Engineering Subtilisin for Peptide Coupling: Studies on the Effects of Counterions and Site-Specific Modifications on the Stability and Specificity of the Enzyme

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Abstract: Several variants derived from the thermostable subtilisin 8397 were made in order to create an enzyme that is more stable toward organic solvents or has a broader specificity for the P_1' residue in amidation or is more effective for peptide segment ligation in aqueous solution. To improve the stability in organic solvents, one of three surface charges was removed each time from 8397 to create the variants: Lys43 → Asn (K43N), Lys256 → Tyr (K256Y), and Asp181 → Asn (D181N). Although the stabilities of these variants in high concentrations of hydrophilic organic solvents were higher than that of the wild-type enzyme, the D181N variant was less stable than the 8397 variant. It appears that removal of isolated surface charges does not necessarily improve the enzyme stability in polar organic solvents. A dramatic change of the enzyme stability in dimethylformamide (DMF) was, however, observed in the presence of different counterions. Subtilisin BPN′ lyophilized from Tris-HCl buffer (50 mM, pH 8.4) and suspended in DMF (solid partially soluble), for example, was completely inactivated in 30 min at 25 °C, while the enzyme still retained about 70% of the original activity in a week if lyophilized from sodium phosphate buffer (50 mM, pH 8.4) (solid completely insoluble in DMF). In general, the enzyme lyophilized from organic buffers deactivates in DMF much faster than that from inorganic buffers. A similar counterion effect was observed with other variants. These studies suggest that subtilisins are very unstable when exposed directly to DMF; the stability is, however, markedly improved if the enzyme is protected by water or salts from contact with the solvent. To use subtilisins and variants in transesterification or aminolysis in organic solvents, water (3–30%) is usually present in order to have significant reactivity, and for transesterifications, it was found that a good rate and yield could be achieved in ethanol containing 30% water. For use in peptide segment ligation in aqueous solution, the active-site serine of subtilisin 8397/C206Q was converted chemically to cysteine, forming thiosubtilisin 8397/C206Q, and the aminolysis:hydrolysis ratio was found to be several orders of magnitude higher than that for subtilisin BPN′ and comparable to that for thiosubtilisin BPN′. The 8397 variant was also modified at the S_1' site via M222A/Y217W mutations to broaden the P_1' specificity.

Introduction

A great deal of interest has been focused in recent years on the use of proteases in peptide synthesis.[1] While solid-phase[2] and solution-phase[3] peptide syntheses have reached a high degree of sophistication, they still require extensive protection and deprotection steps and suffer from racemization and low solubility of many derivatized amino acids and peptides in the solvents used; the methods are therefore not generally applicable to the synthesis of large peptides or proteins or their glycoconjugates. Although enzymatic peptide coupling has been considered to be potentially useful and to have many advantages, including greater stereo- and regioselectivity, reduced need for protecting groups, and mild reaction conditions, it is still not commonly practiced in the laboratory.

Part of the reason is that proteases also catalyze the hydrolysis of both the substrates and the peptide products, and the synthesis of peptide esters for the kinetically controlled peptide ligation in aqueous solution is not trivial. Moreover, many proteases are unstable toward polar organic cosolvents, which often must be used for small protected substrates both to decrease the rate of hydrolysis and increase the solubility of the substrates. Our goal is to create stable enzymes that overcome these problems and can be used in aqueous solution with or without organic cosolvents for kinetically controlled (i.e. aminolysis of esters) peptide synthesis.

Starting with the thermostable subtilisin 8397,[4,5] we have attempted to create variants that are stable toward organic solvents by removing surface charges, an approach that has shown some success in the past with α-lytic protease.[6] The residues chosen for site-directed mutagenesis were those which had no interaction with other nearby charged residues (or at least were not in locally neutral regions), as determined by analysis of the crystal structure, and were not conserved in other subtilisin variants. The residues thus chosen were changed to the amino acids found most commonly in the equivalent positions in the other subtilisin variants surveyed. These variants were studied for their stability and kinetics in buffer/organic cosolvent mixtures. In addition, the

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(2) Merrifield, R. B. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 799. Kent, S. In *Innovation and Perspectives in Solid Phase Synthesis*; Epton, R., Ed.; SPPC Ltd.: Birmingham, U.K., 1992.

(3) Bodanszky, M. *Principles of peptide synthesis*; Spring-Verlag: Berlin, 1984.

(4) For early work on the preparation of thermostable subtilisin 8350, see: Pantoliano, M.; Whitlow, M.; Wood, J. F.; Dodd, S. W.; Hardman, K. D.; Rollence, M. L.; Bryan, P. N. *Biochemistry* **1989**, *28*, 7205. This enzyme is modified from subtilisin BPN′ through the following changes: Met50Phe, Asn76Asp, Gly169Ala, Asn218Ser, Gln206Cys, and Tyr217Lys. The enzyme is stable in high concentrations of DMF and useful for synthesis: 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.

(5) For the preparation and application of subtilisin 8397, see: 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. The sites of modifications are indicated in Figure 9.

(6) Martinez, P.; Arnold. F. *J. Am. Chem. Soc.* **1991**, *113*, 6336. Chen, K.; Arnold, F. H. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 5618.

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Table 1. Kinetic Parameters of Subtilisin Mutants in 100 mM Tris, pH 8.0, T = 25 °C

	wt	8397	K43N	K256Y	D181N
		sAAPFpNA[a]			
k_{cat} (s^{-1})	68	100	60	90	110
K_m (mM)	0.42	0.18	0.19	0.11	0.2
k_{cat}/K_m (mM^{-1} s^{-1})	160	560	315	470	550
		sAAPFSBz[b]			
k_{cat} (s^{-1})	2.8×10^3	1.3×10^3	1.2×10^3	1.3×10^3	1.2×10^3
K_m (mM)	0.62	0.34	0.27	0.24	0.27
k_{cat}/K_m (mM^{-1} s^{-1})	4.5×10^3	3.8×10^3	4.4×10^3	5.4×10^3	4.4×10^3

[a] Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide. [b] Succinyl-Ala-Ala-Pro-Phe thiobenzyl ester.

effect of counterions on the enzyme stability in polar organic solvents such as dimethylformamide (DMF) was also investigated.

In order to increase the ratio of peptide bond formation to substrate hydrolysis (the aminolysis to hydrolysis ratio) in aqueous solution, thiosubtilisin[7-9] has been used. The enzyme, however, only accepts activated peptide esters as substrates. When peptide methyl esters are used as substrates, the reaction must be conducted at higher temperature. The thioenzyme is, however, very unstable at >50 °C ($t_{1/2}$ < 1 h). We therefore converted the active-site serine of stable subtilisin variants to cysteine[10] to be used at high temperatures in peptide coupling with peptide methyl esters. We have also prepared two variants from 8397 that exhibit a broader P_1' specificity in peptide coupling.

Results

Stable Subtilisin Variants in Polar Organic Solvents. Following chromatography on DEAE cellulose, the purity of the mutant subtilisin preparations was greater than 90%, as assayed by SDS-PAGE. Active-site titrations with cinnamoylimidazole demonstrated that approximately 60–70% of the protein was active.

The kinetic constants of the subtilisin variants after charge removal were measured with the hydrolysis of the amide and ester substrates sAAPFpNA and sAAPFSBz. The results are essentially the same as for subtilisin 8397[5] (Table 1), indicating no gross conformational changes, except that small changes for k_{cat} and K_m are observed.

We first attempted to measure the stabilities of the subtilisin mutants in anhydrous organic solvents, since the 8397 variant has been reported to be more stable than the wild-type enzyme under high concentrations of DMF.[5] Aliquots of enzyme were taken from the suspension over a period of time and assayed in aqueous solution. The initial velocity of the enzyme activity was used as a measure of the stability. We found, however, that the stability of the enzymes in anhydrous solvents is extremely dependent on the amount and type of salts associated with the enzyme. The enzyme prepared via lyophilization from a sodium phosphate buffer and suspended in anhydrous DMF is markedly more stable than that prepared from a Tris buffer under the same conditions (i.e. same pH and buffer concentration). The wild-type enzyme with Tris was completely inactivated in 30 min while the enzyme associated with phosphate retained 70% of the activity after 1 week when unstirred (Figure 1). Furthermore, the stability is even dependent on the stirring speed (Figure 2). A similar situation was observed in both 99% DMF and 90% DMF (balance water; see Table 2A); it was found that in most cases the enzyme lyophilized from an inorganic buffer was more stable than that lyophilized from an organic buffer. The concentration of the buffer used in the preparation of the enzyme also affects the stability; subtilisin BPN' prepared from 10 mM sodium phosphate is about 10 times as stable as that prepared from 0.1 mM buffer when suspended in 99% DMF (Table 2B). In fact, when the

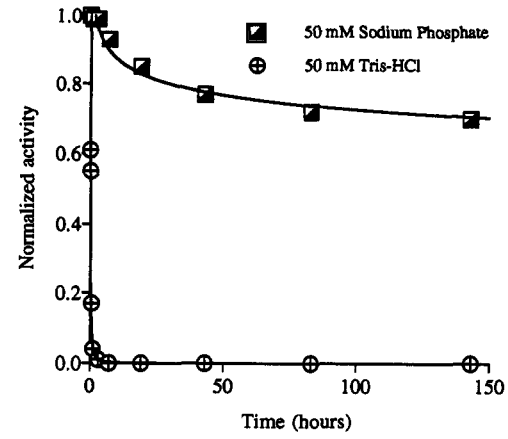


Figure 1. Stability of subtilisin BPN' lyophilized from different buffers at pH 8.4 and suspended in anhydrous DMF (T = 25 °C, without stirring).

