certain degree of conversion (Table III). The reactions were terminated by filtering the enzyme, and the liquid phase was washed with three 150-mL portions of 0.3 M aqueous $NaHCO_3$. The organic phase obtained was dried with $MgSO_4$ and the solvent evaporated in a rotary evaporator. The pure esters were isolated free from the 1-butanol by distillation. Optical rotations and ee values are given in Table III.

Synthesis of (*R*)-(+)-2-Hydroxycaproic Acid ((*R*)-(+)-4). A solution of racemic **4** was prepared in toluene as follows: 2.4 g of (*R,S*)-**4** (0.1 M) was dissolved in 0.18 L of toluene containing 0.3 M 1-butanol and the reaction initiated upon the addition of 9.0 g of CCL. The reaction was stirred at 250 rpm at 30 °C. The reaction was terminated after 30 h by filtering off the CCL. The conversion at this time was 70%. The unreacted acid was extracted into 0.3 M $NaHCO_3$ solution, acidified with HCl, and reextracted into CH_2Cl_2. The solvent was then dried with $MgSO_4$ and then evaporated, leaving behind the pure acid. The (*R*)-(+)-**4** showed $[\alpha]^{25}_D$ + 27.5° (*c* 1, 1 N NaOH).

Chemical Reduction. Preparation of (*S*)-(-)-1,2-Hexanediol ((*S*)-(-)-16). A solution of 1.4 g of (*S*)-(-)-butyl 2-hydroxycaproate dissolved in 100 mL of diethyl ether was slowly added over a period of 1 h with magnetic stirring to 100 mL of diethyl ether solution containing 7.0 g of $LiAl(OCH_3)_3H$. The addition was carried out at 4 °C. When addition was complete, 25 mL of water was added to destroy the unreacted hydride and 100 mL of 4% aqueous sulfuric acid solution was added to extract the salts. The 1,2-hexanediol product remained in the organic phase and was washed three times with 100 mL of sulfuric acid solution. The 1,2-hexanediol was isolated free from 1-butanol by distillation.

The single-pot synthesis of 1,2-hexanediol was carried out in a similar fashion by sequential esterification and reduction steps in diethyl ether. No purification of the butyl ester intermediate was performed; however, the unreacted **4** was removed from the diethyl ether by extraction and the organic phase dried over $MgSO_4$ prior to reduction.

The following data were obtained: bp 91-92 °C (2 mmHg); ^{1}H NMR (360 MHz, $CDCl_3$) δ 4.15-4.08 (1 H, br s, OH), 4.08-3.93 (1 H, br s, OH), 3.70-3.55 (1 H, m, CHOH), 3.42-3.35 (2 H, m, CH_2OH), 1.45-1.30 (6 H, m, 3 CH_2), 0.94-0.88 (3 H, t, CH_3).

Other 1,2-diols were prepared in an analogous manner. For (*S*)-(+)-**13**, the recovery from the reduction step was through the water phase, as the product does not partition into the organic layer. The diol product was then purified by distillation.

(*S*)-(+)-**1,2-Propanediol (13)**: bp 186-187 °C (760 mmHg); ^{1}H NMR ($CDCl_3$) δ 4.70-4.35 (2 H, br s, OH), 3.77-3.69 (1 H, m, CHOH), 3.45-3.20 (2 H, m, CH_2OH), 1.01-0.98 (3 H, t, CH_3).

(*S*)-(-)-**1,2-Butanediol (14)**: bp 195-197 °C (760 mmHg); ^{1}H NMR ($CDCl_3$) δ 4.25-4.20 (2 H, br s, OH), 3.65-3.52 (1 H, m, CHOH), 3.42-3.30 (2 H, m, CH_2OH), 1.45-1.30 (2 H, m, CH_2), 0.93-0.85 (3 H, t, CH_3).

(*S*)-(+)-**3-Methyl-1,2-butanediol (15)**: bp 62-63 °C (4.1 mmHg); ^{1}H NMR ($CDCl_3$) δ 3.73-3.67 (1 H, m, CHOH), 3.53-3.41 (2 H, m, CH_2OH), 1.75-1.66 (1 H, m, CH), 0.98-0.96 (3 H, d, CH_3), 0.93-0.91 (3 H, d, CH_3). Note: the two OH protons were too broad for accurate measurement by NMR.

(*S*)-(-)-**4-Methyl-1,2-pentanediol (17)**: bp 80-82 °C (6.8 mmHg); ^{1}H NMR ($CDCl_3$) δ 4.60-4.35 (2 H, br s, OH), 3.81-3.75 (1 H, m, CHOH), 3.63-3.57 (1 H, m, CH_2OH), 3.42-3.33 (1 H, m, CH_2OH), 1.83-1.75 (1 H, m, CH), 1.42-1.32 (2 H, m, CH_2), 0.96-0.91 (6 H, t, CH_3).

(*S*)-(-)-**3-Phenyl-1,2-propanediol (18)**: bp 135-139 °C (2 mmHg); ^{1}H NMR ($CDCl_3$) δ 7.30-7.15 (5 H, m, C_6H_5), 3.90-3.83 (1 H, m, CHOH), 3.64-3.42 (2 H, m, CH_2OH), 2.74-2.65 (2 H, m, CH_2). Note: the two OH protons were too broad for accurate measurement by NMR.

Enzyme Kinetics. Enzyme kinetics were followed by GC for the formation of the butyl esters. The concentrations of the 2-hydroxy esters were varied from 10 to 200 mM, and the concentration of 1-butanol was fixed at 0.3 M. The concentration of CCL was 50 mg/mL, and the reaction volumes were all 10 mL. All analyses were carried out up to 10% conversion of the acid substrate. The water produced during the reaction with 200 mM substrate and 10% conversion amounts to 20 mM. This concentration of water does not affect the linearity of initial rate (ester produced vs time) measurements.

