Exploitation of Subtilisin BPN′ as Catalyst for the Synthesis of Peptides Containing Noncoded Amino Acids, Peptide Mimetics and Peptide Conjugates

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Abstract: The ability of the serine protease subtilisin BPN′ to catalyze peptide bond formation between fragments containing noncoded amino acids, peptide mimetics, and peptide conjugates in a kinetic approach was explored. It was found that the enzyme accepts numerous of these types of compounds both as acyl donor and acyl acceptor. The results together with specificity studies reported by others provide an active site model as a guideline in the design of enzymatic synthesis of biologically important compounds.

Introduction

Protease-catalyzed peptide bond formation has received a lot of attention for a number of years.[1] Enzymatic peptide coupling is considered to be an attractive alternative for solution and solid phase peptide synthesis, since a high degree of regio- and stereoselectivity can be reached, the reaction conditions are mild, minimal side protection is required, and couplings are generally racemization free. However, a general methodology for enzymatic synthesis of peptides has not yet been achieved, the major limitation being the hydrolytic activity of the protease and the enzyme specificity which often limits the residues between which a peptide bond can be synthesized.

Although numerous papers appeared on the enzymatic synthesis of various peptides,[1] only a few report the enzymatic coupling of noncoded amino acids,[2] peptide mimetics,[3] and peptide conjugates.[4] Since peptide isosteres have become very important for the development of protease inhibitors, new efficient and environmentally friendly methods are required for the synthesis of these types of compounds. We have investigated whether enzymatic coupling, using the commercially available protease subtilisin BPN′ in a kinetic approach, could be potentially useful for this purpose. Subtilisin BPN′ and several variants are well described[5] and frequently applied to peptide synthesis. This serine protease is very specific for L-amino acids in the P1 position (acyl donor), whereas the P1′ residue (nucleophile) is more flexible.[6] For this reason we started our study of P1′ specificity, using Z-Phe-OBn as the acyl donor and various noncoded amino acids, peptide mimetics, and peptide conjugates as acyl acceptors (Figure 1). (Although N-acetylated amino acids are preferred by the enzyme, as detailed in Table 6, Z-protected amino acids are more useful for synthetic purposes as they afford easy deprotection.) We expanded this by using several peptide conjugates as the acyl donors with H-Gly-NH_2 as the acyl acceptor.

Results and Discussion

Before coupling Z-Phe-OBn in the presence of subtilisin BPN′ to several noncoded amino acids we used a standard amino acid (glycinamide **1**) as a nucleophile (Table 1) for a point of reference (80% yield). The noncoded (but naturally occurring) β-alanine amide (**2**) as a P1′ residue was almost an equally good substrate as **1** (70% yield). The α-branched amino acids **3** and **4**, useful for restriction of the conformation of a peptide[6] and of biological importance,[8] and **5**, employed as a galactose mimetic in the development of sialyl Lewis X mimetics,[9] showed little peptide bond formation. In these cases mainly competitive hydrolysis of the benzyl ester was observed. However, when the α-branched amino acids were positioned as P2′ residues with Gly as the P1′ residue, the desired peptides **17** and **18** were obtained in high yields (89% and 75%, respectively), confirming the statement that the specificity of the S2′ subsite is more flexible than of the S1′ subsite.[5] Allylamine (**8**) and the aminoethanol (derivatives) (**9**−**11**) which can be considered as amino acid precursors and have been used in an approach toward the enzymatic synthesis of cyclosporin A analogs,[10] all gave good yields, although the yields were considerably influenced by the O-protecting group (Table 1).

[⊗] Abstract published in *Advance ACS Abstracts,* April 15, 1997.

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(4) Wong, C.-H.; Schuster, M.; Wang, P.; Sears, P. *J. Am. Chem. Soc.* **1993**, *115*, 5893−5901.

(5) (a) Sears, P.; Schuster, M.; Wang, P.; Witte, K.; Wong, C.-H. *J. Am. Chem. Soc.* **1994**, *116*, 6521−6530. (b) Jackson, D. Y., Burnier, J.; Quan, C.; Stanley, M.; Tom, J.; Wells, J. A. *Science* **1994**, *266*, 243.

