Enzymes in Organic Synthesis: Use of Subtilisin and a Highly Stable Mutant Derived from Multiple Site-Specific Mutations

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Abstract: A subtilisin mutant (subtilisin 8350) derived from subtilisin BPN′ via six site-specific mutations (Met50Phe, Gly169Ala, Asn76Asp, Gln206Cys, Tyr217Lys, and Asn218Ser) was found to be 100 times more stable than the wild-type enzyme in aqueous solution at room temperature and 50 times more stable than the wild type in anhydrous dimethylformamide. Kinetic studies using ester, thio ester, and amide substrates, and the transition-state analogue inhibitor Boc-Ala-Val-Phe-CF_3, indicate that both the wild-type and the mutant enzymes have very similar specificities and catalytic properties. The inhibition constant (K_i = 5.0 μM) for the wild-type enzyme is approximately 5 times that of the mutant enzyme (K_i = 1.1 μM), suggesting that the mutant enzyme binds the reaction transition state more strongly than the wild-type enzyme. This result is consistent with the observed rate constants for the corresponding ester and amide substrates; i.e., the k_{cat}/K_m values for the mutant are larger than those for the wild-type enzyme. Application of the mutant enzyme and the wild-type enzyme to organic synthesis has been demonstrated in the regioselective acylation of nucleosides in anhydrous dimethylformamide (with 65–100% regioselectivity at the 5′-position), in the enantioselective hydrolysis of N-protected and unprotected common and uncommon amino acid esters in water (with 85–98% enantioselectivity for the L-isomer), and in the synthesis of di- and oligopeptides via aminolysis of N-protected amino acid and peptide esters. The enzymatic peptide synthesis was carried out under high concentrations of DMF (~50%) to improve substrate solubility and to minimize enzymatic peptide cleavage. Low enantioselectivity was observed in the enzymatic transformation of non-amino acid alcohols and acids.

Enzymes with esterase activities have proven useful as catalysts in the synthesis of peptides,[1] enantioselective transformation of chiral alcohols, amines, and acids,[2] and regioselective acylation of sugars and related compounds.[3] One limitation to the usefulness of most enzymes in this regard, however, is their intrinsic instability in many unnatural environments required to perform any organic reactions.

Advances in methods for introducing specific mutations into a cloned gene and then expressing the altered protein have provided both a powerful experimental tool for studying the relationship between protein structure and functions and a means of creating proteins with improved characteristics for specific uses.[4] One parameter that has proven amenable to modification is protein stability.[5-17] The ability to engineer more stable enzymes should improve their usefulness in synthetic applications.

Several approaches have been used to create a more stable serine protease via single amino acid substitutions in subtilisin from *Bacillus amyloliquefaciens* (subtilisin BPN′). These include the use of site-directed mutagenesis to improve electrostatic, hydrophobic, and H-bonding interactions and to introduce additional disulfide bonds and the use of in vitro random mutagenesis coupled with phenotypic screening to identify stabilizing mutational events.[5-7,17] It was found that combining individual stabilizing mutations results in a cumulative increase in stability. Calorimetric and crystallographic data demonstrate that increases in the free energy of stabilization are often independent and additive.[18] It is therefore possible to create extremely stable versions of subtilisins in a step by step manner. One of the stable versions is subtilisin 8350, which contains six stabilizing mutations. We now have found that in anhydrous dimethylformamide (DMF) the variant enzyme is catalytically competent and has an effective half-life more than 50 times greater than that of the native enzyme. It is also more stable and catalytically more active in many other unnatural environments used in organic transformations. These initial results encouraged us to study the catalytic properties and the active-site requirements of the variant enzyme and to explore

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(1) For recent reviews in peptide synthesis, see: (a) Jakubke, H.-D.; Kuhl, P.; Konnecke, A. *Angew Chem., Int. Ed. Engl.* **1985**, *24*, 85. (b) Morihara, K. *Trends Biotechnol.* **1987**, *5*, 164. (c) Kullmann, W. *Enzymatic Peptide Synthesis*; CRC Press: Boca Raton, FL, 1987. For recent papers, see: (d) Luthi, P.; Luisi, P. L. *J. Am. Chem. Soc.* **1984**, *106*, 7285. (e) Barbas, C. F., III; Wong, C.-H. *J. Chem. Soc., Chem. Commun.* **1987**, 533. (f) West, J. B.; Wong, C.-H. *Tetrahedron Lett.* **1987**, *28*, 1629. (g) Margolin, A. L.; Klibanov, A. M. *J. Am. Chem. Soc.* **1987**, *109*, 3802. (h) Margolin, A. L.; Tai, D. F.; Klibanov, A. M. *Ibid.* **1987**, *109*, 7885. (i) West, J. B.; Scholten, J.; Stolowich, N. J.; Hogg, J. L.; Scott, A. I.; Wong, C.-H. *Ibid.* **1988**, *110*, 3709. (j) Barbas, C. F., III; Matos, J. R.; West, J. B.; Wong, C.-H. *Ibid.* **1988**, *110*, 5162.

(2) For reviews, see: Jones, J. B. *Tetrahedron* **1986**, *42*, 3351. Klibanov, A. M. *CHEMTECH* **1986**, 354. Akiyama, A.; Bednarski, M.; Kim, M. J.; Simon, E. S.; Waldmann, H.; Whitesides, G. M. *Ibid.* **1988**, 627. Shimizu, S.; Yamada, H. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 622. Sih, C. J.; Wu, S.-H. *Top. Stereochem.* **1988**, *19*. Sonnet, P. E. *CHEMTECH* **1988**, 94. Wong, C.-H. *Science* **1989**, *244*, 1145. For recent papers, see: Hemmerle, H.; Gais, H.-J. *Tetrahedron Lett.* **1987**, *28*, 3471. Scilimati, A.; Ngooi, T. K.; Sih, C. J. *Ibid.* **1988**, *29*, 4927. Lam, L. K. P.; Brown, C. M.; Jeso, B. D.; Lym, L.; Toone, E. J.; Jones, J. B. *J. Am. Chem. Soc.* **1988**, *110*, 4409. Lalonde, J. J.; Bergbreiter, D. E.; Wong, C.-H. *J. Org. Chem.* **1988**, *53*, 2323. Drueckhammer, D. G.; Barbas, C. F.; Nozaki, K.; Wong, C.-H.; Wood, C. Y.; Ciufolini, M. A. *Ibid.* **1988**, *53*, 1607. Wang, Y. F.; Lalonde, J. J.; Momongan, M.; Bergbreiter, D. E.; Wong, C.-H. *J. Am. Chem. Soc.* **1988**, *110*, 7200. Laumen, K.; Breitgoff, D.; Schneider, M. P. *J. Chem. Soc., Chem. Commun.* **1988**, 1459. Ohta, H.; Kimura, Y.; Sugano, Y. *Tetrahedron Lett.* **1988**, *29*, 6957. Shimada, M.; Kobayashi, S.; Ohno, M. *Ibid.* **1988**, *52*, 6961. Cotterill, I. C.; Roberts, S. M.; Williams, J. O. *J. Chem. Soc., Chem. Commun.* **1988**, 1628. Gotor, V.; Brieva, R.; Rebolledo, F. *Ibid.* **1988**, 957. Kuhn, T.; Tamm, C.; Riesen, A.; Zehnder, M. *Tetrahedron Lett.* **1989**, *30*, 693. Laumen, K.; Breitgoff, D.; Seemayer, R.; Schneider, M. P. *J. Chem. Soc., Chem. Commun.* **1989**, 148. Johnson, C. R.; Senanayake, C. H. *J. Org. Chem.* **1989**, *54*, 736. Hughes, D. L.; Beryan, J. J.; Amato, J. S.; Reider, P. J.; Grabowski, E. J. J. *Ibid.* **1989**, *54*, 1788. Kitaguchi, H.; Fitzpatrick, P. A.; Huber, J. E.; Klibanov, A. M. *J. Am. Chem. Soc.* **1989**, *111*, 3094. Wang, Y. F.; Chen, S. T.; Liu, K. K. C.; Wong, C.-H. *Tetrahedron Lett.* **1989**, *30*, 1917.

(3) (a) Riva, S.; Chopineau, J.; Kieboom, A. P. G.; Klibanov, A. M. *J. Am. Chem. Soc.* **1988**, *110*, 584. (b) Wang, Y. F.; Lalonde, J. J.; Momongan, M.; Bergbreiter, D. E.; Wong, C.-H. *Ibid.* **1988**, *110*, 7200. (c) Hennen, W. J.; Sweers, H. M.; Wang, Y. F.; Wong, C.-H. *J. Org. Chem.* **1988**, *53*, 4939. (d) Holla, E. W. *Angew Chem., Int. Ed. Engl.* **1989**, *28*, 220.

(4) Fersht, A. R. *Biochemistry* **1987**, *26*, 8031. Bryan, P. N. *Biotechnol. Adv.* **1987**, *5*, 221. Gerlt, G. A. *Chem. Rev.* **1987**, *87*, 1079. Knowles, J. R. *Science* **1987**, *236*, 1252. Benkovic, S. J.; Fierke, C. A.; Naylor, A. M. *Ibid.* **1988**, *239*, 1105. Wells, J. A.; Estell, D. A. *Trends Biochem. Sci.* **1988**, 291.

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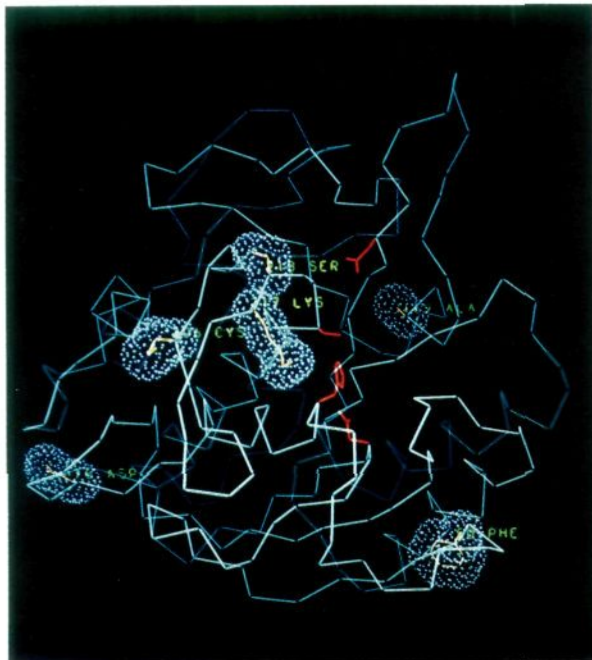


Figure 1. Structure of subtilisin 8350. The six positions of mutation and their improved stabilizing interactions determined by X-ray structure analysis[18] are Met50Phe, hydrophobic; Gly169Ala, hydrophobic; Asn76Asp, Ca^{2+} binding; Gln206Cys, van der Waals contact; Tyr217Lys, H-bonding; Asn218Ser, H-bonding.