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Active-Site-Directed Modification of Subtilisin

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Abstract: This paper describes the development of several competitive inhibitors and mechanism-based inactivators of subtilisin BPN'. Various methyl arylakanesulfonates were prepared and shown to be either competitive inhibitors or selective methylating reagents for the ϵ2-N of the active-site His. Styrene sulfonyl chloride was shown to be a good covalent inactivator and benzyl *N*-(*N*-Boc-L-phenylalanyl)-L-aziridine-2-carboxylate was a strong competitive inhibitor. The second-order rate constant of inactivation and the K_i of the enzyme reacting with the methylating reagents and other inactivators were determined. The methylated enzyme was purified to homogeneity and the kinetic constants for the enzyme-catalyzed ester and amide hydrolyses were determined. It was established that the methylated enzyme lost most of the amidase activity while the esterase activity was still significant and useful for peptide synthesis via aminolysis. A mechanism involving ring flipping of the active-site imidazole, first proposed for methylchymotrypsin activity, was also proposed to explain methylsubtilisin-catalyzed reactions.

Selective modification or inhibition of enzymes at the active site has been a subject of intense investigation. One of our interests in this regard is to develop modified proteases for the aminolytic condensation of peptide segments to form larger peptides without secondary peptide hydrolysis. We have recently developed two strategies to accomplish this goal: one is the use of serine proteases in the presence of water-miscible organic solvents, which selectively inhibit the amide cleavage activities;[1,2] the other is the use of methylchymotrypsin prepared via site-directed methylation.[3] Both approaches provide modified proteases useful for peptide segment coupling. One disadvantage with methylchymotrypsin is that only peptidyl segments that meet the chymotrypsin specificity requirements can be coupled,[2] which presents a limitation of this methodology. We describe here the development of new reagents for selective methylation of subtilisin BPN', a serine protease with a wide range of substrate specificity,[4] at the ϵ-N of active-site His,

(1) Barbas, C. F., III; Matos, J. R.; West, J. B.; Wong, C.-H. *J. Am. Chem. Soc.* **1988**, *110*, 5162, and references cited therein for various approaches to peptide synthesis.

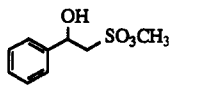
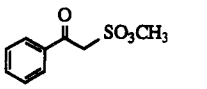
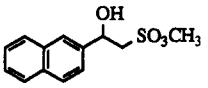
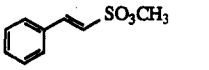
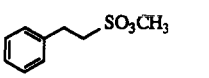
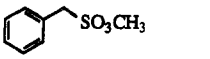
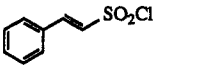
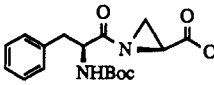
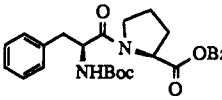
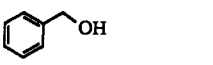
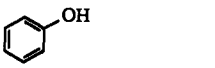
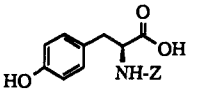
(2) Wong, C.-H.; Chen, S.-T.; Hennen, W. J.; Bibbs, J. A.; Wang, Y.-F.; Liu, J. L.-C.; Pantoliano, M. W.; Whitlow, M.; Bryan, P. N. *J. Am. Chem. Soc.* **1990**, *112*, 945. Zhong, Z.; Liu, J. L.-C.; Dinterman, L. M.; Finkelman, M. A. J.; Mueller, W. T.; Rollence, M. L.; Whitlow, M.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113*, 683.

(3) West, J. B.; Scholten, J.; Stolowich, N. J.; Hogg, J. L.; Scott, A. I.; Wong, C.-H. *J. Am. Chem. Soc.* **1988**, *110*, 3709. West, J. B.; Hennen, W. J.; Bibbs, J. A.; Lalonde, J. L.; Zhong, Z.; Meyer, E. F.; Wong, C.-H. *J. Am. Chem. Soc.* **1990**, *112*, 5313. For other semisynthetic subtilisin, see: Wu, Z. P.; Hilvert, D. *J. Am. Chem. Soc.* **1989**, *111*, 4513. Nakatsuka, T.; Sasaki, T.; Kaiser, E. T. *J. Am. Chem. Soc.* **1987**, *109*, 3808.

(4) Philipp, M.; Bender, M. L. *Mol. Cell. Biochem.* **1983**, *51*, 5.

0002-7863/91/1513-2259$02.50/0

Table I. Inactivators/Inhibitors for Subtilisin BPN'

compound		k_i, s^{-1}	K_I, M	k_i/K_I, s^{-1} M^{-1}
	1	7.68×10^{-4}	0.61	1.26×10^{-3}
	2	4.38×10^{-4}	0.11	3.98×10^{-3}
	3	ND[a]	6.1×10^{-4}	
	4	ND[a]	0.029	
	5		0.18	
	6		0.094	
	7	0.0234	2.94×10^{-3}	7.96
	8		5.6×10^{-4}	
	9		1.7×10^{-3}	
	10		0.11[b]	
	11		0.10[c]	
	12		0.015[d]	

[a] k_i is not determined since the inhibitor inactivates the enzyme very slowly. The enzyme lost only 20% activity in 24 h when enzyme solution ($\sim 10^{-4}$ M) was saturated with inhibitor. K_I was determined by **3** as a competitive inhibitor. [b] Philipp, M.; Bender, M. L. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 478. [c] Glazer, A. N. *J. Biol. Chem.* **1967**, *242*, 433. [d] Philipp, M. Doctoral Dissertation, Northwestern University, Evanston, IL, 1971.

and the investigation of methylsubtilisin (MeS) with regard to its catalysis and energetics. We also describe the development of other new reagents that selectively inhibit or inactivate subtilisin BPN'.