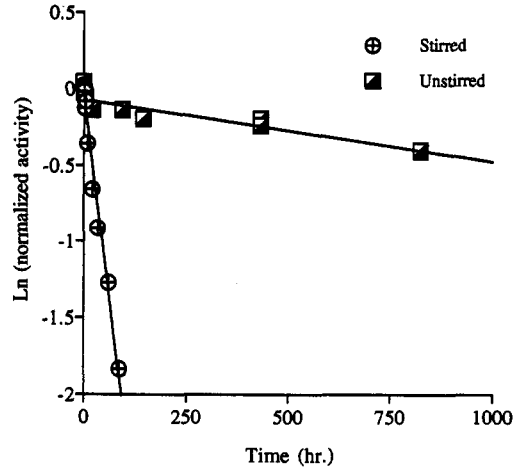


Figure 2. Effect of stirring on the stability of subtilisin 8397 in 100% DMF (enzyme lyophilized from 10 mM sodium phosphate/0.025 mM $CaCl_2$, pH 7.0; assay conducted at T = 25 °C).

Table 2. Stability of Subtilisin BPN' in 90% and 99% DMF (Balance Water) as a Function of the Counterion Type and Concentration

	99% DMF t(50%)[b]	90% DMF t(50%)[b] (min)
(A) Subtilisin Lyophilized from 10 mM of Various Buffers + 0.025 mM $CaCl_2$[a]		
HEPES	<1 min	7.5
Tris	<1 min	7.5
MOPS	<1 min	no data
MES	<1 min	no data
sodium phosphate	5 h	30
potassium phosphate	11 h	36
sodium bicarbonate	<1 min	20
sodium borate	100 h	200
(B) Subtilisin Lyophilized from Specified Concentrations of Sodium Phosphate + 0.025 mM $CaCl_2$, pH 8.0[a]		
10 mM sodium phosphate	5 h	30
1 mM sodium phosphate	5 h	30
0.1 mM sodium phosphate	0.5 h	16

[a] After lyophilization each subtilisin sample was suspended in DMF/water solution at 25 °C with stirring. [b] The decay curves were not, in general, exponential; therefore the number given here is not the half-life but the time required to undergo the *first* 50% decay.

(7) For use of thiosubtilisin in peptide synthesis, see: Nakatsuka, T.; Sasaki, T.; Kaiser, E. T. *J. Am. Chem. Soc.* **1987**, *109*, 3808.

(8) The ratio of aminolysis to hydrolyis is increased by a factor of ~7000: (a) Wu, Z.-P.; Hilvert, D. *J. Am. Chem. Soc.* **1989**, *111*, 4513. (b) Zhong, Z.; Wong, C.-H. *Biomed. Biochim. Acta* **1991**, *10/11*, S9.

(9) Further improvement of aminolysis was observed with a double mutant (Ser221 → Cys, Pro225 → Ala): Abrahmsén, L.; Tom, J.; Burnier, J.; Butcher, K. A.; Kossiakoff, A.; Wells, J. A. *Biochemistry* **1991**, *30*, 4151.

(10) For application of thermostable thiosubtilisin variants to glycopeptide synthesis, see: Wong, C.-H.; Schuster, M.; Wang, P.; Sears, P. *J. Am. Chem. Soc.* **1993**, *115*, 5893.

Table 3. Half-Lives (min) of Subtilisin Mutants in Buffer with Various Concentrations of DMF at $T = 50$ °C

	% DMF		
	0	30	50
1 μM MES, 0.1 μM $CaCl_2$, pH 6.0			
wild type	4.6	4	3
8397	53	82	20
K43N	57	85	23
K256Y	160	90	31
D181N	31	69	13
1 μM HEPES, 0.1 μM $CaCl_2$, 2 mM pBBBA,[a] pH 7.5			
wild type	14	8	3
8397	150	96	24
K43N	110	59	22
K256Y	230	150	28
D181N	120	43	22
100 mM Tris, 50 μM $CaCl_2$, pH 8.0			
wild type	120	40	16
8397	410	1000	208
K43N	1100	852	180
K256Y	2700	1400	220
D181N	140	300	130

[a] *p*-Bromobenzeneboronic acid, a subtilisin inhibitor.

enzymes are dialyzed extensively against distilled water before lyophilization, the activity drops too rapidly to measure when the enzyme is added to solvent. When subtilisin BPN′ (0.5 mg, from Sigma) was dissolved in 1 mL of 50 mM Tris-HCl, pH 8.4, lyophilized, and added to 1 mL of anhydrous DMF, a relatively clear solution was obtained with a very small amount of precipitate. The enzyme prepared from sodium phosphate (50 mM, pH 8.4) is, however, completely insoluble. Further studies indicated that minimal enzyme dissolved in either case; in the case of Tris–enzyme, the salt was dissolving away from the enzyme. These studies suggest that subtilisins are very unstable when exposed directly to DMF. The enzyme stability can be improved if the surface (probably the area containing the polar and charged groups) is protected by salts (the counterions) from contact with the organic solvent. It is, however, difficult to draw informative conclusions from this heterogeneous system regarding the stability of subtilisins in DMF. For this reason, we abandoned studies in anhydrous solvents and focused on stability studies in buffer/cosolvent mixtures in which the enzyme is soluble.

The stabilities were next measured in a homogeneous system, in which the enzymes were dissolved in different buffers with varying amounts of DMF. It was found (Table 3) that the K256Y variant was usually more stable than, the D181N mutant less stable than, and the K43N variant usually similar to the 8397 variant, although all were more stable than the wild type under these conditions. (These trends are not always followed at 0% cosolvent, where peptidase activity is high and thus autohydrolysis is a major factor, even at pH 6.0.[8b]) It was noted, however, that the ratio of the half-lives of the charge variants vs 8397 usually increased as the concentration of DMF was increased from 30% to 50%. It appeared that perhaps DMF, with a dielectric constant ($\epsilon_{25\,°C}$) of 37, was not hydrophobic enough to make the enzyme more stable after surface charge removal, and so cosolvents of lower dielectric constants were used in order to see if a further reduction in the polarity of the medium could stabilize the mutants in a more predictable fashion. The results, listed in Table 4, show that in most cases K256Y is more stable than, D181N less stable than, and K43N about the same as (or slightly more stable than) the 8397 variant and that the relative stabilities do not seem to increase as the polarity of the cosolvent drops.

We then examined the stability of soluble subtilisin BPN′ and the 8397 variant in DMF containing 40% and 20% water and different salts. The results are shown in Figure 3. In all cases, the 8397 variant is more stable than the wild-type enzyme. Interestingly, the enzymes prepared from Tris buffer are, however, more stable than those prepared from phosphate buffer in 80% DMF, a situation opposite to that observed in pure DMF. We also observed that the type of counterion has no effect on the enzyme stability in aqueous solution (assuming pH is held constant) and that the enzyme inactivation in high concentrations of DMF is not due to autolysis.

Table 4. Half-Lives of Charge Mutants in Various Solvents at 50 °C

conditions	wild type	8397	K34N	K256Y	D181N
buffer[a]	2.03 h	6.86 h	19.1 h	46 h	2.3 h
50% ethylene glycol[b]	~16	~130	~120	~200	~100
50% DMSO[b]	8.25	74	70	58	10.7
50% DMF[b]	0.25	3.48	3	3.62	2.1
50% methanol[b]	0.042	0.19	0.23	0.27	0.12
50% acetonitrile[b]	0.032	0.060	0.068	0.086	0.058
50% ethanol[b]	0.05	0.40	0.42	0.57	0.28
50% dioxane[b]	0.12	1.23	1.2	1.52	0.72

[a] 100 mM Tris, 50 μM $CaCl_2$, pH 8.0. [b] Balance made up with buffer (final buffer concentration was 100 mM Tris/50 μM $CaCl_2$).

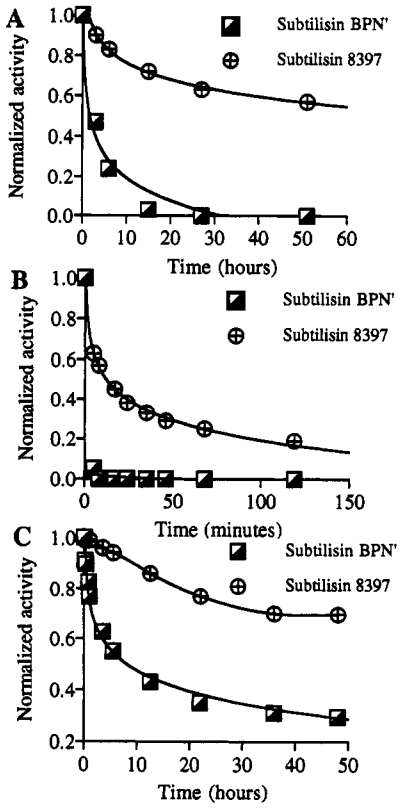


Figure 3. Stability of soluble subtilisin BPN′ (wild type) and subtilisin 8397: (A) prepared from 50 mM sodium phosphate, pH 8.4, and dissolved in 60% DMF, 25 °C; (B) prepared from 50 mM sodium phosphate, pH 8.4, and dissolved in 80% DMF, 25 °C; (C) prepared from 50 mM Tris-HCl, pH 8.4, and dissolved in 80% DMF, 25 °C.