Table I. Active-Site Survey of Subtilisin BPN′ and the Mutant with Chromogenic Substrates

enzyme,[a] substrate	k_{cat}, s^{-1}	K_m, μM	k_{cat}/K_m, M^{-1} s^{-1}
wild, Suc-Ala-Ala-Pro-Phe-pNA[b]	47	172	2.7×10^5
8350, Suc-Ala-Ala-Pro-Phe-pNA[b]	130	160	8.1×10^5
wild, NTCI	0.2	76	2.2×10^3
8350, NTCI[c]	0.6	67	9.6×10^3
wild, Z-Lys-SBzl[c]	46	531	8.7×10^4
8350, Z-Lys-SBzl[c]	33	536	6.1×10^4
wild, Bz-Tyr-OEt[c]	70	1700	4.1×10^4
8350, Bz-Tyr-OEt[c]	233	818	2.9×10^5

[a] Enzyme concentration determined with burst kinetics using *N-trans*-cinnamoylimidazole (NTCI) as an active-site titrant. [b] From ref 18. [c] Each initial rate was measured in 0.1 M phosphate buffer (pH 8.2) with 0.1 M NaCl.

its synthetic utility in regioselective acylation of nucleosides and sugars, in enantioselective transformation of amino acids, alcohols,

(5) Bryan, P. N.; Rollence, M. L.; Pantoliano, M. L.; Wood, J. F.; Finzel, B. C.; Gilliland, G. L.; Howard, A. J.; Poulos, T. L. *Proteins: Struct., Funct., Genet.* **1986**, *1*, 326.
(6) Pantoliano, M. W.; Ladner, R. C.; Bryan, P. N.; Rollence, M. L.; Wood, J. F.; Poulos, T. L. *Biochemistry* **1987**, *26*, 2077.
(7) Pantoliano, M. W.; Whitlow, M.; Wood, J. F.; Finzel, B. C.; Gilliland, G. L.; Poulos, T. L.; Rollence, M. L.; Bryan, P. N. *Biochemistry* **1988**, *27*, 8311.
(8) Cunningham, B. C.; Wells, J. A. *Protein Eng.* **1987**, *1*, 319.
(9) Matthews, B. W.; Nicholson, H.; Becktel, W. J. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 6663.
(10) Perry, L. J.; Wetzel, R. *Science* **1984**, *226*, 555.
(11) Shortle, D.; Lin, B. *Genetics* **1985**, *110*, 539.
(12) Villafranca, J. E.; Howell, E. E.; Voet, D. H.; Strobel, M. S.; Ogden, R. C.; Abelson, J. N.; Kraut, J. *Science* **1983**, *222*, 782.
(13) Hecht, M. H.; Sturtevant, J. M.; Sauer, R. T. *Proteins: Struct., Funct., Genet.* **1986**, *1*, 43.
(14) Liao, H.; McKenzie, T.; Hageman, R. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 576.
(15) Matsumura, M.; Yasumura, S.; Alba, S. *Nature* **1986**, *323*, 356.
(16) Alber, T.; Sun, D.; Wilson, K.; Wozniak, J. A.; Cook, S. P.; Matthews, B. W. *Nature* **1987**, *330*, 41.
(17) Rollence, M. L.; Filpula, D.; Pantoliano, M. W.; Bryan, P. N. *CRC Crit. Rev. Biotechnol.* **1988**, *8*, 217.

Table II. Observed Rate Constants and K_i Constants for the Boc-Ala-Val-Phe-CF_3 Slow-Binding Inhibition of the Carlsberg, BPN′, and 8350 Mutant Subtilisins

enzyme	10^6[I]; 10^3k_{obs}, s^{-1}	K_i, μM[a]
Carlsberg	2.5; 7.5 ± 0.3	
	5.0; 11.5 ± 0.3	1.7 ± 0.2[b]
	7.5; 14.6 ± 0.3	
	10.5; 19.2 ± 0.4	
BPN′	5.0; 9.49 ± 0.28	
	7.5; 12.44 ± 0.28	5.0 ± 0.9[c]
	10.0; 14.80 ± 0.28	
	15.0; 18.00 ± 0.33	
8350 mutant	5.0; 3.17 ± 0.27	
	7.5; 3.89 ± 0.20	
	10.0; 5.07 ± 0.35	1.1 ± 0.3[d]
	15.0; 7.62 ± 0.36	
	20.0; 9.68 ± 0.52	

[a] Progress of inhibition was determined by monitoring the enzyme-catalyzed hydrolysis of Z-Lys-SBzl with varying amounts of Boc-Ala-Val-Phe-CF_3 in pH 8.2 phosphate buffer at 25.0 °C. [b] k_{on} = (2.25 ± 0.11) × 10^3 M^{-1} s^{-1} and k_{off} = (3.56 ± 0.53) × 10^{-3} s^{-1}. [c] k_{on} = (1.17 ± 0.11) × 10^2 M^{-1} s^{-1} and k_{off} = (5.86 ± 0.91) × 10^{-3} s^{-1}. [d] k_{on} = (6.48 ± 0.25) × 10^2 M^{-1} s^{-1} and k_{off} = (7.17 ± 2.18) × 10^{-4} s^{-1}.

and acids, and in synthesis of peptides and polypeptides.

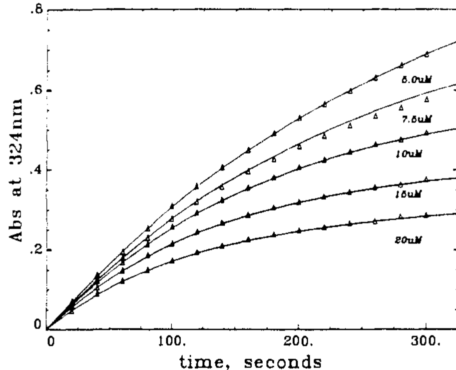
Results and Discussion

Engineering Subtilisin BPN′ and Study of the Catalytic Properties. X-ray crystal structures of wild type and the engineered 8350 mutant have been determined, which allowed a structural comparison of stable mutants with wild type. The positions of the six stabilizing modifications and their improved interactions are shown in Figure 1. Three of these changes occur in an antiparallel β-pair loop involving residues 202–219. The Asn 218 to Ser mutant was identified by screening following random mutagenesis.[5] Lys 217 and Cys 206 are known to occur in many natural alkaline proteases.[19-22] Ala 169 and Phe 50 occur in most other bacterial subtilisins. Asp 76 occurs in subtilisins Carlsberg and Dy,[17] and Ser 218 is present only in the thermophilic proteases thermitase, thermomycolase, and proteinase K.[5-17,23] A thorough characterization of the physical properties of the single-site mutants as well as the 8350 variant will be reported elsewhere.[18]

Shown in Table I is a brief chromogenic substrate survey which compares the activity of subtilisins BPN′ and 8350 for the hydrolysis of amide, ester, and thio ester substrates. In general, the second-order rate constants (k_{cat}/k_m) as well as the first-order rate constants (k_{cat}) reflect that the mutant is a moderately more efficient catalyst than the wild type except in the case of thio ester hydrolysis, where there is no significant difference. The mutant enzyme seems to interact with most substrates in a manner similar to that of the wild-type enzyme, as shown by these kinetic constants, and in some cases (with benzoyl-L-tyrosine ethyl ester) demonstrates more favorable enzyme–substrate interactions. Overall, the mutations not only retain the hydrolytic properties of subtilisin but also slightly improve the catalytic efficiency.

To assess active-site interaction with a transition-state analogue, a slow-binding peptidyl trifluoromethyl ketone inhibitor (Boc-Ala-Val-Phe-CF_3) was designed and prepared for use in kinetic studies. The sequence Ala-Val-Phe was chosen because these residues interact favorably with the corresponding subsites S_3-S_2-S_1 of the enzyme as shown by computer-assisted molecular modeling.[23] Peptidyl trifluoromethyl ketones have been shown to be very specific slow-binding inhibitors of serine proteases.[24-28] The

(18) Pantoliano, M. W.; et al. *Biochemistry*, in press.
(19) Alber, T.; Sun, D.; Wilson, K.; Wozniak, J. A.; Cook, S. P.; Matthews, B. W. *Nature* **1987**, *330*, 41.
(20) Vasantha, N.; Thompson, L. D.; Rhodes, G.; Banner, C.; Nagle, J.; Filpula, D. *J. Bacteriol.* **1984**, *159*, 811.
(21) Wells, J. A.; Ferrari, E.; Henner, D. J.; Estell, D. A.; Chen, E. Y. *Nucleic Acids Res.* **1983**, *11*, 7911.
(22) Bryan, P. N.; Pantoliano, M. W.; Quill, S. G.; Hsiao, H.; Poulos, T. L. *Proc. Natl. Acad. Sci. U.S.a.* **1986**, *83*, 3743.
(23) Jones, T. A. *J. Appl. Crystallogr.* **1978**, *11*, 268.
(24) Imperiali, B.; Abeles, R. A. *Biochemistry* **1986**, *25*, 3760.



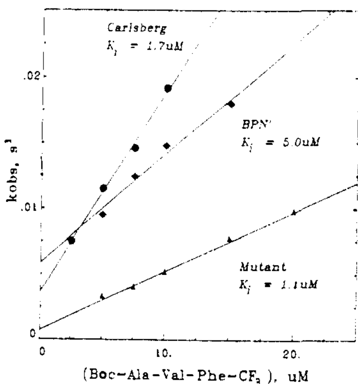


Figure 2. Subtilisin-catalyzed hydrolysis of Z-Lys-SBzl in the presence of the slow-binding inhibitor Boc-Ala-Val-Phe-CF_3. For details, see Experimental Section.