Results and Discussion

Neither the chymotrypsin nor the trypsin methylating reagents would methylate subtilisin;[5] therefore, a new reagent was needed. After evaluation of structural features of synthetic subtilisin substrates[4] and with the aid of computer-assisted molecular modeling,[6] several methyl sulfonate reagents were developed for selective methylation (Table I).

As shown in the table, some methylating reagents (**1** and **2**) irreversibly inactivate subtilisin. A typical time-dependent inactivation was observed as shown in Figure 1 for the semilog plot of remaining enzyme activity vs time in the presence of the inactivator methyl 2-hydroxy-2-phenylethanesulfonate (**1**). A replot of the enzyme half-life ($t_{1/2}$) determined at different inhibitor (I) concentrations vs 1/[I] gave a straight line. The y and x intercepts are $0.69/k_i$ and $-1/K_I$, respectively, where k_i is the pseudo-first-order rate constant of inactivation and K_I is the inhibition constant (In this case, it is equivalent to K_i, the dissociation constant of the EI complex). Some other methylating reagents (**3–6**) were too slow to inactivate the enzyme, but behaved as competitive inhibitors.

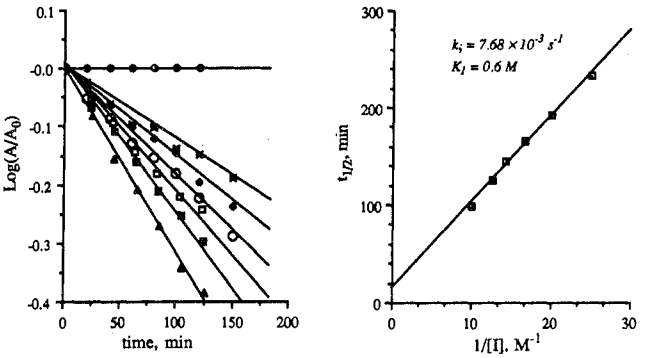


Figure 1. (a) Mechanism-based inactivation of BPN' by **1**. Inhibitor concentrations: ●, 0 mM; * 20 mM; ◆, 25 mM; ○ 30 mM; □, 35 mM; ■, 40 mM; ▲, 50 mM. (b) $t_{1/2}$ vs 1/[I] plot. The x-axis intercept corresponds to $-1/K_I$ and the y intercept is equal to $0.69/k_{inact}$.

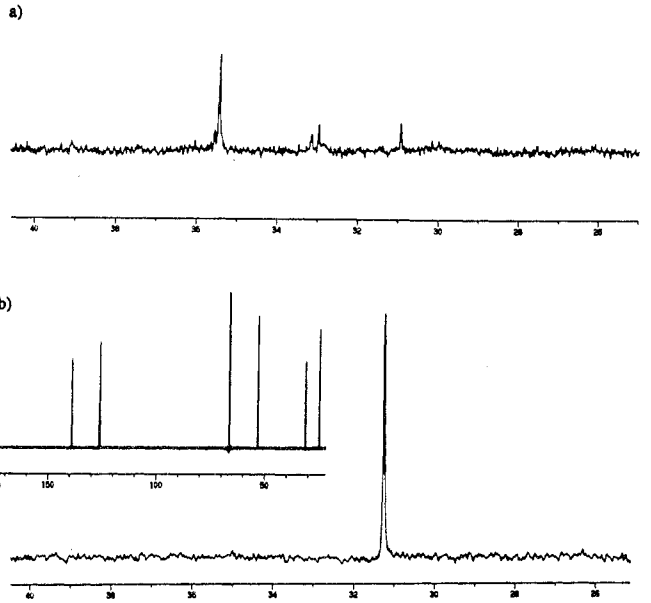


Figure 2. ^{13}C NMR spectra of (a) methylsubtilisin and (b) 1-methyl-L-histidine. The inset is the whole spectrum for 1-methyl-L-histidine. The peak at 66.5 ppm is from dioxane, the internal standard.

To investigate the methylation reaction, subtilisin was treated with **1** containing a carbon-13-enriched methyl group. Following inactivation with the labeled compound, the ^{13}C NMR spectrum of the methylated enzyme was then compared to that of 1-methyl-L-histidine.[7] As shown in Figure 2, for 1-methyl-L-histidine there are four resonances between 20 and 80 ppm including the internal standard at 66.5 ppm (from dioxane). The resonance at 31.3 ppm is due to the methyl group attached to the ϵ2-N of the amino acid as determined by separate DEPT experiments. Figure 2 also shows the resonances between 20 and 40 ppm of subtilisin after reaction with the labeled methylating reagent. The slightly more downfield signal at 35 ppm is probably

(5) Two other reagents were unsuccessful in methylating subtilisin, and these were *p*-nitromethylbenzenesulfonate (for chymotrypsin: Nakagawa, Y.; Bender, M. L. *Biochemistry* **1970**, *9*, 259) and methyl *p*-guanidinobenzenesulfonate (for trypsin: Jackson, M. B.; Bender, M. L. *Biochem. Biophys. Res. Commun.* **1970**, *39*, 1157).

(6) Jones, T. A. *J. Appl. Crystallogr.* **1978**, *11*, 268.

(7) Horsley, W.; Sternlicht, H.; Cohen, J. S. *J. Am. Chem. Soc.* **1970**, *92*, 680. Horsley, W.; Sternlicht, H.; Cohen, J. S. *Biochem. Biophys. Res. Commun.* **1969**, *37*, 47.

(8) West, B. L.; Hennen, W. J.; Lalonde, J. L.; Bibbs, J. A.; Zhong, Z.; Meyer, E. F., Jr.; Wong, C.-H. *J. Am. Chem. Soc.* **1990**, *112*, 5313.