Stable Thiosubtilisin for Peptide Ligation in Aqueous Solution. We next turned our attention to preparing subtilisin variants to be used in aqueous solution for aminolysis. Several methods have been tried to increase the ratio of aminolysis to hydrolysis, including use of organic cosolvents,[5,7] thiolation of the enzyme,[7-10] and methylation of the active-site histidine.[11,12] To compare the effectiveness of these techniques, we constructed[10,11] free energy diagrams from the kinetic data obtained for the reactions of subtilisin variants with succAAPFpNA and succAAPFSBz (Table 1; also refs 8b, 11, and 12). By comparison of the free energies

(11) Zhong, Z.; Bibbs, J. A.; Yuan, W.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113* (6), 2259.
(12) West, J. B.; Hennen, W. J.; Lalonde, J. L.; Bibbs, J. A.; Zhong, Z.; Meyer, E. F.; Wong, C.-H. *J. Am. Chem. Soc.* **1990**, *112*, 5314.

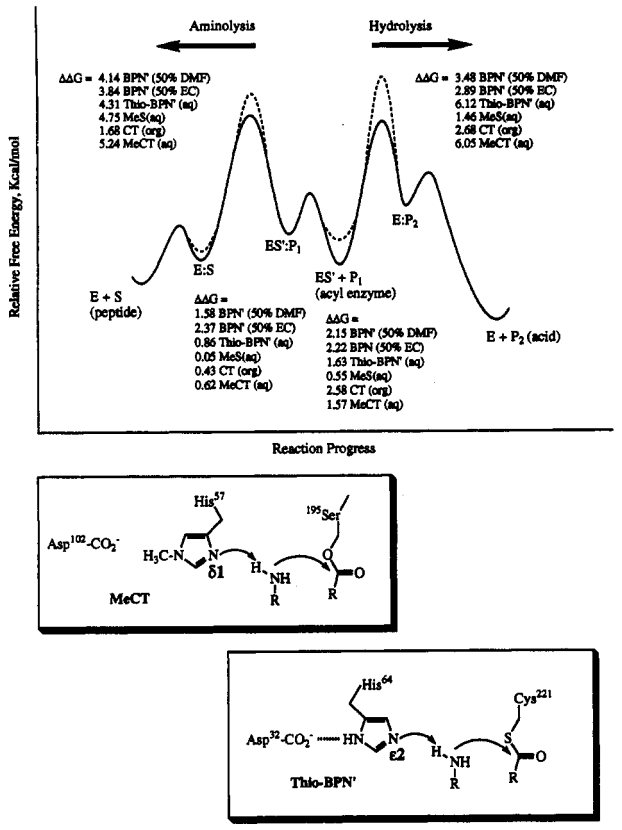


Figure 4. Free energy diagrams for various modified enzymes. Abbreviations: BPN′, subtilisin BPN′; thio-BPN′, the active-site Ser (residue 221) of subtilisin is replaced with Cys; MeS, the active-site His (residue 64) ϵ2-N of BPN′ is methylated; CT, a-chymotrypsin; MeCT, the active-site His (residue 57) ϵ2-N of chymotrypsin is methylated. The bottom section shows aminolysis of the acyl enzymes of MeCT and thio-BPN′.

of activation for aminolysis and hydrolysis of the modified enzyme vs the wild-type enzyme, we gained information regarding whether a given modification was a reasonable technique for improving the aminolysis:hydrolysis ratio. The results are shown in Figure 4. (Data for methylchymotrypsin and chymotrypsin in cosolvent are added for comparison.) These data show that upon thiolation of the enzyme the free energy of activation ($\Delta G^{\ddagger}$) for hydrolysis increases by 4.5 kcal/mol, while that for aminolysis changes by 2.7 kcal/mol. Thus, the $\Delta G^{\ddagger}$ for hydrolysis is increased more than that for aminolysis, and so thiolation is an excellent means of increasing the aminolysis:hydrolysis ratio. In contrast, methylation of the enzyme or addition of an organic cosolvent causes increases in $\Delta G^{\ddagger}$ for hydrolysis of 1.5 and 3.47 kcal/mol, respectively, while those for aminolysis increase by 6.7 and 4.1 kcal/mol, and thus these techniques do not seem to be appropriate ways to increase the ratio of aminolysis to hydrolysis for subtilisin, though they are very effective in the chymotrypsin case.[11]

The implications of the free energy diagram with regard to the enzymatic reaction in 50% seemed paradoxical when compared to the results of coupling experiments in aqueous solution and aqueous/organic cosolvents, in which the ratio of aminolysis to hydrolysis is definitely increased by an amount that cannot be accounted for solely by the decrease in water activity or the increase in nucleophile concentration due to the downshift of the amine pK_a by the addition of organic cosolvents (which favors the uncharged form of the nucleophile). This implies that the ratio of the rate constants of aminolysis and hydrolysis should be increased by the addition of organic cosolvents. The relatively stable cinnamoyl enzyme was therefore generated, and the aminolysis:hydrolysis ratios for deacylation with glycinamide were determined in buffer with or without 50% DMF. It was found

Table 5. Data for Thiosubtilisins

(A) Kinetic Parameters for the Hydrolysis of sAAPFSBz

	thiosubt BPN′	thiosubt 8397/C206Q
k_{cat} (s^{-1})	2.6	5.1
K_m (mM)	0.13	0.05
k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	20	100

(B) Aminolysis:Hydrolysis Ratios of the Cinnamoyl Enzyme

nucleophile	wild type	thiosubt BPN′	thiosubt 8397/C206Q
glycinamide	<50	1.3×10^4	1.2×10^4
leucinamide	<50	1.8×10^3	1.4×10^3

that in buffer alone there was no detectable aminolysis, but in 50% DMF, the aminolysis:hydrolysis ratio was 29. Thus, the ratio of rate constants is increased by the addition of 50% DMF. We surmised that since calculation of the free energy diagram relies on the assumption that the deacylation is rate limiting in the case of ester hydrolysis, perhaps the reason that the higher aminolysis:hydrolysis ratio is not reflected in the free energy diagram is that there may be a change in rate-limiting steps for ester hydrolysis when the reaction is conducted in 50% DMF. We determined the kinetic parameters, both in buffer and in 50% DMF, for hydrolysis of *N-trans*-cinnamoylimidazole, a substrate for which, like ester substrates, deacylation is rate limiting. We found that in water the rate constant for deacylation was equal to k_{cat}, a value of 0.1 s^{-1}. In 50% DMF, however, the deacylation rate only decreased to approximately 0.03 s^{-1}, but k_{cat} was unmeasurable over background hydrolysis, indicating a change in rate-limiting steps.

In order to create an enzyme with a higher aminolysis:hydrolysis ratio in aqueous solution, we made the thiol variant of subtilisin 8397. Repeated attempts at creating thiosubtilisin 8397 by site-directed mutagenesis provided mutants that produced enzyme very poorly (final yield after affinity chromatography was <1.0 mg/L). Removal of cysteine 206 did not increase the level of production. The thiosubtilisin 8397/C206Q was therefore made chemically (see Materials and Methods: Cys206 was removed to facilitate separation of the thiol enzyme from the serine protease by affinity purification). Yield after the reaction was found to be 60% (based on active subtilisin present initially) by titration with Aldrithiol-4, and final yield after thiolsepharose chromatography was 20–30%. Yields could be improved to 50% by use of mercuriagarose instead of thiolsepharose, but mercuriagarose was found to (noncovalently) bind the serine protease very tightly, and extensive washing was unable to remove it completely.

To be certain that the enzymatic activity was comparable to that of thiosubtilisin BPN′, the kinetics of the two enzymes with the ester substrate sAAPFSBz were compared. They were found to have comparable kinetic parameters (Table 5A), with the 8397/C206Q variant being slightly better. These parameters are similar to those observed by Abrahmsén et al.[9]

The aminolysis:hydrolysis ratios were also determined for the two thiol enzymes, as well as for the wild-type serine protease. The results, listed in Table 5B, show that for both nucleophiles studied (leucinamide and glycinamide), the aminolysis:hydrolysis ratios are very high for the thioenzymes and virtually undetectable for the serine protease. Indeed, in the case of leucinamide, the deacylation rate of the wild-type cinnamoyl enzyme actually decreased with added leucinamide, indicating that leucinamide may bind to the active site in a nonproductive way to block entry of water.

Finally, the stabilities of the two thiosubtilisins were compared in buffer containing 5 mM DTT to prevent oxidation of the enzyme, with or without 50% DMF. The results, shown in Figure 5, show that under both conditions thiosubtilisin 8397/C206Q is substantially more stable than thiosubtilisin BPN′.