Figure 3. Computer modeling with the program FRODO indicating the possible structure of the subtilisin–inhibitor complex. Only the diastereomer with C-terminal L-Phe binds to the enzyme. The hydroxyl oxygen of catalytic Ser 221 attacks the *si* face of the trifluoromethyl ketone carbon, generating a tetrahedral oxyanion adduct that is stabilized by H-bonding interactions with Asn 155 and Ser 221 (the oxyanion hole residues) and His 64 (with the fluorine group). This structure is further confirmed by the X-ray crystal structure.[29]

enzyme–inhibitor complex was believed to form a hemiketal adduct between the active site Ser-OH and the carbonyl group of trifluoromethyl ketone. This complex resembles the transition state of hydrolysis catalyzed by the serine protease. The K_i equilibrium constants (where $K_i = k_{off}/k_{on}$, the dissociation constant for the EI complex formed) were found to correlate very nicely to k_{cat}/k_m values for substrates as the acyl portion of the inhibitor or substrate was varied.[24] This correlation suggests that the slow-binding inhibitors set up transition-state binding interactions with the enzyme similar to those with normal substrates. Figure 2 is a plot of the enzyme-catalyzed hydrolysis of Z-Lys-SBzl in the presence of our designed inhibitor. A replot of the observed first-order rate constants as a function of the inhibitor concentration generates a straight line, the slope of which generates the second-order rate constant (k_{on}) for the formation of the enzyme–inhibitor complex and the intercept of which gives the first-order rate constant (k_{off}) for the dissociation of the complex. These plots reveal a typical slow-binding inhibition behavior.[24-28] Collected in Table II are the rate constants for the slow-binding inhibition of three subtilisins (Carlsberg, BPN', and 8350) with the trifluoromethyl ketone inhibitor. Since the K_i values are so similar, the inhibitor–enzyme interactions must also be comparable. These results together with the substrate results strongly suggest that the mutant enzyme and the BPN' wild-type enzyme have very similar specificities and hydrolytic properties. The smaller k_{off} value for the mutant enzyme interacting with the transition-state analogue indicates that the mutant binds to the transition state of the substrate reaction slightly more strongly than the wild type, consistent with the substrate k_{cat}/k_m values for 8350 and the wild-type enzymes.

(25) Stein, R. L.; Strimpler, A. M. *Biochemistry* **1987**, *26*, 2611.
(26) Stein, R. L.; Strimpler, A. M. *Biochemistry* **1987**, *26*, 2682.
(27) Allen, K. N.; Abeles, R. H. *Biochemistry* **1989**, *28*, 135.
(28) Takahashi, L. H.; Radhakrishnan, R.; Rosenfield, R. E., Jr.; Meyer, E. F., Jr.; Trainor, D. A.; Stein, M. *J. Mol. Biol.* **1988**, *201*, 423.

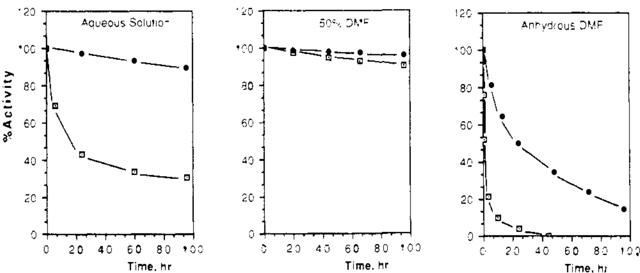


Figure 4. Stability of subtilisins BPN' (□) and 8350 (◆) in aqueous and 50% DMF solution at pH 8.4 and in dry DMF.

Figure 3 shows the structure of the enzyme–inhibitor complex. According to computer modeling, the hydroxyl group of Ser 221 will attack the *si* face of the ketone group, generating a tetrahedral oxyanion with H-bonding interactions with the "oxyanion hole". Another H-bonding interaction is between the F group and the active-site histidyl ϵ-NH group. This structure has been confirmed recently by the X-ray crystal structure of the mutant–inhibitor complex.[29]

Stability. The stability of the mutant enzyme versus the wild-type enzyme is shown in Figure 4. In aqueous solution (50 mM tris-HCl, pH 8.4, and 10 mg/mL), the half-life of the wild-type enzyme is about 20 h compared to ~2000 h for the mutant enzyme. The loss of activity is accompanied by autolysis as determined by fast protein liquid chromatography (FPLC) in a gel filtration column. Small peptide fragments appear increasingly as the incubation time increases. In 50% DMF, both the mutant enzyme and the wild-type enzyme are quite stable. The DMF–water solvent system has been shown to be very useful for peptide synthesis via aminolysis as the serine proteases are more stable, the substrates are more soluble, and the reactions become kinetically irreversible (see below). In anhydrous DMF, the stabilities for both enzymes decrease again. The mutant enzyme has a half-life of about 25 h, while that of the wild-type enzyme is about 30 min. The catalytic activities of both enzymes in DMF are too low to be synthetically useful. With the use of activated esters such as enol esters, however, both enzymes, have moderate activities useful for synthesis (see below).

Regioselective Acylations of Nucleosides. Selective protection of nucleoside functional groups is an interesting subject for study. Successful developments in this area may lead to new methods for synthesis of nucleoside analogues.

Selective chemical acylations of nucleosides have been reported in only a few cases.[30-33] Three steps are usually required to

(29) Whitlow, M.; et al. Unpublished work. The oxyanion may or may not be protonated.
(30) Brown, D. M.; Fasman, G. D.; Magrath, D. I.; Todd, A. R. *J. Chem. Soc.* **1954**, 1448. Brown, D. M.; Todd, A. R.; Varadarajan, S. *Ibid.* **1956**, 2388.
(31) Kamaike, K.; Umura, F.; Kamakage, S.-C.; Ishido, Y. *Nucleic Acids Research Symposium Series*; IRL Press: Oxford, 1985; No. 16, pp 177–180.
(32) Ligouri, A.; Perri, E.; Sindona, G.; Uccella, N. *Tetrahedron* **1988**, *44*, 229–234.

Table III. Regioselective Acetylation of Nucleosides and a Riboside in DMF Catalyzed by Subtilisin 8350

substrate R	substrate B	name	time, days	yield, %
H	thymine	thymidine	1.5	100
OH	uracil	uridine	1	80
			2	90
H	cytosine	2′-deoxycytidine	1	60
			2	80
OH		cytidine	1	60
			2	80
H	adenine	2′-deoxyadenosine	1	50
			2	80
OH		adenosine	1	40
			2	65
H	CH_3O	methyl 2-deoxy-D-ribofuranoside	1	70

prepare the 5′-O-acylribonucleosides.[34,35] Two-step procedures involving selective deacetylation of the di- or triacetates have also been reported,[36-38] but the overall yields are often low.

Enzymatic acylations of certain nucleosides have recently been accomplished with subtilisin Carlsberg[3a] and porcine pancreatic lipase.[39] Using subtilisin Carlsberg as the acylation catalyst provided low yields (21–24%) of the desired 5′-O-acyl derivatives with moderate to good (55–85%) regioselectivity.[3a] The utility of lipase catalysis was limited to those reactions where relatively nonpolar organic solvents could be used. Unfortunately, most nucleosides are insoluble under such conditions.

We found that subtilisin 8350 efficiently catalyzed the regioselective acetylation of ribonucleosides in DMF with isopropenyl acetate as acylating reagent (Table III). Small portions (<5%) of diacetylated byproducts were observed. The reactions were highly regioselective when large amounts of enzyme and extended reaction times (48 h) were used to obtain higher yields of the desired 5′-O-acetyl derivatives. The formation of diacetyl byproducts may be attributed to the stereoelectronic effects that have been observed in the differential reactivities of the hydroxyl groups, and hence the product distributions, obtained during acylation and deacylation of nucleosides.

Formation of 5′-O-acetylcytidine has not been previously reported. This may be attributed to the functionality and low reactivity inherent in this nucleoside. Using subtilisin 8350 in DMF, we were able to prepare 5′-O-acetylcytidine in good yield with high regioselectivity (Table III).

The utility of a recently reported efficient deoxygenation[40] leading to the anti-HIV 2′,3′-dideoxynucleosides[41] suffered from

Scheme I. Protocol for Synthesis of N-Protected Peptide Esters for Use in Subtilisin-Catalyzed Aminolysis To Form Large Peptides

the losses associated with preparing the 5′-O-acetyl-2′-deoxynucleosides. For example, 5′-O-acetylthymidine was obtained from thymidine in approximately 50% yield over two steps.[36] Lipase-catalyzed acetylations of 2-deoxyribose derivatives have been found to be much less regioselective than the enzymatic acetylations of the corresponding ribose derivatives. Subtilisin 8350, however, was a highly regioselective catalyst and allowed good to excellent yields of the 5′-O-acetyl-2′-deoxynucleosides to be obtained. For example, the enzymatic acetylation of thymidine using subtilisin 8350 in anhydrous DMF at 45 °C provided a quantitative yield of 5′-O-acetylthymidine after 36 h. Thus enzymatic acetylation followed by deoxygenation[41] would allow the overall yield of 3′-deoxythymidine from thymidine to be doubled while at the same time employing fewer steps. Similar enhancements of yields were observed (see Table III) for the enzymatic preparation of 5′-O-acetyl derivatives of 2′-deoxycytidine and 2′-deoxyadenosine over the previously reported[36] two-step procedures. It is interesting to note that when protease N (from Amano) was used as catalyst for all the reactions under the same conditions, the products obtained were identical with the same degree of selectivity and yield. More enzyme (about five times), however, is required to obtain the same yields at the same reaction time.

Kinetic Constants in Organic Cosolvents. To investigate the effect of DMF and dioxane on the enzymatic catalysis, the kinetic parameters (k_{cat} and K_m) for subtilisin BPN′ catalyzed hydrolysis of ester and amide substrates were determined. It was observed that the organic solvents affect both catalysis (k_{cat}) and binding (K_m) (Table IV). Both k_{cat} and k_{cat}/K_m for the amide as well an ester hydrolysis decrease as the volume of organic solvents increases. The rate of decrease for the amide hydrolysis, however, is faster than that for the ester hydrolysis. Studies are in progress to examine the pH–reaction profile and to use different substrates to probe binding and catalysis.