Table II. Kinetic Constants for Native and Methylated Subtilisin (MeS)

	k_{cat}, s^{-1}	K_m, μM	k_{cat}/K_m, M^{-1} s^{-1}	k_2, s^{-1}	K_S, M^{-1}
Suc-AAPF-pNA					
native BPN′	47	172	2.73×10^5	53.25	1.95×10^{-4}
MeS	0.109	213	5.12×10^2	0.109	2.13×10^{-4}
Suc-AAPF-SBzl					
native BPN′	400	100	4×10^6		
MeS	85	254	3.35×10^5		

Table III. Free Energy for Native and Methylated Subtilisins Action on Suc-Ala-Ala-Pro-Phe-X (Where X = pNA and SBzl)

	relative free energy,[a] kcal/mol				
enzymes	$\Delta G°$ (ES)	$\Delta G^‡$ (k_2)	$\Delta G^‡$ (K_S)	$\Delta G^‡$ (k_{cat}/K_m)	$\Delta G°$ (ES′)
native	3.12	15.08	13.89	16.62	2.73
MeS	3.17	18.75	14.81	18.09	3.28

[a] All free energies calculated are relative to the energy of free enzyme assigned as 0 kcal/mol.

Scheme I. General Reaction Scheme for Serine Protease Action on Substrate (S)[a]

$$E + S \underset{K_S}{\rightleftharpoons} ES \xrightarrow{k_2} ES' + P_1 \xrightarrow{k_3} E + P_2$$

due to the different environment in the enzyme active site. Similar ^{13}C NMR results were also observed with chymotrypsin after reaction with ^{13}C-labeled methyl *p*-nitrophenylsulfonate. Some other compounds were also prepared and evaluated as inactivators as shown in Table I. Styrenesulfonyl chloride (**7**) and *N*-(*N*-Boc-Phe)-aziridine-2-carboxylic acid benzyl ester (**8**) are particularly interesting. According to the inhibition kinetics, the former is an inactivator and is believed to react with the active-site serine residue. The latter is a competitive inhibitor, which binds to the active site ~30 times more strongly than the corresponding Phe-Pro peptide. Surprisingly, the aziridine ring is not subjected to nucleophilic attack in the active site.

Several amide substrates were tested as substrates with methylsubtilisin. The rates were extremely low and difficult to measure. The only amide substrate that showed appreciable hydrolysis was *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide. Therefore, the corresponding thioester Suc-Ala-Ala-Pro-Phe-SBzl was chosen for a kinetic comparison. The results are summarized in Table II. When k_{cat} was considered, the methylated enzyme hydrolyzed the thioester at 22% of the native enzyme rate and the amide substrate at only 0.2% of the native enzyme rate. A slight change in substrate affinity was also observed. After methylation, the K_m value for the ester increased more than that for the amide substrate. Similar trends in k_{cat} and K_m were also observed with subtilisin in the presence of water-miscible organic solvents.[2]

As seen with native subtilisin and other serine proteases,[9] methylsubtilisin demonstrated pre-steady-state burst kinetics with the ester substrate but not with the amide, indicating that with ester substrates deacylation is still rate limiting, whereas with amide substrates, acylation is rate limiting. This is consistent with the methylchymotrypsin results where the acyl enzyme intermediate in ester hydrolysis was detected at room temperature with NMR.[3]

With data from Table II, the simple rate constants as shown in Scheme I can be determined. The rate-limiting step with esters is k_3. Thus, k_2 and K_S were calculated from k_3(ester) and k_{cat}(amide).[10] The change in free energy upon substrate binding

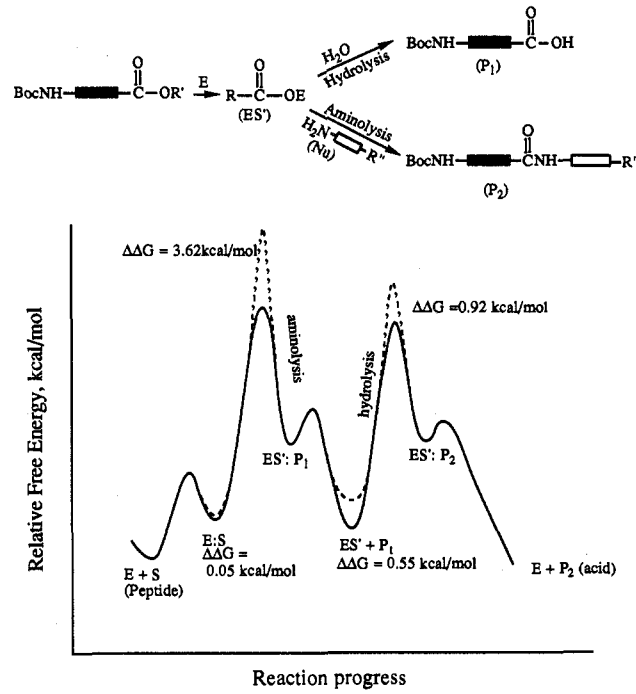


Figure 3. Free energy diagrams for the native (solid line) and methylated (dashed line) subtilisin reactions.

and activation can be determined from K_S, k_2, and k_3(ester) (Table III). A free energy diagram was generated from these data (Figure 3).

From the free energy diagram generated for native and methylsubtilisin,[10] it is obvious why methylsubtilisin has very little amidase activity. As shown in Table III and Figure 3, the free energy required for acylation with methylsubtilisin is very high (18.75 kcal/mol) compared to that with the native enzyme (15.08 kcal/mol) even though the substrate has a better affinity for the methylated enzyme (as reflected in K_S). Apparently, the substrate binds to the methylsubtilisin in a way that is less favorable for acylation. This result indicates why there is very little peptide (or product) hydrolysis after formation with methylchymotrypsin and methylsubtilisin. The change in the free energy of activation for acylation between the native and methylated enzyme is approximately 3.67 kcal/mol.