Synthetic Application. We have reported the application of subtilisin 8397 to various synthetic transformations,[5,10] including

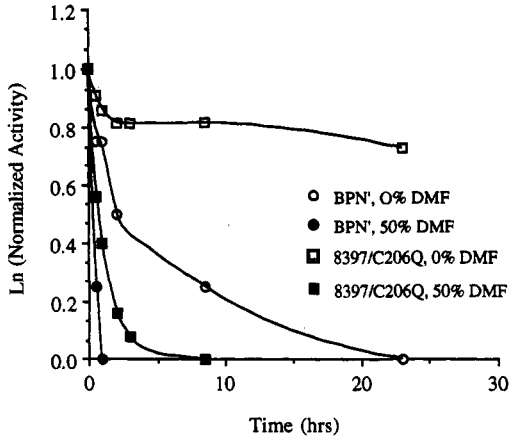
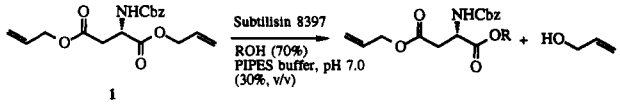


Figure 5. Deactivation of thiosubtilisins; 10 mM Tris/5 mM DTT, pH 8.0, 50 °C.

Table 6. Subtilisin 8397-Catalyzed Transesterification of *N*-Carbobenzoxyaspartic Acid Diallyl Ester



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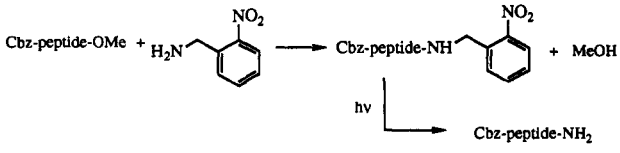
ROH	product	reaction time (h)	yield (%)
CH_3OH	**2**	5	75
C_2H_5OH	**3**	6	75
$CH_3CH(OH)CH_3$	**4**	4	70
cis-$HOCH_3CH_2{=}CH_2CH_2OH$	**5**	5	60
$C(CH_3)_3OH$		no transesterification	

Table 7. Subtilisin 8397-Catalyzed Acylation of *o*-Nitrobenzylamine in 70% Ethanol with N-Protected Amino Acid or Peptide Esters



acyl donor ester[a]	reaction product[b]	yield[c] (%)
Cbz-Ala-Ser-OMe	Cbz-Ala-Ser-oNBA	65
Ac-Tyr-OEt	Ac-Tyr-oNBA	84
Cbz-Ala-Phe-OMe	Cbz-Ala-Phe-oNBA	72
Cbz-Ala-His-OMe	Cbz-Ala-His-oNBA	80

[a] [acyl donor ester] = 0.25 M. [b] [*o*-nitrobenzylamine] = 0.75 M. [c] Based on acyl donor ester.

enantioselective hydrolysis of amino acid esters, polymerization of amino acid and peptide esters, regioselective acylation of sugars and nucleosides, and condensation of peptide fragments containing natural and unnatural amino acids such as D-amino acids and glycosyl amino acids. We have also reported the use of thiosubtilisin 8397/C206Q as a peptide ligase in aqueous solution for the condensation of glucopeptides.[10] We report here two other types of reactions useful in peptide chemistry: one is the regioselective transesterification of N-Cbz-aspartic acid diallyl ester (Table 6), and the other is transacylation of N-protected amino acid and peptide esters to the amino group of *o*-nitrobenzylamine (Table 7). The former case provides a new route to various selectively protected aspartic acid derivatives, as the remaining allyl ester can be easily removed with Wilkinson's catalyst for further transformations. The latter case provides a useful route to peptide amides, as the *o*-nitrobenzyl group can be selectively deprotected with light.[13] These two types of enzymatic reactions were carried out in 70% ethanol solution.

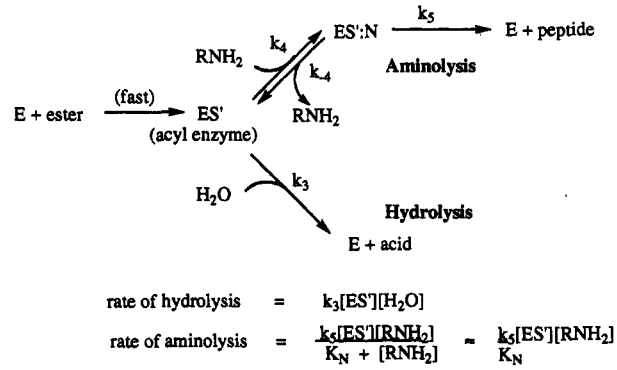
(13) For carboxypeptidase Y-catalyzed acylation, see: Henricksen, D. B.; Breddam, K.; Moller, J.; Buchardt, O. *J. Am. Chem. Soc.* **1992**, *114*, 1876.

Table 8. Kinetic Parameters for the Subtilisin-Catalyzed Hydrolysis of Suc-Ala-Ala-Pro-Phe-pNA (0.1 M Tris-HCl, pH 8.0, 25 °C)

enzyme	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})
8397[a]	74	0.097	7.6×10^5
8397 M222A[b]	126	0.283	4.4×10^5
8397 M222A/Y218W[b]	84	0.074	1.1×10^6

[a] Data from ref 11; 0.1 M sodium phosphate, pH 8.2, 0.1 M NaCl, 25 °C. [b] 0.1 M Tris-HCl, pH 8.0, 25 °C.

Scheme 1



$$\text{rate of hydrolysis} = k_3[ES'][H_2O]$$

$$\text{rate of aminolysis} = \frac{k_5[ES'][RNH_2]}{K_N + [RNH_2]} \approx \frac{k_5[ES'][RNH_2]}{K_N}$$

Alteration of P_1' Specificity. As demonstrated in the acyl-transfer experiments using amino acid amide acceptors,[14] the structure–activity relationships of the S_1'–P_1' interaction of the recently isolated subtilisin 72 are significantly different from those obtained from α-chymotrypsin. Whereas both enzymes show a similar specificity toward Gly-NH_2, introduction of side chains to the acceptor (e.g. Ala-NH_2, Leu-NH_2) reduces binding by subtilisin by 1–2 orders of magnitude. The opposite is observed for chymotrypsin. Evaluation of the three-dimensional structure of subtilisin BPN'[14b] has led us to suggest that the S_1'–P_1' interaction is subject to steric hindrance, since the S_1' binding site is filled by the bulky side chain of Met 222. Despite the fact that Met 222 is conserved in most subtilisins known to date,[15] a study investigating replacement with all proteinogenic amino acids[16] showed that the overall activity is retained after exchange to certain amino acids such as Cys or Ala. Therefore, we decided to change the 8397 variant at position 222 and the neighboring position 217 and evaluate the effects of the mutations on acceptor binding.

The k_{cat} and K_m values for subtilisin 8397 and the M222A and M222A/Y217W variants are similar, indicating that the mutations do not greatly affect catalysis (Table 8). According to Scheme 1 the specificity of an acyl enzyme ES' toward an acceptor N corresponds to the second-order rate constant k_5/K_N of its conversion to the acyl-transfer product. Since k_3 is constant for a given acyl donor, k_5/K_N is proportional to k_5/K_Nk_3 and can be derived from the initial reaction rates or the progress kinetics of an appropriate acyl-transfer reaction.[17] Parameters for wild-type and mutant subtilisins were obtained in two reaction systems. In the first system (Figure 6), Mal-Tyr-OMe serves as donor substrate for the acyl transfer to a series of amino acid amides. The acceptor specificity is given as $\log(k_5/K_Nk_3)$, representing the relative free energies for the interaction of free Mal-Tyr-subtilisin with free nucleophile. The specificity of the wild-type subtilisin toward amino acid amides follows the same pattern as described above for subtilisin 72. In contrast, subtilisin 8397/

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(15) Siezen, R. J.; de Vos, W. M.; Leunissen, J. A. M.; Dijkstra, B. W. *Protein Eng.* **1991**, *4*, 719.

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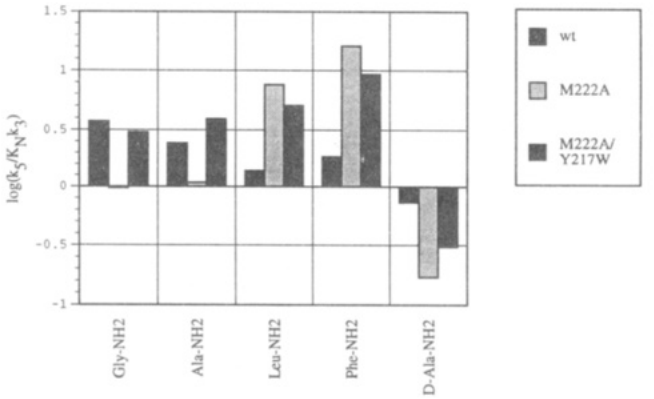

Figure 6. Specificity of Mal-Tyr-subtilisin 8397 and its mutants M222A and M222A/Y217W for amino acid amide acceptors (1 mM Mal-Tyr-OMe, 500 mM acceptor, pH 8.0).