Synthesis of Peptides. Methionine methyl ester was used as a test substrate in aqueous solution containing 0–50% organic cosolvent. Both the wild-type and engineered subtilisins were tested. Both enzymes catalyzed the polymerization reaction and similar results were obtained in each case. In an aqueous triethylamine-buffered (pH 7.5) solution of methylmethionine hydrochloride (0.75 M) and subtilisin 8350, an insoluble polymer was formed. Analysis of the polymer by ^1H NMR and size-exclusion chromatography established that approximately 20–30 methionine residues were incorporated into the polymer. When 10% DMF was added to the reaction mixture, a polymer containing approximately 40 methionine residues was formed. Interestingly, the degree of polymerization can be controlled by the concentration of DMF; the polymer length increases with increase of the organic solvent. In 50% DMF, for example, a polymer containing more than 50 methionine residues was obtained. It was observed that, under this condition, the enzyme showed insignificant amidase activities while the esterase activities remain significant, consistent with the kinetic studies described above and with previous findings for other serine proteases.[1j] The lack of amidase activities and the improvement of product solubility under the high concentration of DMF are perhaps the major reasons for the higher degree of polymerization. Previous attempts to

(33) Mitsunobu, O.; Kimura, J.; Fujisawa, Y. *Bull. Chem. Soc. Jpn.* **1972**, *45*, 245. Shimokawa, S.; Kimura, J.; Mitsunobu, O. *Ibid.* **1976**, *49*, 3357–3358.
(34) See ref 31.
(35) Mitzuna, Y.; Endo, T.; Ikeda, K. *J. Org. Chem.* **1975**, *40*, 1385.
(36) Ihido, Y.; Nakazaki, N.; Sakairi, N. *J. Chem. Soc., Perkin Trans. 1* **1979**, 2088.
(37) Brown, D. M.; Hanes, L. J.; Todd, A. R. *J. Chem. Soc.* **1950**, 3299.
(38) Brown, D. M.; Todd, A.; Varadarajan, S. *J. Chem. Soc.* **1956**, 2388.
(39) Hennen, W. J.; Wong, C.-H. Unpublished results.
(40) Prisbe, E. J.; Martin, J. C. *Synth. Commun.* **1985**, *15*, 401–409.
(41) Hitsuya, H.; Broder, S. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 1911.

Table IV. Influence of Dioxane and DMF on the Kinetic Constants for Subtilisin BPN′ Catalyzed Hydrolysis

cosolvent	$10^4 k_{cat}$, min^{-1}	K_m, mM	k_{cat}/K_m, M^{-1} min^{-1}
Bz-Arg-OEt hydrolysis[a]			
none	330 ± 20	1.42 ± 0.09	23.5 ± 0.7
40% dioxane	220 ± 10	3.61 ± 0.15	6.1 ± 0.1
50% DMF	90 ± 20	2.86 ± 0.85	3.1 ± 0.2
Z-Arg-pNA hydrolysis[b]			
none	1.1 ± 0.1	1.2 ± 0.2	0.09 ± 0.01
40% dioxane	0.020 ± 0.002	0.67 ± 0.11	0.004 ± 0.001
Ala-Ala-Phe-pNA hydrolysis[a]			
none	2.68 ± 0.10	0.127 ± 0.010	2.1 ± 0.1
40% dioxane	0.186 ± 0.046	0.574 ± 0.180	0.033 ± 0.002
50% DMF	0.027 ± 0.01	0.079 ± 0.010	0.034 ± 0.003

[a] Each reaction was carried out in a pH 8.0 Tris buffer with 5% v/v MeOH. [b] Each reaction was carried out in a pH 8.0 sodium phosphate buffer with 0.1 M NaCl with 5% v/v CH_3CN.

Table V. Use of Subtilisin 8350 in Synthesis of Peptides

entry	acyl donor	nucleophile	product	reaction time, min	yield, %
1	Z-Tyr-OMe	D-Arg-OMe	Z-Tyr-D-Arg-OMe[a]	30	70
2	Z-Leu-OMe	Ala-NH_2	Z-Leu-Ala-NH_2[b]	25	59
3	Boc-Leu-OMe	Phe-NH_2	Boc-Leu-Phe-NH_2[c]	35	46
4	Boc-Leu-OMe	Leu-NH_2	Boc-Leu-Leu-NH_2[d]	10	48
5	Z-Val-OMe	D-Leu-OMe	Z-Val-D-Leu-OMe[e]	30	52
6	Boc-Met-Leu-OMe	Leu-NH_2	Boc-Met-Leu-Leu-NH_2[f]	15	68
7	Boc-Met-Leu-OMe	Phe-$NHCH_2Ph$	Boc-Met-Leu-Phe-$NHCH_2Ph$[g]	10	95
8	Fm-Met-Leu-OMe	Phe-$NHCH_2Ph$	Fm-Met-Leu-Phe-$NHCH_2Ph$[h]	25	70
9	Z-Tyr-Gly-Gly-Phe-OMe	Leu-NH_2	Z-Tyr-Gly-Gly-Phe-Leu-NH_2[i]	30	57
10	Boc-Tyr(OBzl)-Gly-Gly-OEt	Phe-Leu-NH_2	Boc-Tyr(OBzl)-Gly-Gly-Phe-Leu-NH_2[j]	25	83
11	Boc-Tyr(OBzl)-Gly-Gly-Hpy	Phe-Leu-NH_2	Boc-Tyr(OBzl)-Gly-Gly-Phe-Leu-NH_2[j]	45	85
12	Boc-Tyr-D-Ala-Phe-Gly-OEt	Tyr-Pro-Ser(OBzl)-NH_2	Boc-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser(OBzl)-NH_2[k]	20	80
13	Boc-Tyr-D-Ala-Phe-Gly-Hpy	Tyr-Pro-Ser(OBzl)-NH_2	Boc-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser(OBzl)-NH_2[k]	25	82
14	Boc-Tyr-D-Ala-Phe-OMe	Gly-Tyr-Pro-Ser(OBzl)-NH_2	Boc-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser(OBzl)-NH_2[k]	60	25
15	Met-OMe	Met-OMe	polymethionine[l]	180	23

[a] MP 121–124 °C, identical with that prepared previously.[1j] [b] Mp 175–177 °C; $[\alpha]^{25}_D = -26.5°$ (*c*, EtOH) [lit.[m] mp 185–190 °C; $[\alpha]^{25}_D = -26°$ (*c* 1, EtOH)]. [c] Mp = 83–85 °C; $[\alpha]^{25}_D = -23.6°$ (*c* 1, MeOH). Anal. Calcd for $C_{20}H_{31}N_3O_4$: C, 63.63; H, 8.28; N, 11.13. Found: C, 63.28; H, 8.20; N, 11.20. [d] Mp = 192–194 °C; $[\alpha]^{25}_D = -18.5°$ (*c* 1, DMF). Anal. Calcd for $C_{13}H_{33}N_3O_4$: C, 59.45; H, 9.68; N, 12.23. Found: C, 59.50; H, 9.77; N, 12.30. [e] Mp = 144–146 °C; $[\alpha]^{25}_D = +23.5°$ (*c* 2, EtOH) [lit.[n] mp 130–131 °C; $[\alpha]^{25}_D = +22°$ (*c* 1, EtOH)]. [f] Mp = 155–157 °C; $[\alpha]^{25}_D = -23.6°$ (*c* 1, DMF). Anal. Calcd for $C_{22}H_{42}N_4O_5S$: C, 55.68; H, 9.22; N, 11.81. Found: C, 55.66; H, 9.20; N, 11.80. [g] Mp 208–210 °C; $[\alpha]^{25}_D = -49.2°$ (*c* 1, MeOH); amino acid analysis, Leu(1) 1.0, Met(1) 0.9, Phe(1) 1.0. Anal. Calcd for $C_{32}H_{46}N_4O_5S$: C, 64.18; H, 7.74; N, 9.36. Found: C, 64.20; H, 7.76; N, 9.40. [h] Mp 218–220 °C; $[\alpha]^{25}_D = -36.6°$ (*c* 1, MeOH) (lit.[45] mp 228–230 °C). [i] See footnote *m*. [j] Mp 192–194 °C; $[\alpha]^{25}_D = -6.2°$ (*c* 1, DMF); amino acid analysis, Tyr(1) 0.89, Gly(2) 2.0, Phe(1) 1.0, Leu(1) 1.0. Anal. Calcd for $C_{40}H_{52}N_6O_8$: C, 64.49; H, 7.04; N, 11.28. Found: C, 64.50; H, 7.01; N, 11.30. [k] Mp 153–155 °C; $[\alpha]^{25}_D = -26.2°$ (*c* 1, DMF); amino acid analysis, Tyr(2) 1.88, Ala(1) 1.0, Gly(1) 1.01, Phe(1) 0.92, Pro(1) 0.89, Ser(1) 0.88. Anal. Calcd for $C_{52}H_{64}N_8O_{12}$: C, 64.71; H, 6.68; N, 8.71. Found: C, 64.70; H, 6.66; N, 8.80. [l] Molecular weight = 4000–5000. [m] Kubota, M.; Ogawa, H.; Yajima, H. *Chem. Pharm. Bull.* **1976**, *24*, 2435. [n] Okada, K.; Kurosawa, S.; Hinamoto, M. *Ibid.* 1974, *22*, 2136.

obtain polyamino acids via protease catalysis were not successful; only a polymer of about 8 amino acids was prepared.[42]

The aminolytic activities of the mutant enzyme in 50% DMF were used in the kinetically controlled synthesis of several di- and oligopeptides with a variety of amines (3 equiv) as nucleophiles, including D- and L-amino acid derivatives and peptide amides (Table V). The yields of the reactions ranged from 50% to 95%, and the reaction times were relatively short. The peptide substrates were prepared by solution-phase or by solid-phase synthesis with a styrene-bound oxime resin.[43] The unique feature of the solid-phase synthesis is that the N-protected peptide ester can be prepared directly from the peptide-resin. Reaction with a nucleophile such as amino acid methyl ester or 1-hydroxypiperidine gives the N-protected peptide ester, which can be used for the enzymatic coupling (Scheme I). With this chemoenzymatic method, unnatural amino acids can be incorporated into the peptide fragments at a position away from the C-terminus (the P_1 site). Several peptides prepared are biologically active. For example, Tyr-D-Arg is an enkephalin releasing factor[44] and Met-Leu-Phe-$NHCH_2C_6H_5$ is a chemotactic peptide.[45] The analgesic peptides Leu-enkephalinamide[46] (entries 10 and 11) and dermorphin[47] (entries 12–14) were prepared in relatively high yields. Given the broad substrate specificity of subtilisin, many biologically active peptide derivatives, particularly those containing unusual amino acids, perhaps can be made by this chemoenzymatic approach.