For serine protease assisted peptide synthesis in a kinetically controlled approach, the acyl enzyme intermediate partitions between hydrolysis to form an acid product or aminolysis to form the amide bond. This partitioning is one of the keys to peptide coupling. Assuming amide bond formation is the reverse of amide bond cleavage, and the principle of microscopic reversibility must apply, the incoming amine nucleophile must bind in the active site[15] where the previous amine leaving group is bound prior to

(9) Walsh, C. *Enzymatic Reaction Mechanisms*; W. H. Freeman and Co.: San Francisco, 1979; Chapter 3. Fersht, A. *Enzyme Structure and Mechanism*, 2nd ed.; W. H. Freeman and Co.: New York, 1985; pp 197–207.

(10) With serine proteases, it has been established that with ester substrates the rate-limiting chemical step is deacylation[9,11] of the acyl enzyme (ES′), or k_3 in Scheme I. By use of k_3 and the k_{cat} determined for the amide substrate, k_2 can be determined based on $1/k_2 = 1/k_{cat}(\text{amide}) - 1/k_3$. Once k_2 is calculated, K_S, the ES dissociation constant, is determined by dividing k_2 by the second-order rate constant, k_{cat}/K_m (for the amide substrate). All the first-order rate constants were converted to free energies of activation by using the Eyring equation or transition-state theory.[12] The second-order rate constants and equilibrium constants were normalized to a 1-μM standard state before relative energies were calculated.[13] The equilibrium constants were converted to calories by using the thermodynamic relationship, $\Delta G = -RT \ln K$. The relative energy difference for ES′, as compared to the free enzyme, can be estimated[14] by subtracting the free energy of activation for the ester k_{cat}/K_m ($\Delta G^‡(k_{cat}/K_m)$ from the free energy of activation for k_{cat} ($\Delta G^‡(k_{cat})$), that is, $\Delta\Delta G°$ (ES′) = $\Delta G^‡(k_{cat}) - \Delta G^‡(k_{cat}/K_m)$.

(11) Stein, R. L.; Strimpler, A. M.; Hori, V. J.; Powers, J. *Biochemistry* **1987**, *25*, 1301.

(12) Maskill, H. *The Physical Basis of Organic Chemistry*; Oxford University Press: Oxford, 1985. Jencks, W. P., *Catalysis in Chemistry and Enzymology*; Publications Inc.: New York, 1969, 1975.

(13) Albery, W. J.; Knowles, J. R. *Biochemistry* **1976**, *15*, 5631.

(14) Fersht, A. *Enzyme Structure and Mechanism*, 2nd ed.; W. H. Freeman and Co.: New York, 1985; Chapter 12.

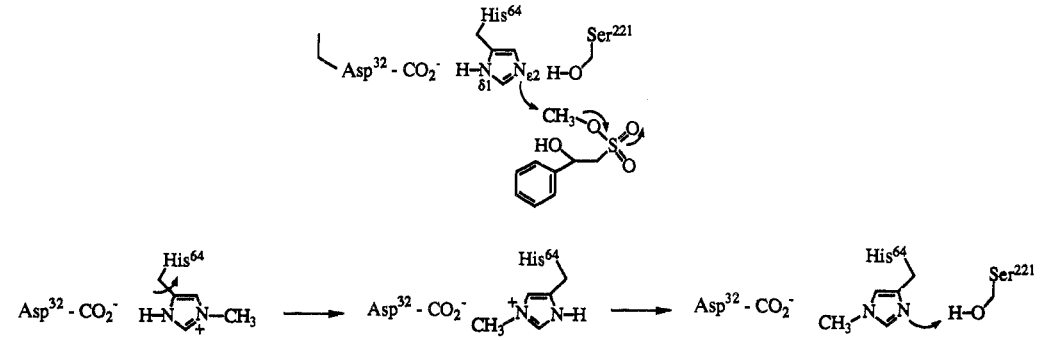


Figure 4. Mechanisms for subtilisin BPN′ reacting with **1** and for methylsubtilisin-catalyzed reaction.

leaving (P_1'). By methylating the active-site histidine, it is possible that the critical water molecule needed for rapid hydrolysis has been displaced because the active site becomes more hydrophobic. Crystal structures of chymotrypsin and methylchymotrypsin have shown that indeed a critical water molecule has been removed from the methylchymotrypsin active site.[16] Methylation decreases the stability of the acyl enzyme. The acyl enzyme of methylsubtilisin is less stable than that of the native enzyme by 0.55 kcal/mol. As a result, the binding of the amine nucleophile to the methylsubtilisin acyl enzyme is more favorable; that is, it is easier to form a ES′–nucleophile complex. Since more amine nucleophile is binding to the acyl enzyme, more aminolysis rather than hydrolysis events are taking place. The combination of a less stable acyl enzyme and a more hydrophobic active site as a result of methylation seems to favor aminolysis over hydrolysis.

These results together with that from methylchymotrypsin[2] lead us to propose mechanisms for the methylation and aminolysis catalyzed by methylsubtilisin (Figure 4). After methylation, the imidazole group may undergo ring flipping to use δ1-N as a general base for catalysis. This ring-flipping mechanism was first proposed by Henderson[17] and supported by others for methylchymotrypsin activity.[8,17] It is this modification that weakens the amide cleavage activity more severely than the ester cleavage activity.

In summary, with methylenzyme-catalyzed peptide formation, an ester reacts with methyl enzyme quickly to form an acyl enzyme intermediate, which then partitions between an amine nucleophile (aminolysis) and a water molecule (hydrolysis). As compared to the native enzyme, the methyl enzyme seems to favor the aminolysis reaction, which becomes almost irreversible due to the increased binding of the peptide product to the enzyme as shown by K_m. Similar situations were observed when serine proteases were treated with water-miscible organic solvents.[2] Preliminary studies on other aminolyses using methylsubtilisin[18] indicate that the modified enzyme is a useful catalyst for peptide synthesis. Work is in progress to use methylsubtilisin for synthesis of large peptides.