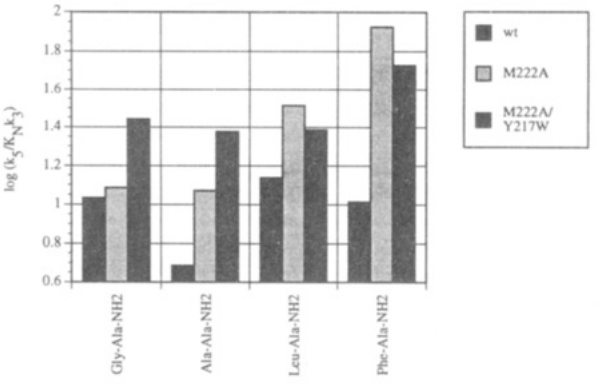

Figure 7. Specificity of Suc-Ala-Ala-Pro-Phe-subtilisin 8397 and its mutants M222A and M222A/Y217W for dipeptide amide acceptors differing in P1′ (1mM Suc-Ala-Ala-Pro-Phe-SBn, 100 mM acceptor, pH 8.0).

M222A shows a significant preference for bulky amino acid amides such as Phe-NH_2 and Leu-NH_2, while its affinity toward Gly-NH_2 and Ala-NH_2 is markedly reduced as a result of the mutation. The double mutant 8397/M222A/Y217W shows high specificities for all L-amino acid amides under investigation, thus combining the advantageous properties of both wild type and M222A. Comparison of the data for L-Ala-NH_2 and D-Ala-NH_2, respectively, reveals that the stereoselectivity of the S1′ site increases in the order wild type < M222A < M222A/Y217W. In the second reaction system, acyl transfer from the donor Suc-Ala-Ala-Pro-Phe-SBn to a series of dipeptide amides of the general structure X-Ala-NH_2 was studied (Figure 7). Although the specificity of the wild type is obviously not related to the structure of the P_1' acceptor residue, M222A again prefers bulky amino acids in this position. While introduction of the additional mutation Y217W results in a slightly decreased affinity for Phe and Leu in P_1', the binding of Gly and Ala is improved. Consequently, M222A/Y217W represents a highly efficient acyl-transfer catalyst with a broad acceptor specificity.

Discussion

In order to carry out peptide synthesis in aqueous solution, it is often necessary to add substantial amounts of water-miscible cosolvent, both in order to increase the solubility of the substrates and to reduce the amount of hydrolysis by lowering the concentration of water, by increasing the activation energy of hydrolysis over that of aminolysis (see results), and by increasing the effective (deprotonated) amine concentration. However, many enzymes are destabilized under such conditions. Also, it is often necessary to carry out the reaction at an elevated temperature to make the reaction proceed at an acceptable rate without using highly activated ester substrates. This is particularly true in the case of thiosubtilisin, where the k_{cat} values for various substrates are several orders of magnitude lower than those of the serine protease.[9] For these reasons, it would be beneficial to create a subtilisin variant that is stabilized toward thermal and cosolvent-induced denaturation.

Previous work has produced subtilisin 8397 containing five mutations that stabilize the protein in a variety of ways. The space-filling model of this variant is shown in Figure 8, in which the surface charges are highlighted. Further removal of each of three other surface charges of this variant did not increase the stability of DMF as expected.

As mentioned briefly in the introduction, removal of surface charges is expected to stabilize enzymes toward organic cosolvents. The rationale behing this can be explained by considering the "denatured" form of the protein one expects to find in a solvent of low dielectric constant. In the folded state, one predicts that the hydrophilic residues will be on the surface and the hydrophobic residues will be buried. In the denatured form (in an organic solvent), one expects the protein to have "flipped inside out", so that the hydrophobic residues face the organic solvent and the charged residues are now buried. There is evidence that the irreversible deactivation of subtilisin occurs through a two-step mechanism[4]

$$\underset{\text{(natured)}}{\text{N}} \overset{K_{eq}}{\rightleftarrows} \underset{\text{(partially unfolded)}}{\text{D}} \overset{k}{\rightarrow} \underset{\text{(denatured)}}{\text{I}}$$

where the first step occurs on a much more rapid time scale than the second. There are two lines of thought regarding this deactivation. Ohta et al.[18a] report that the actual unfolding of subtilisin is irreversible and that the 77-amino acid fragment derived from the N-terminus of the proenzyme is required as a "chaperone" for the refolding. According to this scheme, then, the "D" state would be a partially unfolded enzyme and the "I" state would be the completely unfolded version. In contrast, Bryan and co-workers[18b] report that subtilisin is reversibly unfolded and that the two transitions represent the unfolded (D) and the chemically inactivated (I) forms of the enzyme. One expects that the intermediate D has an increased number of hydrophobic residues on the surface and has buried many of the charged residues. Assuming that denaturation of charge mutants still occurs through the same intermediate, removing certain surface charges will lower the free energy change of the first step, since the free energy of the N state will be lowered (fewer charged residues in a hydrophobic environment) and the free energy of the D state will be raised (because now certain hydrophobic residues, charged in the wild-type enzyme, are being buried) (Figure 9). This will decrease the free energy drop between the two states and shift the equilibrium of the first step toward the left, reducing the percent of molecules in the D state and thus slowing the D to I transitions, given by

$$\frac{dI}{dt} = (k)(K_{eq})[N]$$

In addition, if the irreversible step is generated by the unfolding of the enzyme, as suggested by Ohta and co-workers, then it seems likely that the transition state might be a structure that "looks like" the final state, that is, a structure in which even more of the hydrophilic residues are buried but in which the enzyme is in a strained conformation which can then relax to the final inactivated state (I). If this is the case, then not only would the rate of the initial reversible step be decreased by removal of surface charges but the second irreversible step would be slowed dramatically also and for the same reasons.

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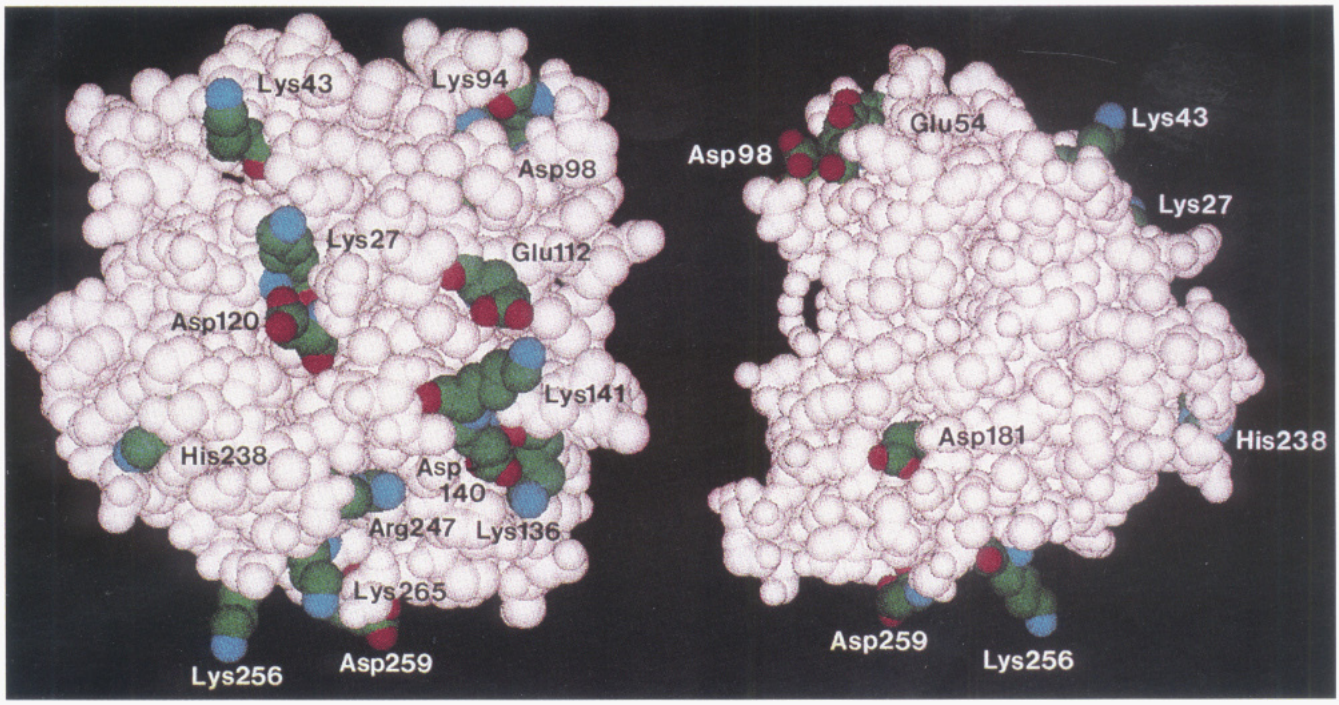

Figure 8. Space filling models of subtilisin 8397 highlighting surface charges. The sites of mutation and their improved effects are as follows: Met 50 Phe (hydrophobic interaction), Asn 76 Asp (Ca^{2+} binding), Gly 169 Ala (conformational restriction), Asn 218 Ser (hydrogen bonding), Gln 206 Cys (van der Waals). Further surface charge mutations were made to subtilisin 8397 (K43N, D181N, or K256Y).