Enantioselective Synthesis. We have also extended the use of subtilisin 8350 to the enantioselective transformation of many other chiral and prochiral compounds. The results are summarized in Table VI. It is obvious from the limited number of compounds tested that the mutant enzyme is highly enantioselective in the hydrolysis of racemic N-protected or unprotected amino acid esters. Of all natural and unnatural amino acids tested, only the L-isomers were hydrolyzed. At approximately 50% conversion, both hydrolyzed products and unreacted substrates can be obtained in >40% yield and >91% ee. The enzyme, however, is not very selective in the hydrolysis of compounds other than amino acids. It is worth noting that α-methyl-DL-tryptophan methyl ester is not a substrate for the enzyme, although tryptophan methyl ester is a very good substrate. Replacement of the α-amino group of an amino acid with another functionality (e.g., OH, OCH_3, CH_3) reduces the enantioselectivity substantially (entries 9–12, 17–18, 20). The enzyme also showed poor selectivity in the trans-esterification of vinyl acetate to a number of hydroxyl compounds

(42) Jost, R.; Brambilla, E.; Monti, J. C.; Luisi, P. L. *Helv. Chim. Acta* **1980**, *63*, 375.
(43) Kaiser, E. T. *Acc. Chem. Res.* **1989**, *22*, 47 and references cited therein.
(44) Takagi, H.; Shiomi, H.; Ueda, H.; Amano, H. *Eur. J. Pharmacol.* **1979**, *55*, 109.
(45) Freer, R. J.; et al. *Biochemistry* **1982**, *21*, 257.
(46) Hughes, J.; et al. *Nature* **1975**, *258*, 577.
(47) Montecucchi, P. C.; et al. *Int. J. Pept. Protein Res.* **1981**, *17*, 275.

Table VI. Use of Subtilisin 8350 in Enantioselective Synthesis

	substrate $R_3CH(NHR_1)CO_2R_2$: R_1	R_2	R_3			R_1NH–$CH(R_3)$–CO_2R_2	+ R_1NH–$CH(R_3)$–CO_2H
1	CH_3CO	CH_3	CH_2CH_2-	40	49	95 [a]	98 [a]
2	CH_3CO	CH_3		120	50	>98 [b]	>98 [b]
3	CH_3CO	CH_3	HO	90	50	>98 [c]	>98 [c]
4	H	$C_6H_5CH_2$	CH_2-	35	52	92 [d]	85 [d]
5	H	CH_3	HO, CH_2-	25	48	91 [e]	95 [e]
6	H	CH_3	CH_2-	20	49	90 [e]	93 [e]
7	EtOCO	CH_3	O	35	49	95 [f]	95 [f]
8	H	CH_3CH_2	CH_3CH_2-	40	50	95 [g]	polymer [g]
9	OH, CO_2CH_3			300	39	OH, CO_2CH_3 (26) [e]	+ OH, CO_2H (40) [e]
10	$PhCH_2S$, CH_3, CO_2CH_3			120	44	$PhCH_2S$, CH_3, CO_2CH_3 (42) [h]	+ $PhCH_2S$, CH_3, CO_2H (53) [h]
11	OCH_3, CO_2CH_3			2 days	55	OCH_3, CO_2CH_3 (31) [e]	+ OCH_3, CO_2H (38) [e]
12	OH, CO_2CH_3			60	47	OH, CO_2CH_3 (22) [e]	+ OH, CO_2H (25) [e]
13	HO, NHCbz, OH + O, O			13 days in THF	50		HO, NHCbz, OAc (37) [i]
14	HO, OH + O, O			14 days in THF	30		AcO, OH (24) [i]
15	Cl, OH + O, O			5 days in THF	no reaction		
16	O, CO_2CH_3, HO + O, O			5 days in THF	no reaction		
17	CO_2CH_3			5 days	no reaction		
18	H_3CO, CO_2CH_3			5 days	no reaction		
19	CO_2CH_3, NHAc, N, H			5 days	no reaction		
20	Cl, OAc			5 days	no reaction		

[a] *N*-Acetyl-D-homophenylalanine methyl ester: 91% yield; mp 61–62 °C; $[\alpha]^{25}_D = -2.11°$ (*c* 1, EtOH) [lit.[53] mp 56–57 °C; $[\alpha]^{25}_D = -2.08°$ (*c* 1.85, EtOH)]. The *N*-acetyl-L-acid: 80% yield; mp 179–180 °C; $[\alpha]^{25}_D = +24.2°$ (*c* 1, EtOH) [lit.[53] mp 173–174 °C; $[\alpha]^{25}_D = +25.5°$ (*c* 1, EtOH)]. [b] *N*-Acetyl-D-phenylglycine methyl ester: 83% yield; mp 108–109 °C; $[\alpha]^{25}_D = -176.2°$ (*c* 1, EtOH) [lit.[53] mp 112–113 °C; $[\alpha]_D = -175.8°$ (*c* 0.9, EtOH)]. *N*-Acetyl-L-phenylglycine: 80% yield; mp 184–185 °C; $[\alpha]^{25}_D = +220.4°$ (*c* 1, CH_3OH) [lit.[53] mp 188–190 °C; $[\alpha]^{25}_D = +219.3$ (*c* 1, CH_3OH)]. [c] *N*-Acetyl-D-*p*-hydroxyphenylglycine methyl ester: 72% yield; mp 170–172 °C; $[\alpha]^{25}_D = -192.0°$ (*c* 1, CH_3OH) [lit.[54] $[\alpha]^{25}_{578} = -198.4°$ (*c* 1, CH_3OH)]. *N*-Acetyl-L-*p*-hydroxyphenylglycine: 74% yield; mp 248–250 °C; $[\alpha]^{25}_D = +172.0°$ (*c* 1, H_2O). ^1H NMR data are identical with the reported values.[54] This compound was further hydrolyzed to *p*-hydroxyphenylglycine: $[\alpha]^{25}_D = +156°$ (*c* 1, 1 N HCl) [lit.[55] $[\alpha] = +157°$ (*c* 1, 1 N HCl)]. [d] D-Leucine benzyl ester: mp 132–134 °C; $[\alpha]^{25}_D = -0.50°$ (*c* 5, EtOH) [lit.[56] mp 155–156 °C; $[\alpha]^{25}_D = -0.47°$ (*c* 1, EtOH)]. [e] Based on the rotation compared to that from Aldrich. [f] Compared to those reported previously.[57] [g] D-Homophenylalanine ethyl ester was hydrolyzed to D-homophenylalanine: mp 224–226 °C; $[\alpha]^{25}_D = -43°$ (*c* 2, 1 N HCl). ee was determined on the basis of the rotation compared to the authentic sample from Aldrich. The molecular weight of the polymer was 600–1200 as determined by the reaction volume from a LH-20 column. [h] After deprotection with Na/NH_3, the compound was converted to 3(*S*)-benzoyl-2-methylpropionic acid. The rotation and NMR data were determined and compared to the reported values.[58] [i] $[\alpha]^{25}_D = +1.1°$ (*c* 1, $CHCl_3$), compared to the *R*-enantiomer prepared from *N*-(benzyloxycarbonyl)-D-serinol [$[\alpha]^{25}_D = +2.9°$ (*c* 1, $CHCl_3$)]. [j] Based on the rotation and NMR data reported previously.[59]

in organic solvents. This type of reaction, however, is very effective and enantioselective when lipases are used as catalysts.[2,3b] Low selectivities were also obtained when the wild-type enzyme was used as catalyst (data not shown).

In summary, we have illustrated in this study that the techniques of site-directed mutagenesis can be used to prepare an enzyme mutant useful for organic synthesis. The stability of subtilisin in DMF as well as in aqueous solution can be significantly improved without the loss of specificities and catalytic properties. The proteolytic activity can be easily controlled by addition of water-miscible organic solvents such as DMF. Under such a condition, the serine protease is stable and the remaining esterase activity is high enough for synthetic application. The enzymatic procedures described are particularly useful for kinetic resolution of amino acids, for regioselective acylation of sugar-related compounds, and for synthesis of peptides.

Experimental Section

General. ^1H and ^{13}C NMR were obtained at 200 and 50 MHz, respectively, on a Varian XL-200 spectrometer. Mass spectra were obtained on a Hewlett-Packard 5995C quadrupole gas chromatograph–mass spectrometer operating at 70-eV HRMS. Thin-layer chromatography was done on 0.25-mm layers of silica gel (60 Å) that contained a fluorescent indicator. Column chromatography was done on Baker flash chromatography silica gel (230–400 mesh). Fast protein liquid chromatography (FPLC) was done on a Pharmacia system comprised of two P-500 pumps, a GP-250 gradient programmer, and a single-path UV-1 monitor with a Sepharose 12 GPC column. The FPLC column eluent was 50 mM sodium formate, pH 4.8, at a flow rate of 0.5 mL/min.

Engineering Subtilisin BPN′. The subtilisin gene from *Bacillus amyloliquefaciens* (which encodes subtilisin BPN′) has been previously cloned, sequenced, and expressed at high levels from its natural promoter sequences in *Bacillus subtilis*.[21,22] This has enabled us to introduce mutations in vitro into the plasmid-encoded subtilisin gene and conveniently analyze their effects on the stability properties of the altered enzyme. Mutant genes were recloned into a pUB110-based expression plasmid[5] and used to transform *B. subtilis*. The *B. subtilis* strain used as the host contains a chromosomal deletion of its subtilisin gene and therefore produces no background wild-type activity. Most mutant enzymes are efficiently expressed from this vector and are secreted into the culture medium at a concentration of about 1 g/L. Subtilisin is the major secreted protein in this system and comprises almost 80% of the total extracellular protein. Wild-type and 8350 enzymes were purified essentially as has been described.[23] Both preparations contained ~20% enzyme and 80% salt and were used directly for synthesis.

Enzyme Assay. Ten milligrams of lyophilized enzyme powder in 4 mL of anhydrous DMF or aqueous solution was stirred at room temperature to maintain homogeneity. Periodically, 100-μL aliquots were removed, diluted with 400 μL of diluted HCl (pH 3.5), and shaken to dissolve the enzymes. A 50-μL portion of the diluted enzyme solution was added to a cuvette containing 187 μM Z-Lys-SBzl, 270 μM 4,4′-dithiodipyridine (DTDP), and 900 μL of 0.2 M phosphate buffer containing 5% v/v DMF (pH 8.0). The initial reaction rates were determined from time-dependent plots of the increasing absorbance at 324 nm.