Experimental Section

Reagents. All inorganic salts, buffer components, and solvents were reagent grade and used as received. Subtilisin BPN′, Suc-Ala-Ala-Pro-Phe-pNA and Suc-Ala-Ala-Pro-Phe-SBzl were purchased from Sigma and used as received. Other chemicals and $^{13}CH_3OH$ (99% atom ^{13}C) were purchased from Aldrich and used as received.

Methyl 2-Hydroxy-2-phenylethanesulfonate (1). To 10 mL of anhydrous THF was added 1.1 g of $CH_3SO_3CH_3$ (10 mmol) at −78 °C. A 1.6 M solution of *n*-butyllithium in hexane/THF (6.25 mL) was added dropwise through a syringe with stirring. The reaction mixture was allowed to stir for an additional 20 min and then 1.06 g of benzaldehyde in 2 mL of THF was added dropwise. The reaction was stopped after 30 min by quenching with 1 N HCl. The solution was extracted with ethyl acetate (150 mL) and washed with 1 N HCl (3 × 100 mL) and H_2O (2 × 100 mL). The organic phase was dried over Na_2SO_4 and the solvent was evaporated in vacuo. The residue was subjected to silica gel chromatography to give 1.7 g (78%) of pure **1** as a thick oil: ^1H NMR ($CDCl_3$) δ 3.22 (s, 1 H), 3.38 (d, 1 H, J = 15 Hz), 3.50 (dd, 1 H, J_1 = 14.5 Hz, J_2 = 15 Hz), 3.95 (s, 3 H), 5.30 (d, 1 H, J = 9.5 Hz); HRMS (M^+) calcd 216.0457, found 216.0434.

Methyl 2-Oxo-2-phenylethanesulfonate (2). The procedure for the synthesis of **1** was applied substituting distilled benzoyl chloride for benzaldehyde. The final product was recrystallized from ether/ethyl acetate (1.28 g, 68%) as a white solid: mp 77.5–78 °C; ^1H NMR ($CDCl_3$) δ 4.02 (s, 3 H), 4.73 (s, 2 H), 7.51–7.56 (m, 2 H), 7.64–7.66 (m, 1 H), 7.99–8.02 (m, 2 H); HRMS (M^+) calcd 214.0300, found 214.0482.

Methyl 2-Hydroxy-2-naphthylethanesulfonate (3). By employment of the same procedure for the synthesis of **1** with 2-naphthaldehyde in place of benzaldehyde, compound **3** was prepared on a 10-mmol scale. The final product was recrystallized from CH_2Cl_2/ethyl acetate (2.0 g, 75%) as white solid: mp 63.5–64 °C; ^1H NMR ($CDCl_3$) δ 3.46–3.61 (m, 3 H), 3.97 (s, 3 H), 5.48 (d, 1 H, J = 8.5 Hz), 7.46–7.52 (m, 3 H), 7.84–7.88 (m, 4 H); HRMS (M^+) calcd 266.0430, found 266.0633.

General Procedure for the Preparation of Methyl Alkanesulfonate.[19] To a methylene chloride solution containing 1.0 equiv of methanol and 1.1 equiv of triethylamine cooled in an ice bath was added dropwise a methylene chloride solution containing 0.95 equiv of sulfonyl chloride. After the addition was completed, the reaction mixture was allowed to stir until the TLC showed complete consumption of sulfonyl chloride. The triethylamine hydrochloride was filtered and washed with CH_2Cl_2. The combined organic solution was extracted with 1 N HCl three times and H_2O twice. The solvent was dried over Na_2SO_4 and evaporated in vacuo to yield pure product.

Methyl Styrenesulfonate (4). By use of the general procedure described above, compound **4** was prepared on a 2-mmol scale. The final product was recrystallized from hexane/CH_2Cl_2 (0.35 g, 90%) as white solid: mp 66–66.5 °C; ^1H NMR ($CDCl_3$) δ 3.86 (s, 3 H), 6.72 (d, 1 H, J = 15.6 Hz), 7.41–7.57 (m, 5 H), 7.62 (d, 1 H, J = 15.5 Hz).

2-Phenylethanesulfonyl Chloride. A 1.38-g sample of phenethyl mercaptan (10 mmol) and 5.32 g of *N*-chlorosuccinimide (40 mmol) dissolved in 40 mL of methylene chloride were layered with 20 mL of H_2O. The mixture was stirred for 2 h at room temperature until TLC showed that the reaction was complete. This reaction mixture was extracted with 200 mL of CH_2Cl_2 and the organic layer was washed with saturated $NaHCO_3$ (3 × 100 mL) and H_2O (2 × 100 mL). After being concentrated in vacuo, the residue was purified by silica gel chromatography to give the title compound as a thick oil (1.53 g, 75%): ^1H NMR ($CDCl_3$) δ 2.84–2.86 (m, 2 H), 2.92–2.94 (m, 2 H), 7.2–7.3 (m, 5 H).

Methyl 2-Phenylethanesulfonate (5). This compound was prepared on a 1-mmol scale (0.18 g, 90%): ^1H NMR ($CDCl_3$) δ 3.17 (dd, 2 H, J_1 = 9.8 Hz, J_2 = 10 Hz), 3.38 (dd, 2 H, J_1 = J_2 = 9.8 Hz), 7.18–7.34 (m, 5 H).

Methyl α-Phenylmethanesulfonate (6). This compound was prepared on a 10-mmol scale and recrystallized from CH_2Cl_2/hexanes (1.6 g, 86%) as white solid: mp 62.5–63 °C; ^1H NMR ($CDCl_3$) δ 3.66 (s, 3 H), 4.27 (s, 2 H), 7.3–7.34 (m, 5 H).