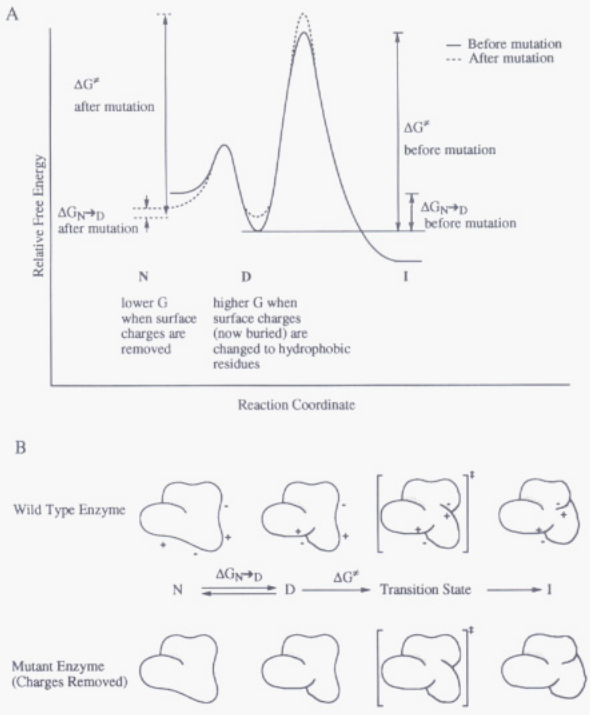

Figure 9. (A) Hypothetical free energy diagram for deactivation of subtilisin in organic solvents. (B) Pictorial illustration of subtilisin deactivation in organic solvents.

The inability of surface charge removal to stabilize lyophilized enzyme suspended in pure DMF may be attributed to the fact that the enzyme is not truly exposed to DMF but is encased in a shell of buffer salts and water. It is not surprising, therefore, that the effects of charge removal are masked under these conditions. The effect of counterions on the stability of subtilisins in dry DMF appears to override the effect resulting from site-directed mutagenesis. Complete removal of the salt destabilizes the enzymes to the point that the deactivation curve cannot be easily measured. For this reason, we carried out stability studies in homogeneous systems. In water/DMF mixtures, the mutant enzymes have stabilities quite different from that of the 8397 variant, and not all have increased stability, even at 50% (v/v) DMF. The relative stabilities do, however, increase with increase of DMF, and so it seemed reasonable that if one could put the enzymes in a medium of even lower dielectric constant, one might find that the stabilities of all the charge mutants became higher than that of subtilisin 8397. This was not found to be the case. Following the logic of the argument above, it is possible that in the transition from the N to the D state certain regions of the protein are not perturbed and thus removing surface charges in those regions will have little effect on protein stability. This may be the case for the K43N mutant, which has stability similar to that of the 8397 variant. It is also possible that certain charged residues may help destabilize the D state: perhaps they are moved further away from their counterions, or perhaps they are pushed out into the solvent where they cannot H-bond with other residues. If one such charge is removed, the D state will be stabilized, increasing the deactivation rate. This might explain why the D181N mutant shows reduced stability toward organic cosolvents. What is clear is that random surface charge removal, while it may provide some stable mutants (as in the case of K256Y), does not always stabilize in a predictable fashion.

In order to create a peptide ligase, one would like to increase the ratio of the second-order rate constants, $(k_5/K_N)/k_3$. Addition of organic cosolvents and thiolation of the active-site since serine were shown in the results section to be effective means of accomplishing this. Thiosubtilisin 8397/C206Q has been shown[10] to be able to catalyze peptide synthesis in water (without cosolvent) at 50 °C, conditions under which the serine proteases favor hydrolysis and thiosubtilisin BPN′ is unstable. It was found to couple glycopeptides with a yield of 60% when an acyl acceptor: donor ratio of 5:1 was used.

The investigation of acyl-transfer reactions catalyzed by subtilisin 8397 and its mutants M222A and M222A/Y217W demonstrates that the acceptor specificity of subtilisin BPN′ can be significantly improved by site-directed mutagenesis. Data obtained using amino acid amide and dipeptide amide nucleophiles indicate that their interaction with the S_1' site of the wild-type enzyme is governed by different binding modes. In contrast, 8397/M222A prefers bulky, hydrophobic residues in the same

position independent of the type of acceptor. In terms of binding, the preference of Phe over Gly corresponds to a relative free energy difference of approximately 1.5 kcal/mol. Since the mutation has the potential of increasing the conformational flexibility of the side chain of Y217 defining the boundary of the S_1' binding site, this energy difference could be due to an improved interaction of Phe with Y217. The additional mutation Y217W was introduced to alter the hydrophobic area for acceptor binding. Whereas M222A/Y217W indeed binds the P_1' residues Gly and Ala more tightly than M222A, binding of Phe and Leu is not further improved. This observation might be attributed to conformational constraints introduced by the bulky P_1'-Trp residue in position 217. In any event, the 8397/M222A/Y217W variant is better than the wild-type subtilisin 8397 in catalyzing kinetically controlled peptide synthesis reactions. The P_1' acceptor binding is more flexible and is improved by an average of 0.7 kcal/mol. It appears to be useful for peptide synthesis. Modification of other positions recently reported by Jones et al. also altered the substrate specificity in hydrolysis.[19]

In conclusion, this paper describes the first study of counterion effects on the stability of enzyme in organic solvents and the detailed examination of surface charge modification of subtilisin BPN′ that affects the enzyme stability in high concentrations of DMF. An improvement of enzymatic aminolysis in aqueous solution was accomplished using peptide methyl esters as substrates and a thermostable thiosubtilisin variant as catalyst. A new subtilisin variant was also developed from 8397 via M222A/Y217W mutations to broaden the P_1' specificity.

Materials and Methods

The subtilisin-deficient *Bacillus subtilis* strain 4935 and the subtilisin 8397-containing shuttle vector pGX5097 were obtained from the Genex Corporation; oligonucleotide primers were ordered from Genosys, Woods Hole, TX. Sure competent *Escherichia coli* and all DNA-modifying enzymes were orderd from Sigma. Potassium thioacetate, *p*-(chloromercurio)benzoic acid, and Aldrithiol-4 (4,4′-dipyridyl disulfide) were obtained from Aldrich, while (phenylmethyl)sulfonyl fluoride (PMSF) and wild-type subtilisin BPN′ were ordered from Sigma.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed using the overlap extension method.[20] Briefly, four oligonucleotide primers were constructed: one corresponding to the sense sequence at the start of the subtilisin gene and incorporating the *Bam*HI restriction site (pZ11, below); one corresponding to the antisense sequence at the end of the gene, incorporating the *Sal*I restriction site (pZ06-A); and two that correspond to the sense and antisense sequences about the mutation site, incorporating the mutated codon

pZ11: 5′-CAG GCG GAT CCG AAA TCA AAC GGG GAA AAG

pZ06-A: 5′-
CAG GTC GAC ATT ATG GAG CGG ATT GAA CAT GCG GAG

D181N-F: 5′-GTA GGC GCT GTT AAC AGC AGC AAC CAA

D181N-R: 5′-TTG GTT GCT GCT GTT AAC AGC GCC CAT

K43N-F: 5′-CCT GAT TTA AAC GTA GCA GGC GGA

K43N-R: 5′-TCC GCC TGC TAC GTT TAA ATC AGG

K256Y-F: 5′-ACC ACT ACA TAT CTT GGT GAT

K256Y-R: 5′-ATC ACC AAG ATA TGT AGT GGT

C206Q-F: 5′-GTA TCT ATC CAA AGC ACG CTT

C206Q-R: 5′-AAG CGT GCT TTG GAT AGA TAC

M222A-F: 5′-GGTACGTCAGCGGCATCTCCG

M222A-R: 5′-CGGAGATGCCGCTGACGTACC

Y217W-F: 5′-TACGGGGCGTGGAGTGGTACG

Y217W-R: 5′-CGTACCACTCCACGCCCCGTA

The polymerase chain reaction (PCR) was performed using Taq DNA polymerase (Stratagene) in two batches, each with two primers and the plasmid PGX5097 as a template to produce two fragments corresponding to the front and the back of the subtilisin gene, with overlapping sequences at the mutation site. These two fragments were combined and allowed to anneal, and the resulting hybrid structure was amplified further. The gene was then cleaved with *Bam*HI and *Sal*I and ligated into the cleaved and dephosphorylated PGX5097 plasmid. (PCR, endonuclease digestion, dephosphorylation, and ligation protocols were derived from Sambrook, Fritsch, and Maniatis.[21] *E. coli* were transformed with the resultant plasmid according to the procedure recommended by Stratagene and were selected on LB plates containing 50 μg/mL ampicillin. Plasmid DNA from the resulting colonies was sequenced to verify the presence of the mutation and then inserted into the protease-deficient *B. subtilis* 4935 (Genex) using the procedure suggested by Harwood and Cutting.[22]

Isolation of Subtilisin 8397. Subtilisin 8397 was isolated by the procedure of Carter and Wells,[23] omitting the final cation-exchange column. In brief, *B. subtilis* carrying the subtilisin 8397-bearing plasmid pGX5097 was grown in AM3 medium (Difco) + 15 μg/mL kanamycin for 2 days. The cells were removed by centrifugation, and the supernatant was precipitated with 40% and 80% ammonium sulfate. The 80% fraction was dialyzed against distilled water, and 100 mM MES/50 mM $CaCl_2$ (pH 6.0) was added at a 1:10 ratio. The subtilisin was run through DEAE cellulose (DE-52, Whatman) equilibrated with 10 mM MES/5 mM $CaCl_2$ and then was dialyzed against 4 × 4 L of distilled water and lyophilized to dryness. Final yield was approximately 10 mg of subtilisin per liter of medium.