Substrate Kinetics. The enzyme kinetic constants were determined by measuring initial rates spectrophotometrically with varying substrate concentrations. These initial rates were fit to the Michaelis–Menten equation by using a nonlinear least-squares computer program. Each initial rate was measured in 0.1 M sodium phosphate buffer (pH 8.2) with 0.1 M NaCl at 25.0 °C. The initial rates for *N*-benzoyl-L-tyrosine ethyl ester (BTEE) were monitored at 260.5 nm (where $\epsilon = 542\ M^{-1}\ cm^{-1}$). The initial rates for Z-Lys-SBzl were monitored at 324 nm with DTDP coupling reagent[48,49] (where $\epsilon = 15\,000\ M^{-1}\ cm^{-1}$). The initial rates for *N-trans*-cinnamoylimidazole (NTCI) were monitored at 335 nm (where $\epsilon = 9040\ M^{-1}\ cm^{-1}$). The wild-type and mutant enzyme concentrations were determined with burst kinetics using NTCI as the active-site titrant.[50] These burst kinetics were measured in 0.1 M sodium phosphate buffer (pH 7.0) with 0.1 M NaCl.

For the organic solvent effects on subtilisin BPN′ catalysis, the enzymatic kinetic constants were determined spectrophotometrically with Bz-Arg-OEt, Ala-Ala-Phe-*p*-nitroanilide (Ala-Ala-Phe-pNA), and Z-Arg-pNA as substrates. For the control reactions, the initial rates were measured in 0.1 M Tris buffer (pH 8.0) with 5% v/v methanol. For each organic solvent experiment, the desired volume of buffer was replaced with organic solvent. The extinction coefficients (ϵ in $M^{-1}\ cm^{-1}$) for the Bz-Arg-OEt reactions were 542 for the control reactions, 655 for the 40% dioxane reactions, and 960 for the 50% DMF reactions. For the amide hydrolysis, the ϵ values were 10 700 for the control reactions, 13 300 for the 40% reactions, and 13 900 for the 50% DMF reactions. All initial rates for each substrate were fit to the Michaelis–Menten equation by using the nonlinear least-squares computer program ENZFITTER.

Inhibition Kinetics. For all the Boc-Ala-Val-Phe-CF_3 inhibition reactions, the pH was maintained at 8.2 with 0.1 M sodium phosphate buffer containing 0.1 M NaCl. The substrate for all of these inhibition studies was Z-Lys-SBzl. The Z-Lys-SBzl concentration for the Carlsberg inhibition was 235 μM (0.47 K_m), while it was 213 μM (0.40 K_m) for the BPN′ inhibition and 235 μM (0.44 K_m) for the BPN′ mutant inhibition. For each concentration of Boc-Ala-Val-Phe-CF_3 examined, the substrate, the DTDP coupling reagent (270 μM), and Boc-Ala-Val-Phe-CF_3 at the desired concentration all in the buffer solution were incubated in the reaction vessel for 10 min at 25.0 °C. After incubation, the enzyme solution was injected into this vessel to start the reaction. These progress curves were monitored at 324 nm for 300 s.

For these progress curves, the first-order conversion of the initial rate of substrate hydrolysis, v_0, to the steady-state rate of EI dissociation, v_s, was measured.[24] The first-order rate constant k_{obs} was then generated:

$$P = v_s t - (v_s - v_0)[1 - \exp(-k_{obs}t)]/k_{obs} + A_0 \qquad (1)$$

In this equation, P represents the concentration of product released as the enzyme turns over the substrate, t is the time, and A_0 is the initial absorbance. These progress curves were fit to eq 1 by using a nonlinear least-squares computer program. The resulting k_{obs} values were then fit to eq 2 for calculation of K_i ($K_i = k_{off}/k_{on}$). The k_{obs} vs [I] plot is shown in Figure 2 for each enzyme.

$$k_{obs} = k_{off} + k_{on}[I]/(1 + [S]K_m) \qquad (2)$$

Stability Study. Reaction media were (a) 0.05 M Tris-HCl/DMF (1:1 v/v), pH 8.4, (b) 0.05 M Tris-HCl, pH 8.4, and (c) anhydrous DMF. For the aqueous and 50% DMF reactions, 20 mg of the respective lyophilized enzyme was placed in a vial and, at time zero, 2 mL of the particular medium was added and the reaction stirred. For the DMF reaction, 50 mg of each enzyme was placed in a small vial and the vial was sealed with a septum and purged with dry argon. At time zero, 2 mL of anhydrous DMF (Aldrich) was added via transfer needle. At various times, aliquots were removed (0.1 mL for the aqueous and 50% reactions, 0.05 mL for the DMF reaction) for assay. The 100% activity point was taken to be 1 min after the addition of the solvent to allow for complete dissolution of the enzyme (the enzyme was not soluble in 100% DMF). For activity assays, the aliquots were dissolved in 2 mL of 0.2 M phosphate, pH 7.8, containing 1 mM *N*-(*p*-toluenesulfonyl)-L-arginine methyl ester. Hydrolysis of the ester was followed spectrophotometrically at 247 nm.

Synthesis of the Peptidyl Trifluoromethyl Ketone Boc-Ala-Val-Phe-CF_3. To 2-hydroxy-3-amino-4-phenyl-1,1,1-trifluorobutane hydrochloride[51] (506 mg, 2 mmol) in 15 mL of ethyl acetate was added diisopropylethylamine (2 mmol) with stirring. The mixture was cooled to 0 °C and Boc-Ala-Val-OH (2 mmol), hydroxybenzotriazole (HOBt, 375 mg, 3 mmol) and dicyclohexylcarbodiimide (DCC, 432 mg, 2.2 mmol) were added. The mixture was stirred at 0 °C for 1 h and then at room temperature for 5 h. The urea formed in the reaction was filtered off and the filtrate was diluted with ethyl acetate (100 mL). The solution was washed with water (15 mL), 10% citric acid (3 × 15 mL), water (2 × 15 mL), 5% sodium bicarbonate (3 × 15 mL), and water (2 × 15 mL). The organic phase was dried over anhydrous sodium sulfate and then evaporated under reduced pressure to give an amorphous white material (916 mg), which was further purified on a silica gel column (ethyl acetate:*n*-hexane = 3:1) to obtain pure Boc-Ala-Val-Phe-CH(OH)CF_3; mp 190–191 °C. This compound was then oxidized with $KMnO_4$ according to the procedure described previously and purified by silica gel column chromatography (ethyl acetate:*n*-hexane = 5:1) to give the title compound (291 mg, 72% yield); mp 154–156 °C. Anal. Calcd for $C_{23}H_{30}N_3O_5$: C, 56.90; H, 6.23; N, 8.66. Found: C, 56.80; H, 6.40; N, 8.55.

Representative Procedure for Regioselective Acylation of Nucleosides in Dry DMF. 5′-*O*-Acetylcytidine: To a solution of 243 mg (1 mmol) of cytidine in 4 mL of dry DMF were added 1.1 mL (10 equiv) of isopropenyl acetate and 260 mg of pulverized subtilisin 8350. The suspension was shaken at 45 °C in a sealed bottle. After 24 h, an additional 130 mg of enzyme was added to the reaction mixture. After the ap-

(48) Grassetti, D. R.; Murray, J. F. *Arch. Biochem. Biophys.* **1967**, *119*, 41.

(49) Green, D. J.; Shaw, E. *Anal. Biochem.* **1979**, *93*, 223.

(50) Bender, M. L.; Begue-Canton, M. L.; Blakeley, R. L.; Brubacher, L. J.; Feder, J.; Guhter, 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.

(51) Imperiali, B.; Abeles, R. H. *Tetrahedron Lett.* **1986**, *27*, 135.

propriate times, as indicated in Table III, the reaction mixture was filtered and the filtrate was evaporated to dryness. The residue was purified by silica gel chromatography using a gradient of ethyl acetate/methanol (9:1 → 3:1) as the eluent. After the product fractions were pooled and evaporated in vacuo, 5′-O-acetylcytidine was obtained as a white amorphous solid: ^{1}H NMR (DMSO-d_6) δ 2.05 (s, 3 H, OAc), 3.90 (pseudo t, 1 H, J = 5.7 Hz), 3.96 (t, 1 H, J = 5.7 Hz), 3.97 (t, 1 H, J = 5.7 Hz), 4.15 (dd, 1 H, J_1 = 12.0 Hz, J_2 = 5.7 Hz), 4.26 (dd, 1 H, J_1 = 12.0 Hz, J_2 = 3.1 Hz), 5.74 (d, 1 H, J = 7.4 Hz), 5.76 (d, 1 H, J = 3.6 Hz, H-1′), 7.18 (br d, 2 H, J = 21.5 Hz, 4-NH_2), 7.58 (d, 1 H, J = 7.4 Hz); ^{13}C NMR (DMSO-d_6) δ 20.58, 63.86, 69.83, 73.45, 80.52, 90.23, 94.35, 141.64, 155.53, 165.97, 170.58; LRMS m/z 285, 175, 151, 112, 43; HRMS m/z calcd for $C_{11}H_{15}N_3O_6$ 285.09609, found 285.09882.

5′-O-Acetylthymidine: yield 100%; ^{1}H NMR (DMSO-d_6) (partial) δ 2.59 (3 H, s, OAc), 3.85–3.90 (1 H, m, H-3′), 4.13–4.26 (3 H, m, H-4′, -5′, and -5″), 6.18 (1 H, t, J = 7.2 Hz, H-1′) [lit.[36] ^{1}H NMR 2.59 (3 H, s), 3.8–4.0 (1 H, m), 4.1–4.3 (3 H, m), 616 (1 H, t)]; ^{13}C NMR (DMSO-d_6) δ 12.08, 20.78, 38.58, 63.96, 70.31, 83.61, 83.81, 109.78, 135.95, 150.46, 163.71, 170.26; HRMS m/z $M^{•+}$ calcd for $C_{12}H_{16}N_2O_6$ 284.10084, found 284.10098; mp 151–152 °C (lit.[36] mp 151.5–152 °C).

5′-O-Acetyluridine: yield 90%; ^{1}H NMR (DMSO-d_6) δ 2.06 (3 H, s), 3.94 (1 H, q, J = 5.1 Hz), 3.99 (1 H, dt, J = 5.3, 3.4 Hz), 4.08 (1 H, q, J = 5.2 Hz), 4.16 (1 H, dd, J = 12.0, 5.6 Hz), 4.24 (1 H, dd, J = 12.0, 3.3 Hz), 5.29 (1 H, d, J = 5.5 Hz), 5.48 (1 H, d, J = 5.5 Hz), 5.68 (1 H, d, J = 8.1 Hz), 5.75 (1 H, d, J = 5.0 Hz), 7.64 (1 H, d, J = 8.1 Hz), 11.36 (1 H, s); ^{13}C NMR (DMSO-d_6) δ 20.71, 63.81, 69.84, 72.72, 81.11, 88.76, 102.10, 140.85, 150.70, 163.14, 170.127; HRMS m/z calcd for $C_7H_{11}O_5$ 175.06063, found 175.06068; mp 162–164 °C (lit.[38] mp 163–164 °C). Anal. Calcd for $C_4H_5N_2O_2$ (base + 2H) 113.02882, found 113.02884.