Benzyl *N*-(*N*-(*tert*-Butyloxycarbonyl)phenylalanyl)-L-aziridine-2-carboxylate (Boc-Phe-Azr-OBzl) (8). The reaction mixture containing 4 mL of anhydrous DMF, 265 mg of Boc-Phe (1.0 mmol), 203 mg of

(15) Riechmann, L.; Kasche, V. *Biochim. Biophys. Acta* **1985**, *830*, 164. Stein, R. L.; Strimpler, A. M. *Biochemistry* **1987**, *26*, 2238, and references cited therein.

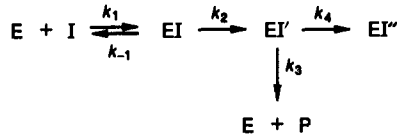
(16) Wright, C. S.; Hess, G. P.; Blow, B. M. *J. Mol. Biol.* **1972**, *63*, 9295.

(17) Henderson, R. *Biochem. J.* **1971**, *124*, 13. Scholten, J. D.; Hogg, J. L.; Raushel, F. M. *J. Am. Chem. Soc.* **1989**, *110*, 8246.

(18) The following exemplify the use of methylsubtilisin in peptide synthesis. Each reaction was carried out in a 5-mL solution (50 mM Tris/DMF, 1:1, pH 8.8) containing 5 mg of enzyme. The products were identical with those reported previously.[2,3] Details for synthesis will be published separately. Boc-Met-Leu-OMe (0.1 M) + Phe-$NHCH_2Ph$ (0.2 M) $\xrightarrow{50\ h}$ Boc-Met-Leu-Phe-$NHCH_2Ph$ (70%); Boc-Tyr-Gly-Gly-Phe-OCH_2CN (0.1 M) + Leu-NH_2 (0.2 M) $\xrightarrow{1\ h}$ Boc-Tyr-Gly-Gly-Phe-Leu-NH_2 (85%).

(19) True, W. E.; Vrencur, D. J. *J. Org. Chem.* **1970**, *35*, 1226.

Scheme II. Mechanism-Based Inactivation



$K_I = [(k_{-1} + k_2)/k_1][(k_3 + k_4)/(k_2 + k_3 + k_4)]$; $K_i = [E][I]/[EI]$; and $k_{inact} = k_i = k_2k_4/(k_2 + k_3 + k_4)$. When k_2 is rate determining, $K_I = K_i$ and

$$\frac{d(\ln E)}{dt} = \frac{k_{inact}[I]}{K_I + [I]} \qquad t_{1/2} = \frac{0.69}{k_{inact}} + \frac{0.69K_I}{k_{inact}[I]}$$

HOBt (1.5 mmol), 206 mg of DCC (1.0 mmol), 101 mg of L-aziridine-2-carboxylic acid benzyl ester,[20] and 140 mL of triethylamine (1.0 mmol) was stirred at room temperature overnight. The solution was diluted with 50 mL of ethyl acetate and extracted with saturated $NaHCO_3$ (3 × 50 mL) and H_2O (2 × 50 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was subjected to silica gel chromatography to give 220 mg (52%) of pure oily product: ^1H NMR (DMSO-d_6) δ 1.38 (s, 9 H), 2.50 (t, 1 H, J = 1.7 Hz), 2.70 (dd, 1 H, J_1 = 1.24 Hz, J_2 = 5.7 Hz), 3.09 (dd, 2 H, J_1 = 4.0 Hz, J_2 = 13.9 Hz), 3.26 (q, 1 H, J = 2.94 Hz), 4.25–4.36 (m, 1 H), 5.16 (s, 2 H), 7.21–7.41 (m, 10 H); HRMS (M^+) calcd 424.1999, found 424.2039.

Purification of Subtilisin BPN′ for Inhibition Studies. A 6-mg sample of subtilisin BPN′ (Sigma) was dissolved in 0.6 mL of ammonium acetate buffer (0.1 M with 2 M $(NH_4)_2SO_4$) and subjected to FPLC chromatography equipped with a phenyl Superose column (5 cm × 5 mm). The major peak eluting at 13.5 mL was collected (from 11.5 to 15.5 mL) for inhibition and inactivation kinetic studies. The enzyme concentration in these fractions is ~20 μM.

Methylation of Subtilisin BPN′ and Its Purification. In each methylation reaction, 7 mg of subtilisin BPN′ was dissolved in 0.62 mL of Tris-HCl buffer solution (0.1 M Tris-HCl with 2 M $(NH_4)_2SO_4$, pH 8.2). A 13-mg sample of methyl 2-hydroxy-2-phenylethanesulfonate in 80 μL of DMF was added with stirring. The reaction mixture was stirred for 3 h prior to FPLC purification.

To purify the methylated subtilisin BPN′, 500 μL of the methylation mixture was injected onto the phenyl Superose FPLC. The protein mixture was eluted by NH_4OAc buffer (0.1 M, pH 6.8) with a linear gradient of $(NH_4)_2SO_4$ from 1.7 to 0 M in 35 min and a flow rate of 0.5 mL/min. The methylated subtilisin was eluted at 11.5 mL while native subtilisin was eluted at 13.5 mL. The fractions from 10.5 to 12.5 mL were collected. These fractions were then concentrated and rechromatographed three times to ensure the final methylated subtilisin was free of native enzyme. All the eluting procedures were the same except that only the 10.5–11.5-mL fractions were collected for the kinetic studies.

Confirmation of Methylated Subtilisin. By use of ^{13}C-enriched methylating reagent, ~2 mg of pure [^{13}C]methylsubtilisin was obtained after FPLC. This enzyme was dissolved in 0.7 mL of D_2O with 1 μL of dioxane as internal standard for ^{13}C NMR study.

