrate in DMSO (30 μL), DMSO (120 μL) and H_2O (1.29 mL), and the reaction mixture was incubated for 5 min at 25 °C. The reaction was initiated by addition of 10 μL of SC stock solution ($1.4x10^{-6}$ M in 0.1 M NaH_2PO_4 buffer pH 7.5). The final volume of the reaction mixture was 3 mL, with a final concentration of 0.1 M NaH_2PO_4, 0.5 M NaCl, 4.5 nM of SC, 0.25 mM of substrate and 5% DMSO. The progress curve with inhibitor was obtained with 100-120 μL of the inhibitor solution (0.14-0.27 M in DMSO) added, under the same reaction conditions as above. The progress of hydrolysis of each assay was recorded directly into a PC. The differences between the times taken for the absorbance at the following percentage of conversion (15,18, 21, 24, 27, 30, 33, 36 and 39%) to reach the same value were

obtained by comparison of progress curves in the absence and in the presence of inhibitor. These differences were plotted against $\log(s_0/s)$, where s_0 is the initial concentration of substrate, and s the remaining substrate concentration, and the inhibition and kinetic constants determined from the slope using the equation $K_i = [I] \cdot K_M / V_{max} \cdot 1 / \text{slope}$.[15]

For CT, all kinetic measurements were performed using degassed water to minimize the inhibition of the enzyme by CO_2, and the kinetic data determined under the same reaction conditions as for SC. K_M and K_{cat} for the standard substrate Suc-Ala-Ala-Pro-Phe-PNA were again determined with the Grafit program under the following reaction conditions: substrate concentration (0.025, 0.033, 0.050, 0.100, 0.200, 0.500 and 1.000 mM), 0.1 M NaH_2PO_4 buffer pH7.5, 0.5 M NaCl, 5% DMSO and 2.1 nM of the enzyme. The progress curve without inhibitor was obtained from the reaction mixture of pH 7.5 buffer containing 0.2 M NaH_2PO_4 and 1.0 M NaCl (1.50 mL), 25 mM substrate in DMSO (30 μL), DMSO (120 μL) and H_2O (1.32 mL) incubated for 5 min at 25 °C. The reaction was initiated by the addition of 30 μL of CT (1.6×10^{-6} M in 1.0 mM HCl), with a final reaction mixture volume of 3 mL containing 0.1 M NaH_2PO_4, 0.5 M NaCl, 16 nM of the enzyme, 0.25 mM of substrate and 5% DMSO. The progress curve with inhibitor was also obtained by adding 100-120 μL of the inhibitor solution (0.14-0.17 M in DMSO) under the same reaction condition as above. The progress of hydrolysis of each assay was recorded directly into a PC, and the inhibition constants calculated as for SC. The results are recorded in Table 1.

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