5′-O-Acetyl-2′-deoxycytidine: yield 80%; ^{1}H NMR (DMSO-d_6) (partial) δ 2.04 (3 H, s, OAc), 3.85–3.95 (1 H, m, H-4′), 4.10–4.22 (3 H, m, H-3′, -5′, and -6″), 6.20 (1 H, t, H-1′) [lit.[36] 2.03 (3 H, s), 3.8–4.1 (1 H, m), 4.1–4.4 (3 H, m), 6.20 (1 H, t)]; ^{13}C NMR (DMSO-d_6) δ 20.59, 39.65, 64.12, 70.51, 83.73, 85.04, 94.49, 141.09, 155.40, 165.95, 170.65; mp 182–184 °C (lit.[36] mp 185–186 °C).

5′-O-Acetyl-2′-deoxyadenosine: yield 80%; ^{1}H NMR (DMSO-d_6/D_2O) (partial) δ 1.99 (3 H, s, OAc), 4.01 (1 H, dt, J = 6.3, 4.0 Hz, H-4′), 4.14 (1 H, dd, J = 11.7, 6.3 Hz, H-5′), 4.27 (1 H, dd, J = 11.7, 4.2 Hz, H-5″), 4.49 (1 H, dt, J = 6.2, 4.1 Hz, H-3′), 6.36 (1 H, t, J = 6.6 Hz, H-1′) [lit.[36] ^{1}H NMR (partial) 2.00 (3 H, s), 3.9–4.1 (1 H, m), 4.2–4.4 (2 H, m), 4.4–4.6 (1 H, m), 6.40 (1 H, t)]; ^{13}C NMR (DMSO-d_6/D_2O) 20.78, 38.77, 64.35, 70.97, 83.77, 84.48, 119.60, 140.23, 149.69, 153.30, 156.60, 171.09; HRMS m/z $M^{•+}$ calcd for $C_{12}H_{15}N_5O_4$ 293.11240, found 293.11290.

5′-O-Acetyladenosine: yield 65%; ^{1}H NMR (DMSO-d_6) (partial) δ 2.01 (3 H, s, OAc), 4.05–4.10 (1 H, m, H-4′), 4.17 (1 H, dd, J = 11.8, 6.2 Hz, H-5′), 4.25 (1 H, t, J = 5.0 Hz, H-3′), 4.32 (1 H, dd, J = 11.8, 4.3 Hz, H-5″), 4.66 (1 H, t, J = 5.0 Hz, H-2′), 5.91 (1 H, d, J = 5.0 Hz, H-1′) [lit.[36] ^{1}H NMR (partial) 4.3–4.7 (3 H, m), 4.57 (1 H, q), 4.82 (1 H, t), 6.05 (1 H, d)]; HRMS m/q $M^{•+}$ calcd for $C_{12}H_{15}N_5O_5$ 309.10732, found 309.10737; mp 141–143 °C (lit.[52] mp 143 °C). The large geminal coupling constants (11.7–11.8 Hz) in 5′-O-acetylthymidine, 5′-O-acetyl-2′-deoxyadenosine, and 5′-O-acetyladenosine verify the H-5′ and H-5′ assignments and further confirm the site of acetylation.

Methyl 5-O-acetyl-2-deoxyribofuranoside: yield 70%; ^{1}H NMR ($CDCl_3$) (partial) δ 3.95–4.12 (3 H, m), 4.13–4.24 and 4.29–4.38 (1 H, 2 m), 4.98–5.08 (1 H, m) [lit.[3c] ^{1}H NMR 3.96–4.11 (3 H, m), 4.11–4.24 and 4.28–4.39 (1 H, 2 m), 4.98–5.07 (1 H, m)].

Polymethionine: A solution of 150 mg of methionine methyl ester hydrochloride in 1 mL of water containing 10% DMF was adjusted to pH 7.5–8.0 with triethylamine. Subtilisin 8350 (5 mg, 1.7×10^{-7} mol) was added and the reaction stirred at room temperature. Within 10 min a white precipitate began to form. After 1 h the reaction mixture was transferred to a centrifuge vial and the precipitate collected by centrifugation. The sediment was washed with five 0.5-mL portions of distilled water. The residue was then dried in vacuo. The ^{1}H NMR spectrum of the dried polymethionine in deuteriotrifluoroacetic acid revealed a 13:1 ratio of the integration values for the α-proton resonances (4.15 ppm) and the signal for the C-terminal methyl ester (3.12 ppm). This leads to a degree of polymerization of approximately 40 monomer units per polymer. The water-insoluble methionine polymer (20 mg) was suspended in 1 mL of 30% H_2O_2. A clear homogeneous solution formed within 10 min. After the solution was stirred for 1 h at room temperature, 3 mg of catalase was added, and the reaction mixture was allowed to stand overnight to destroy the excess H_2O_2. The clear solution was diluted with 10 volumes of water, and 2-μL portions were injected into a Protein Pak HPLC size-exclusion column using 0.05% aqueous sodium azide as the mobile phase at a flow rate of 1 mL/min. The product eluted as a narrow band with a retention time of 11 min. This corresponds to a standardized molecular weight of 6000–7000 and a degree of polymerization of approximately 40 subunits.

Enzymatic Resolution of N-Protected DL-Amino Acid Esters. To a stirred suspension of substrate (2 mmol) in a mixture of potassium phosphate buffer (0.2 M, pH 8.5, 10 mL) and acetone (5 mL) was added subtilisin 8350 (50 mg). The resulting mixture was stirred at 40 °C, and the progress of hydrolysis was monitored by TLC until 50% of the substrate was hydrolyzed. The reaction was then quenched by addition of 1 N HCl (10 mL), and the mixture was extracted with ethyl acetate (3 × 15 mL). The combined ethyl acetate extracts were evaporated, and the products were further purified by silica gel column chromatography (n-hexane:ethyl acetate = 3:1) to give N-protected D-amino acid ester as the first product and N-protected L-amino acid as the second product. The results are summarized in Table VI.

Enzymatic Resolution of DL-Amino Acid Esters. A solution of racemic amino acid ester (0.1 mmol) and the enzyme (50 mg) in sodium bicarbonate buffer (0.2 M, pH 8.2) was incubated at 35 °C for 1 h. The free amino acid precipitated was collected by filtration. The unreacted D-amino acid ester was extracted into ethyl acetate, and the organic solution was dried and evaporated to give the D-amino acid ester. The results are summarized in Table VI.

Enzymatic Peptide Bond Formation. A kinetically controlled approach was utilized for peptide synthesis. In a typical reaction, 1 mmol of an acyl donor, 3 mmol of a nucleophile, and 5 μmol of subtilisin 8350 in a 1:1 mixture of water and dimethylformamide (pH 8.5–9.0, adjusted with triethylamine) was incubated at 20–35 min. The product was then isolated by extraction with ethyl acetate or 1-butanol and purified by gel filtration chromatography through Sephadex LH-20 using metanol as the mobile phase. The results are summarized in Table VI.

General Procedure for Subtilisin-Catalyzed Resolution of Esters. A solution of substrate (1 to ~3 mmol) in phosphate buffer solution (30 to ~90 mL, 0.05 M, pH 7) was mixed with 50 to ~100 mg of subtilisin at room temperature with stirring. The reaction was monitored by GC. Once the required extent of conversion was reached, the pH of the reaction solution was adjusted to 2.0 with 1 N HCl. The products were extracted with ethyl acetate or dichloromethane. The combined extracts were dried over anhydrous sodium sulfate and the solvent was removed in vacuo. The products were separated by silica gel column chromatography.

The stereochemistry of products was determined on the basis of optical rotation. The optical purity of unreacted esters was determined by ^{1}H NMR spectroscopy in the presence of Eu(hfc)$_3$.

The acids were converted to the corresponding methyl esters by treatment with CH_2N_2 and then analyzed by the same procedure. All results are summarized in Table VI.

General Procedure for Subtilisin-Catalyzed Transesterification. A solution of diol (1 mmol) and vinyl acetate (4 equiv) in 4 mL of THF was mixed with 200 mg of subtilisin. After 13 days (or 14 days), the enzyme was filtered off and the solvent was removed by evaporation in vacuo. The ester product and the unreacted diol were separated by chromatography on a silica gel column (ethyl acetate:n-hexane = 1:4 → 1:1). To determine the optical purity, the monoesters were treated with (+)-2-methoxy-2-(trifluoromethyl)phenylacetyl chloride [(+)-MTPA chloride],[60] and the resulting (+)-MTPA esters (15 and 21 mg) were analyzed by ^{1}H NMR spectroscopy in the presence of Eu(hfc)$_3$ (29 and 84 mg, respectively). The results are summarized in Table VI.

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(52) Michelson, A. M.; Szabe, L.; Todd, A. R. *J. Chem. Soc.* **1956**, 1546.
(53) Pattabiraman, T. N.; Lawson, W. B. *Biochem. J.* **1972**, *126*, 645.
(54) Hermann, S.; Gunter, S. K.; Alfred, A.; Michael, P. *Biotechnol. Bioeng.* **1985**, *27*, 420.
(55) Clark, J. C.; Phillipps, G. H.; Steer, M. R. *J. Chem. Soc., Perkin Trans. I* **1976**, 475.
(56) Chen, S.-T.; Wang, K. T.; Wong, C.-H. *J. Chem. Soc., Chem. Commun.* **1986**, 1514.
(57) Drueckhammer, D. G.; Barbas, C. F., III; Nozaki, K.; Wong, C.-H.; Wood, C. Y.; Ciufolini, M. A. *J. Org. Chem.* **1988**, *53*, 1607.
(58) Hayashi, K.; Ozaki, Y.; Nunami, K. I.; Uchida, T.; Kato, J.; Kinashi, K.; Yoneda, N. *Chem. Pharm. Bull.* **1983**, *31*, 570.
(59) Wang, Y.-F.; Chen, C. S.; Girdankas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1984**, *106*, 3695.
(60) Dale, J. A.; Dull, D. L.; Mosher, H. S. *J. Org. Chem.* **1969**, *34*, 2543.