Kinetic Studies of Mechanism Based Inactivation. To 800 μL of Tris-HCl buffer (0.1 M with 0.1 M NaCl, pH 8.2) was added 100 μL of purified subtilisin BPN′, giving ~2 μM enzyme solution. Inactivator solution in DMF (100 μL) was added, which made the inactivation solution contain 10% DMF. The enzyme activity was assayed at certain time intervals, normally every 20 min, depending on the inactivation rate. The logarithm of remaining enzyme activity was plotted vs time. A similar procedure was applied at several different inactivator concentrations in order to obtain different half-life values. The replot of $t_{1/2}$ vs $1/[I]$ is linear, where the y-axis intercept corresponds to $0.69/k_i$ while the x-axis intercept is equivalent to $-1/K_I$.

The enzyme activity was assayed on a Beckman DU-70 spectrophotometer with Suc-Ala-Ala-Pro-Phe-pNA as substrate. For each assay, to a cuvette containing 970 μL of Tri-HCl buffer (0.1 M, pH 8.2) were added 10 μL of substrate (18 mM in stock) and a 20-μL enzyme aliquot taken from the inhibitor solution. The initial rate of hydrolysis (monitored at 410 nm, ϵ = 8737 M^{-1}) was measured to calculate the relative enzyme activity. Scheme II illustrates the kinetic analyses of inactivation.

Determination of Enzyme Concentration. Burst kinetics were used to determine the enzyme concentrations.[20] In a typical assay, 5 μL of titrant solution was added to 975 μL of Tris-HCl buffer (0.1 M, pH 7.0 with 0.1 M NaCl). After 60 s, 20 μL of enzyme solution was injected into the cuvette and the initial burst (ΔA) was measured by extrapolating to t = 60 s. The enzyme concentration was then calculated by

$$E = \frac{\Delta A \times 1000}{20 \times \epsilon}\ \text{M}$$

For the native enzyme, *N-trans*-cinnamoylimmidazole was used as titrant and the reaction was monitored at 335 nm, where ϵ = 9040 M^{-1}. Z-Tyrosine p-nitrophenyl ester was used to titrate the active-site concentration of methylsubtilisin at 405 nm with ϵ = 9367 M^{-1}.

Kinetics of Enzyme Reaction with Suc-Ala-Ala-Pro-Phe-X (X = pNA and SBzl). The concentration of the pNA substrate ranged from 50 to 150 μM. In a typical assay, a calculated amount of substrate solution in DMF was added to 940 μL of Tris-HCl buffer (0.1 M with 0.1 M NaCl, pH 8.2) and the total volume was adjusted to 980 μL with DMF. After the addition of 20 μL of enzyme solution, the reaction was monitored at 410 nm, where ϵ = 8737 M^{-1}. After all the initial rates were acquired, data were processed by ENZFITTER software[21] on an IBM PS2/30-286 personal computer to yield k_{cat} and K_m.

In the assay of thiobenzyl ester substrate, 5 μL of dithiodipyridine (0.16 M in DMF as stock solution) was added as coupling reagent and the reaction was monitored at 324 nm (ϵ = 15 000 M^{-1}).[22] Data were processed by ENZFITTER to give k_{cat} and K_m.

Competitive Inhibition Kinetics. The initial rate of subtilisin-catalyzed hydrolysis of Suc-Ala-Ala-Pro-Phe-pNA was measured at five different substrate concentrations, ranging from 50 to 150 μM at different constant inhibitor concentration. The replot of the slopes of the Lineweaver–Burk plots vs [I] gave the $-K_I$ as the x-axis intercept.

Registry No. 1, 131353-61-2; **2**, 131353-62-3; **3**, 131353-63-4; **4**, 13719-30-7; **5**, 10307-30-9; **6**, 5877-96-3; **7**, 4091-26-3; **8**, 131353-64-5; **9**, 70462-58-7; Suc-AAPF-pNA, 70967-97-4; Suc-AAPF-SBzl, 80651-95-2; Boc-ML-OMe, 66880-59-9; Boc-YGGF-OCH_2CN, 131353-65-6; Boc-MLF-NHBzl, 124044-60-6; Boc-YGGFL-NH_2, 79396-86-4; Boc-Phe-OH, 13734-34-4; H-His-OH, 71-00-1; H-Phe-NHBzl, 6455-20-5; H-Leu-NH_2, 687-51-4; $CH_3SO_3CH_3$, 66-27-3; PhCHO, 100-52-7; PhCOCl, 98-88-4; $PhCH_2CH_2SO_2Cl$, 4025-71-2; $PhCH_2SO_2Cl$, 1939-99-7; $PhCH_2CH_2SH$, 4410-99-5; subtilisin, 9014-01-1; 2-naphthaldehyde, 66-99-9; L-aziridine-2-carboxylic acid benzyl ester, 67413-26-7.

(20) Bender, M. L.; Begue-Canton, M. L.; Blakeley, R. L.; Bruchacher, L. J.; Feder, J.; Gunter, C. R.; Kezdy, F. J.; Killheffer, J. V.; Marshall, T. H.; Miller, C. G.; Roeske, R. W.; Stoops, J. K. *J. Am. Chem. Soc.* **1966**, *88*, 5890.

(21) ENZFITTER is a nonlinear regression data analysis program for the IBM PC written by R. J. Leatherbarrow, BIOSOFT, P. O. Box 580, Miltown, NJ 08850. For a review of the software, see: Bruckenstein, S. *J. Am. Chem. Soc.* **1988**, *110*, 4100.

(22) McRae, B. J.; Kurachi, K.; Heimark, R. L.; Fujikawa, K.; Davie, E. W.; Powers, J. C. *Biochemistry* **1981**, *20*, 7196.

(23) This research was supported by NSF Grant CHE 8996249.