Preparation of Thiosubtilisin. Thiosubtilisin was prepared according to the method of Phillipp, Polgar, and Bender,[24] with modifications. In brief, 100 mg of subtilisin in 4 mL of 100 mM sodium phosphate, pH 7, was reacted with 1 mg of PMSF in 0.1 mL of 1,4-dioxane. After 1 h, 400 mg of potassium thioacetate was added, and the mixture was stirred at room temperature for 3 days. β-Mercaptoethanol was added to a concentration of 1.5% and allowed to stand for 15 min to reduce any disulfide bonds that might have formed. The thiosubtilisin was then dialyzed extensively under nitrogen and titrated with both *p*-(chloromercurio)benzoic acid (PCMBA)[25] and 4,4′-dipyridyl disulfide ("Aldrithiol-4").[26] The volume was doubled with 100 mM phosphate buffer (pH 7), 5 mL of activated thiolsepharose was added, and the mixture was shaken mildly overnight to allow complete binding. (Note: it was found that (mercaptopropyl)sepharose did NOT effectively bind thiosubtilisin, most likely due to lack of a spacer arm between the resin and the activated thiol.) The resin was then extensively washed with approximately 4 L of 50 mM phosphate/5% DMF (pH 7) to remove all noncovalently bound wild-type subtilisin. (The DMF helped prevent binding of the protein to the mercuriobenzene group via hydrophobic interactions and was found to be superior to 0.1% Triton X-100 in this regard.) Thiosubtilisin was eluted with 100 mM β-mercaptoethanol in the same buffer, dialyzed against distilled water, lyophilized, and stored under nitrogen at −20 °C. Thiosubtilisin was assayed for wild-type-enzyme contamination by adding 10 mM Aldrithiol-4, which irreversibly inhibits thiosubtilisin but not wild-type subtilisin,[24] to a portion of the enzyme preparation and then assayed for activity.

Stability Assay in Dimethylformamide (Heterogeneous). Method A. Subtilisin BPN′ (either wild type or 8397) was dissolved at 0.5 mg/mL in either 50 mM Tris-HCl or 50 mM sodium phosphate, pH 8.4, and then 2 mL of the solution was transferred into a small glass bottle, quickly frozen in a −78 °C bath, and lyophilized overnight to generate a white solid. The solid was kept in a desiccator at 4 °C. At 25 °C, 1 mL of anhydrous DMF was added to a bottle with wild-type solid subtilisin BPN′ (1 mg of the protein per bottle). The protein was quickly dispersed in DMF by vigorous shaking with a vortexer. Next, the mixture was kept at 25 °C without stirring and the enzyme activity was assayed at various time intervals. When the subtilisin was lyophilized from sodium phosphate, a good dispersion was obtained. When the enzyme was prepared from Tris-HCl, most of the solid dissolved and a relatively clear solution was obtained with a very small amount of precipitate. To assay the enzyme activity, 20 μL of the DMF suspension was removed using a micropipet with the top of the tip cut off (in order to take the suspended

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(26) Brocklehurst, K.; Little, G. *Biochem. J.* **1973**, *133*, 67.

sample) and was injected directly into 1.0 mL of substrate buffer (100 mM Tris-HCl, pH 8.0, with 0.5 mM sAAPFpNA). The hydrolysis of the substrate was followed at 410 nm for 2 min.

Method B. Subtilisin was dissolved to a concentration of 10 mg/mL in 1 mM $CaCl_2$, and 50 μL of this solution was mixed with 2 mL of various buffers (generally either 10 mM or 100 mM, to give a 1000:1 or 10 000:1 molar ratio of salt to enzyme, respectively).

(1) Stirred Assays. The solution was frozen on dry ice and lyophilized to dryness. The resulting solid was placed in a siliconized 1-dram glass vial with a small teflon stir bar and covered with 3 mL of dry solvent under nitrogen. After about 15 min of rapid stirring (at room temperature), the dispersion was fine enough to sample reproducibly. Time points were taken by removing 50 μL of the suspension with a wide-bore pipet tip and then dissolving that in 450 μL of 100 mM Tris buffer (pH 8). The sample was assayed immediately (although no change of activity was noticed after dissolution in buffer) by mixing 25 μL of the sample with 975 μL of 0.5 mM sAAPFpNA in 100 mM Tris, pH 8, and monitoring the change in absorbance.

(2) Unstirred Assays. One hundred microliters of the enzyme solution was aliquoted into each of 20 tubes, frozen on dry ice, and lyophilized to dryness. One hundred microliters of DMF was added (under nitrogen), and the samples were left at room temperature until sampling. For each time point, 900 μL of 100 mM Tris buffer (pH 8) was added to each of two tubes, and the activity of the resulting solution was assayed immediately (as above).

Stability Assay in Aqueous/Organic Solvent (Homogeneous Solution). Buffer and various percentages of cosolvent were mixed together and preheated to 50 °C. Enzyme solution (50 μL) was added (1 mg/mL of 1 mM $CaCl_2$) to 1 mL of the preheated solution. At various times, 50-μL samples were removed and placed on ice until they were assayed (assay: 25 μL of enzyme sample was added to 975 μL of 0.5 mM sAAPFpNA in 100 mM Tris, pH 8, and monitored at 410 nm).

Stability Assay of the Enzyme in 60% and 80% DMF Solution. At 25 °C, 1 mL of either 60% or 80% DMF solution was added to the lyophilized enzyme (either wild-type subtilisin BPN′ or subtilisin 8397, 1 mg per bottle). The protein was then quickly dispersed by vigorously shaking with a vortexer. For the subtilisin prepared via lyophilization from sodium phosphate, the solid was partly dissolved. The undissolved solid was removed by centrifuging the mixture and transferring only the solution portion into another glass bottle. For the subtilisin with Tris-HCl as counterions, the protein was completely dissolved in 60% or 80% DMF. In both cases, the solutions were kept at 25 °C without stirring, and the enzymatic activity was assayed at various time intervals. The activity was assayed by injecting 20 μL of the solution into 1.0 mL of the substrate buffer and following the hydrolysis of the substrate at 410 nm for 2 min.

Kinetic Assays. Kinetic rates were measured spectrophotometrically at 25 °C in 100 mM Tris-HCl, pH 8.0, with 4% DMF. The hydrolysis of the amide substrate succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (AAPF-pNA) was observed at 410 nm, while the hydrolysis of the thioester analog, succinyl-Ala-Ala-Pro-Phe-S-benzyl ester (AAPFSBz), was measured either at 340 nm (when using Aldrithiol-4) or directly at 250 nm. Aldrithiol-4, a common chromophore for monitoring this reaction, was used at a concentration of 10 mM for assays with the serine proteases, but for assays with thiosubtilisin it was avoided due to its reaction with the active-site cysteine.[24]

Subtilisin active sites were titrated by spectrophotometrically (λ = 310 nm) measuring the burst observed with *N-trans*-cinnamoylimidazole in 100 mM sodium acetate, pH 5.0.[11] Thiosubtilisin active sites were titrated with *p*-nitrophenyl trimethylacetate.[24] Briefly, a small amount of enzyme was added to 1 mL of 100 mM sodium phosphate, pH 7.0, containing 250 μM titrant, and the burst was observed at 405 nm. Alternatively, the thiol groups were titrated by measuring the increase in absorbance at 340 nm when thiosubtilisin was mixed with Aldrithiol-4[26] and comparing it to a standard curve made with cysteine. Both titration methods gave the same numbers.

The aminolysis:hydrolysis ratio of the various subtilisins was measured by the method of Polgar and Bender.[27] Briefly, 15–30 mg of subtilisin was dissolved in 1 mL of 100 mM sodium acetate (pH 5), and 1 mg of *N-trans*-cinnamoylimidazole (NTCl) in 100 μL of DMF was added. After the enzyme was allowed to react for 1 min, the mixture was cooled on ice and loaded onto a G-25 sephadex column equilibrated with acetate buffer (pH 5) at 4 °C. The peak protein fractions were pooled and kept on ice until assayed. Deacylation rates were determined by adding 100 μL of the cinnamoyl enzyme to 900 μL of 100 mM borate, pH 9.0, with varying amounts of either glycinamide or leucinamide. The pseudo-first-order decays were measured spectrophotometrically at 310 nm for subtilisin and 320 nm for thiosubtilisin and were curve fitted to a single exponential decay using least squares analysis.

The rate of disappearance of cinnamoyl enzyme is a result of both aminolysis and hydrolysis reactions and can be described by (see Jakubke in ref 1)

$$d(ES')/dt = k_3(H_2O)(ES') + k_4/K_N(\text{glycinamide})_{eff}(ES')$$

where k_4 is the maximal rate of deacylation, K_N is a Michaelis constant for deacylation, and $(\text{glycinamide})_{eff}$ is the concentration of glycinamide in the free base form.

$$(\text{glycinamide})_{eff} = (\text{glycinamide})_{total}/(1 + 10^{pK_a - pH})$$

The pK_a values of glycinamide and leucinamide are taken to be 8.0.[28] A plot of the disappearance of cinnamoyl enzyme vs $(\text{glycinamide})_{eff}$ will then produce a line (at glycinamide concentrations well below K_N) with slope $(k_4/K_N)(ES')$ and intercept $(k_3)(H_2O)(ES')$. The slope divided by the intercept and multiplied by the concentration of water (55.6 M) provides the desired ratio.