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Registry No. $CF_3CH(OH)CH(NH_2)CH_2Ph$, 124044-49-1; BOC-Ala-Val-OH, 60209-59-8; BOC-Ala-Val-NHCH(Bzl)CH(OH)CF_3, 124044-50-4; BOC-Ala-Val-Phe-CF_3, 124044-51-5; $H_2C{=}C(Me)OAc$, 108-22-5; Bz-Arg-OEt, 971-21-1; Z-Arg-pNA, 29542-03-8; Ala-Ala-Phe-pNA, 61043-41-2; Z-Tyr-OMe, 13512-31-7; Z-Leu-OMe, 51021-87-5; BOC-Leu-OMe, 63096-02-6; Z-Val-OMe, 24210-19-3; BOC-Met-Leu-OMe, 66880-59-9; Fm-Met-Leu-OMe, 124044-52-6; Z-Tyr-Gly-Gly-Phe-OMe, 98254-08-1; BOC-Tyr(Bzl)-Gly-Gly-OEt, 87423-40-3; BOC-Tyr(Bzl)-Gly-Gly-Hpy, 124044-53-7; BOC-Tyr-D-Ala-Phe-Gly-OEt, 78330-92-4; BOC-Tyr-D-Ala-Phe-Gly-Hpy, 124044-54-8; BOC-Tyr-D-Ala-Phe-OMe, 124044-55-9; Met-OMe·HCl, 2491-18-1; D-Arg-OMe, 65160-70-5; Ala-NH_2, 7324-05-2; Phe-NH_2, 5241-58-7; Leu-NH_2, 687-51-4; D-Leu-OMe, 23032-21-5; Phe-$NHCH_2Ph$, 6455-20-5; Phe-Leu-NH_2, 38678-59-0; Tyr-Pro-Ser(Bzl)-NH_2, 124044-56-0; Gly-Tyr-Pro-Ser(Bzl)-NH_2, 124044-57-1; Z-Tyr-D-Arg-OMe, 102683-28-3; Z-Leu-Ala-NH_2, 62074-75-3; BOC-Leu-Phe-NH_2, 33900-15-1; BOC-Leu-Leu-NH_2, 124044-58-2; Z-Val-D-Leu-OMe, 53941-41-6; BOC-Met-Leu-Leu-NH_2, 124044-59-3; BOC-Met-Leu-Phe-$NHCH_2Ph$, 124044-60-6; Fm-Met-Leu-Phe-$NHCH_2Ph$, 124044-61-7; Z-Tyr-Gly-Gly-Phe-Leu-NH_2, 71591-21-4; BOC-Tyr(BzD)-Gly-Gly-Phe-Leu-NH_2, 81638-88-2; BOC-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser(Bzl)-NH_2, 124044-62-8; (±)-$AcNHCH(CH_2CH_2Ph)COOMe$, 36061-01-5; (±)-AcNHCH-(Ph)COOMe, 36061-00-4; (±)-$AcNHCH(C_6H_4$-p-$OH)COOMe$, 72651-18-4; DL-Leu-OBzl, 80089-22-1; DL-Tyr-OMe, 18869-47-1; DL-Phe-OMe, 15028-44-1; (±)-$H_2NCH(CH_2CH_2Ph)COOEt$, 46460-24-6; (±)-PhCH(OH)COOMe, 4358-87-6; (±)-$PhCH_2SCH_2CH(Me)$-COOMe, 124044-63-9; (±)-PhCH(OMe)COOMe, 56143-21-6; (±)-$CH_3CH(OH)COOMe$, 2155-30-8; (±)-$CH_3CH(C_6H_4$-p-Bu-i)COOMe, 114376-60-2; (±)-$Cl(CH_2)_3CH(OAc)CH_3$, 124044-65-1; ZNHCH-$(CH_2OH)_2$, 71811-26-2; (±)-$Cl(CH_2)_3CH(OH)CH_3$, 69459-96-7; AcO-$CH{=}CH_2$, 108-05-4; (R)-$AcNHCH(CH_2CH_2Ph)COOMe$, 36060-87-4; (R)-AcNHCH(Ph)COOMe, 36060-85-2; (R)-$AcNHCH(C_6H_4$-p-OH)-COOMe, 72691-40-8; D-Leu-OBzl, 46741-65-5; D-Tyr-OMe, 3410-66-0; D-Phe-OMe, 21685-51-8; (R)-$H_2NCH(CH_2CH_2Ph)COOEt$, 124044-66-2; (S)-PhCH(OH)COOMe, 21210-43-5; (R)-$PhCH_2SCH_2CH$-(Me)COOMe, 56751-44-1; (S)-PhCH(OMe)COOMe, 26164-27-2; (R)-$CH_3CH(OH)COOMe$, 17392-83-5; (S)-$AcNHCH(CH_2CH_2Ph)$-COOH, 96613-91-1; (S)-AcNHCH(Ph)COOH, 42429-20-9; (S)-$AcNHCH(C_6H_4$-p-OH)COOH, 37784-24-0; Leu-OH, 61-90-5; Tyr-OH, 60-18-4; Phe-OH, 63-91-2; (R)-PhCH(OH)COOH, 611-71-2; (S)-$PhCH_2SCH_2CH(Me)COOH$, 73672-15-8; (R)-PhCH(OMe)-COOH, 3966-32-3; (S)-$CH_3CH(OH)COOH$, 79-33-4; (R)-ZNHCH-$(CH_2OH)CH_2OAc$, 124044-68-4; (S)-$H_2NCH(C_6H_4$-p-OH)COOH, 32462-30-9; (R)-$H_2NCH(CH_2CH_2Ph)COOH$, 82795-51-5; (R)-$PhCOSCH_2CH(Me)COOH$, 74407-70-8; (S)-$PhCOSCH_2CH(Me)$-COOH, 72679-02-8; subtilisin, 9014-01-1; thymidine, 50-89-5; uridine, 58-96-8; 2'-deoxycytidine, 951-77-9; cytidine, 65-46-3; 2'-deoxyadenosine, 958-09-8; adenosine, 58-61-7; methyl 2-deoxy-β-D-ribofuranoside, 51255-18-6; 5'-O-acetylthymidine, 35898-31-8; 5'-O-acetyluridine, 6773-44-0; 5'-O-acetyl-2'-deoxycytidine, 72560-70-4; 5'-O-acetylcytidine, 18531-23-2; 5'-O-acetyl-2'-deoxyadenosine, 72560-67-9; 5'-O-acetyladenosine, 2140-25-2; methyl 5'-O-acetyl-2-deoxy-β-D-ribofuranoside, 116466-99-0; polymethionine (homopolymer), 26062-47-5; polymethionine (SRU), 26854-80-8; (±)-N-(ethoxycarbonyl)-2-(2-furyl)glycine methyl ester, 113531-30-9; (±)-methyl 2-(6-methoxynaphth-2-yl)propanoate, 42589-75-3; DL-N-acetyl-α-methyltryptophan methyl ester, 124044-64-0; *cis*-cyclopenten-3,5-diol, 29783-26-4; (±)-methyl 7-(3-hydroxy-5-oxocyclopent-1-en-1-yl)heptanoate, 40098-26-8; (R)-N-(ethoxycarbonyl)-2-(2-furyl)glycine methyl ester, 113452-70-3; (S)-N-(ethoxycarbonyl)-2-(2-furyl)glycine, 124044-67-3; poly(L-homophenylalanine), homopolymer, 124044-69-5; poly(L-homophenylalanine), SRU, 124044-70-8; (3R)-*cis*-5-acetoxycyclopenten-3-ol, 60176-77-4.

Microwave Spectra of Isotopic Species and Substitution Structure of Cyclohexane

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Abstract: The rotational spectra of cyclohexane-*1,1*-d_2, cyclohexane-^{13}C-*1,1*-d_2, cyclohexane-d_1 (equatorial and axial), and cyclohexane-*1,1,2,2,3,3*-d_6 have been measured between 8 and 26 GHz with a pulsed microwave Fourier transform spectrometer. From their analysis the rotational constants and the quartic centrifugal distortion constants have been determined. The complete substitution structure has been deduced from these rotational constants. Measurements of the Stark effect have provided information on the dipole moment induced by deuterium substitution.

Carbocyclic compounds have challenged chemists for a long time to derive their structures and conformations. Early electron diffraction studies established that the chair form is the most stable conformer for saturated six-memered rings in general and for cyclohexane in particular.[1,2] Several later investigations by gas electron diffraction have improved the structure of cyclohexane.[3-7] The best values for the structural parameters are $\angle CCC = 111.4\ (2)^\circ$, r_g(C–C) = 1.536 (2) Å, and r_g(C–H) = 1.121 (4) Å with the assumption of equal bond lengths for axial and equatorial hydrogens.[6] Complementary information on the structure of cyclohexane was obtained from ab initio calculations.[8,9]

Due to its D_{3d} symmetry, the chair conformation of cyclohexane does not possess a permanent electric dipole moment, and so the powerful method of microwave spectroscopy could not be used to determine the substitution structure. However, some structural parameters followed from rotational constants which were obtained from the analysis of the pure rotational Raman spectrum of C_6H_{12} and C_6D_{12}.[10]

(1) Pauling, L.; Brockway, L. O. *J. Am. Chem. Soc.* **1937**, *59*, 1223–1236.
(2) Hassel, O.; Ottar, B. *Arch. Math. Naturvidensk.* **1942**, *B45*, 1–7.
(3) Davis, M.; Hassel, O. *Acta Chem. Scand.* **1963**, *17*, 1181.
(4) Alekseev, N. V.; Kitaigorodskii, A. I. *Zh. Strukt. Khim.* **1963**, *4*, 163–166.
(5) Geise, H. J.; Buys, H. R.; Mijlhoff, F. C. *J. Mol. Struct.* **1971**, *9*, 447–454.
(6) Bastiansen, O.; Fernholt, L.; Seip, H. M.; Kambara, H.; Kuchitsu, K. *J. Mol. Struct.* **1973**, *18*, 163–168.
(7) Ewbank, J. D.; Kirsch, G.; Schäfer, L. *J. Mol. Struct.* **1976**, *31*, 39–45.
(8) Chiu, N. S.; Ewbank, J. D.; Schäfer, L. *J. Mol. Struct.* (*Theochem*) **1982**, *86*, 397–399.
(9) Wiberg, K. B. *J. Am. Chem. Soc.* **1983**, *105*, 1227–1233.

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