For aminolysis of maleyl-Try-OMe (Mal-Tyr-OMe), to 0.1 mL of 500 mM appropriate amino acid amide hydrochloride in 0.25 M KOH was added Mal-Tyr-OMe (1 mM). After addition of 1–10 μM enzyme, the initial rates of product formation were measured by HPLC. Calculation of $\log(k_5/K_N k_3)$ was performed as described.[17] Acyl transfer using Suc-Ala-Ala-Pro-Phe-SBz was performed using 1 mM donor ester and 100 mM dipeptide amide hydrochloride in 50 mM KOH.

A. Transesterification Reactions. Typically, 0.1 mmol of **1** (Table 5) is dissolved in 200 μL of the appropriate alcohol and 100 μL of 0.3 M Pipes buffer (pH 7). The reaction is started by addition of 1 mg of subtilisin 8397 and monitored by TLC and/or GC. After complete conversion of **1**, the reaction mixture is extracted with ethyl acetate. The ethyl acetate phase is washed with saturated sodium biocarbonate and water. After removal of the ethyl acetate, pure products are obtained.

B. Cbz-Asp(OAll)-OAll (1). To a solution of 1.92 g (5 mmol) of aspartic acid diallyl ester were added stepwise 0.066 g (0.5 mmol) of DMAP and 1.08 mL (10 mmol) of DMEA in 10 mL of methylene chloride and 0.64 mL (4.5 mmol) of benzyl chloroformate. After 12 h at 25 °C the reaction mixture was washed with 0.1 M HCl, water, saturated sodium bicarbonate, and water and concentrated. After crystallization from ethyl acetate/hexane, the product is obtained 69% (1.2 g) yield: ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 2.89 (dd, 1H, J = 4.58, 17.18 Hz), 3.09 (dd, 1H, J = 4.53, 17.15 Hz), 4.56–4.60 (m, 2H), 4.62–4.72 (m, 3H), 5.13 (s, 2H), 5.26–5.47 (m, 4H), 5.75–5.96 (m, 3H), 7.27–7.38 (m, 5H); HRMS calcd for $C_{18}H_{21}N_1O_6$ + H^+ 480.0423, found 480.0423.

C. Cbz-Asp(OAll)-OMe (2). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 2.85 (dd, 1H, J = 4.56, 17.22 Hz), 3.04 (dd, 1H, J = 4.48, 17.16 Hz), 3.72 (s, 3H), 4.53–4.57 (m, 2H), 4.58–4.67 (m, 1H), 5.09 (s, 2H), 5.18–5.31 (m, 2H), 5.73 (d, 1H, J = 8.55 Hz), 5.77–5.91 (m, 1H), 7.24–7.34 (m, 5H).

D. Cbz-Asp(OAll)-OEt (3). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 1.26 (t, 3H, J = 7.09 Hz), 2.88 (dd, 1H, J = 4.57, 17.08 Hz), 3.07 (dd, 1H, J = 4.58, 17.10 Hz), 4.21 (q, 2H, J = 7.11 Hz), 4.56–4.60 (m, 2H), 4.60–4.67 (m, 1H), 5.12 (s, 2H), 5.21–5.35 (m, 2H), 5.78 (d, 1H, J = 8.22 Hz), 5.81–5.95 (m, 1H), 7.27–7.38 (m, 5H); HRMS calcd for $C_{17}H_{21}N_1O_6$ + H^+ 336.1447, found 336.1447.

E. Cbz-Asp(OAll)-O^{t}Pro (4). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 1.19–1.28 (m (2d), 6H), 2.87 (dd, 1H, J = 4.60, 17.03 Hz), 3.05 (dd, 1H, J = 4.59, 17.06 Hz), 4.53–4.73 (m, 3H), 5.02–5.12 (m, 1H), 5.12 (s, 2H), 5.21–5.34 (m, 2H), 5.76 (d, 1H, J = 8.11 Hz), 5.81–5.96 (m, 1H), 7.28–7.40 (m, 5H).

F. Cbz-Asp(OAll)-O-*cis*-2-butene-1,4-diyl (5). ^{1}H-NMR ($CDCl_3$, 300 MHz) δ 2.87 (dd, 1H, J = 4.56, 17.16 Hz), 3.06 (dd, 1H, J = 4.46, 17.20 Hz), 4.23 (d, 2H, J = 6.54 Hz), 4.54–4.60 (m, 2H), 4.60–4.68 (m, 1H), 4.71–4.78 (m, 2H), 5.15 (s, 2H), 5.15–5.34 (m, 2H), 5.55–5.66 (m, 1H), 5.75–5.96 (m, 3H), 7.27–7.40 (m, 5H); HRMS calcd for $C_{19}H_{23}N_1O_7$ + Cs^+ 510.0529, found 510.0539.

Acylation of *O*-Nitrobenzylamine for the Preparation of Peptide Amide. N-protected peptide ester (0.2 mmol) and 0.112 g (0.6 mmol) of *o*-nitrobenzylamine were dissolved in 0.5 mL of ethanol. After addition

(27) Polgar, L.; Bender, M. *Biochemistry* **1967**, *6* (2), 610.

(28) Stryer, L. *Biochemistry*, 3rd ed.; W. H. Freeman and Co.: New York, 1988; p 21.

of 0.056 mL (0.4 mmol) of triethylamine and 0.2 mL of water, reactions were started by addition of 10 mg of crude subtilisin 8397 and followed by TLC. After 2–3 days the solvents were evaporated *in vacuo*. The residue was dissolved in ethyl acetate and washed with 0.1 M HCl, saturated sodium bicarbonate, and brine. The solution was concentrated and dried *in vacuo*. The crude material was crystallized from ethyl acetate to yield the pure products, which were characterized by HRMS and ^{1}H-NMR, respectively.

A. Cbz-Ala-Ser-oNBa. ^{1}H-NMR (500 MHz, CD_3OD) δ 1.37 (d, 3H, J = 7.0 Hz), 3.80 (dd, 2H, J = 5.0, 11.0 Hz), 3.90 (dd, 2H, J = 5.0, 11.0 Hz), 4.13 (q, 1H, J = 7.0 Hz), 4.42 (t, 1H, J = 5.0 Hz), 4.65 (d, 1H, J = 16.5 Hz), 4.72 (d, 1H, J = 16.5 Hz), 5.00 (s, 2H), 7.25–7.35 (m, 5H), 7.40–7.50 (m, 1H), 7.60–7.65 (m, 2H), 8.04 (d, 1H, J = 7.5 Hz); HRMS calcd for $C_{21}H_{24}N_4O_7$ + CS^+ 577.0699, found 577.0699.

B. Ac-Tyr-oNBA. ^{1}H-NMR (500 MHz, CD_3OD) δ 1.95 (s, 3H), 2.85 (dd, 1H, J = 7.5, 13.5 Hz), 2.96 (dd, 1H, J = 7.5, 13.5 Hz), 4.08–4.13 (m, 1H), 4.54 (dd, 1H, J = 6.0, 16.5 Hz), 4.66 (dd, 1H, J = 6.0, 16.5 Hz), 6.65–6.71 (m, 2H), 6.99–7.05 (m, 2H), 7.17 (d, 1H, J = 7.5 Hz), 7.46 (t, 1H, J = 7.0 Hz), 7.58 (t, 1H, J = 7.5 Hz), 8.02 (d, 1H, J = 8.0 Hz), 8.27 (d, 1H, J = 7.5 Hz), 8.48 (t, 1H, J = 6.5 Hz); HRMS calcd for $C_{18}H_{19}N_3O_5$ + Cs^+ 490.0379, found 490.0376.

C. Cbz-Ala-Phe-oNBA. ^{1}H-NMR (500 MHz, $CDCl_3$) δ 1.32 (d, 3H, J = 7.0 Hz), 2.95–3.05 (m, 1H), 3.05–3.15 (m, 1H), 4.15 (q, 1H, J = 7.0 Hz), 4.47–4.55 (m, 1H), 4.60–4.73 (m, 2H), 4.99 (d, 1H, J = 12.0 Hz), 5.07 (d, 1H, J = 12.0 Hz), 5.11–5.19 (m, 1H), 6.58 (d, J = 7.5 Hz), 7.00–7.09 (m, 2H), 7.10–7.20 (m, 3H), 7.30–7.40 (m, 4H), 7.42–7.52 (m, 2H), 7.60 (t, 1H, J = 7.0 Hz), 8.05 (d, 1H, J = 8.0 Hz); HRMS calcd for $C_{27}H_{28}N_4O_6$ + Cs^+ 637.1063, found 637.1067.

D. Cbz-Ala-His-oNBA. ^{1}H-NMR (500 MHz, CD_3OD) δ 1.32 (d, 3H, J = 6.0 Hz), 3.08 (d, 2H, J = 6.5 Hz), 4.06 (q, 1H, J = 7.0 Hz), 4.58–4.64 (m, 3H), 4.94 (d, 1H, J = 7.5 Hz), 5.00 (d, 1H, J = 7.5 Hz), 6.82 (s, 1H), 7.25–7.35 (m, 5H), 7.43–7.49 (m, 1H), 7.52 (s, 1H), 7.58–7.62 (m, 1H), 8.03 (d, J = 8.5 Hz); HRMS calcd for $C_{24}H_{26}N_6O_6$ + Cs^+ 627.0968, found 627.0965.

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