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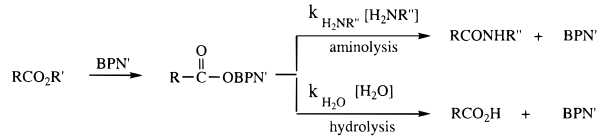
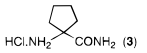
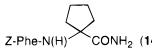
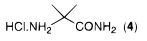
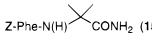
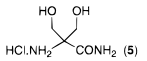
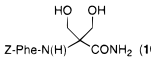
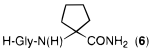
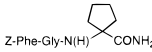
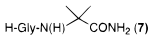
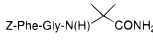
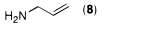
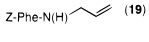
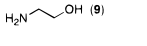
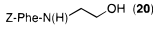
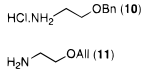
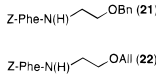
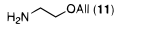
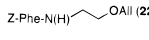


Figure 1. Kinetic approach (aminolysis) to enzymatic amidation catalyzed by subtilisin BPN′.

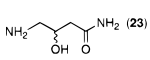
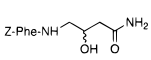
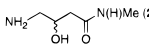
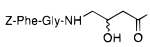
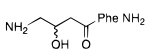
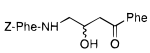
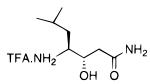
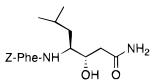
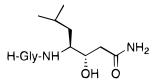
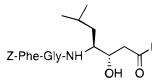
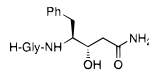
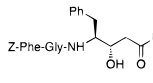
Table 1. Subtilisin BPN′ Catalyzed Coupling of Peptide Fragments Containing Noncoded Amino Acids and Amino Acid Precursors

acyl donor	acyl acceptor[a]	product[b]	Yield
Z-Phe-OBn	HCl.H-Gly-NH$_2$ (**1**)	Z-Phe-Gly-NH$_2$ (**12**)	80%
Z-Phe-OBn	HCl.H-β-Ala-NH$_2$ (**2**)	Z-Phe-β-Ala-NH$_2$ (**13**)	70%
Z-Phe-OBn	 (**3**)	 (**14**)	6%
Z-Phe-OBn	 (**4**)	 (**15**)	0%
Z-Phe-OBn	 (**5**)	 (**16**)	20%
Z-Phe-OBn	 (**6**)	 (**17**)	89%
Z-Phe-OBn	 (**7**)	 (**18**)	75%
Z-Phe-OBn	 (**8**)	 (**19**)	74%
Z-Phe-OBn	 (**9**)	 (**20**)	70%
Z-Phe-OBn	 (**10**)	 (**21**)	62%
Z-Phe-OBn	 (**11**)	 (**22**)	40%

[a] All substrates were prepared using standard peptide chemistry, employing the EDCI/HOBt and the mixed anhydride coupling methods in combination with Boc or Z protection and removal by TFA or HCl and hydrogenolysis, respectively.[33] Hydoxylmethylserine amide (**5**) was synthesized from serine according to Otani and Winitz.[34] The *O*-protected amino alcohols (**10**) and (**11**) were prepared by alkylation of the corresponding Boc amino alcohol, followed by removal of the Boc group. [b] The reaction was performed in DMF/water (8/3, v/v), using 0.18 M acyl donor and 0.55M acyl acceptor in the presence of BPN′ (9 mg/mL). Under the same conditions without addition of the protease no hydrolysis nor aminolysis took place.

Statine and derivatives (**23**−**28**) have a long history as peptide bond isosteres and have been frequently used in the development of protease inhibitors of, for example, renin[11] and HIV protease.[12] Although these isosteres are potentially inhibitors of subtilisin BPN′, we reasoned that some peptide bond formation might occur if the isostere was placed at or especially after the P1′ residue in the acyl acceptor. Indeed, the statine analog of GlyGly (**23**) was a good acyl acceptor, yielding 83% of coupled product (Table 2). By changing the amide to a methylamide (**24**), the yield of formed peptide (**37**) dropped dramatically (30%). Replacing the amide with a Phe amide (**25**) gave again upon enzymatic coupling a good yield (78%). Statine amide (**26**) itself was not a good substrate, and only a trace amount of the desired product was obtained. Placing statine or an analog (4-amino-3-hydroxy-5-phenylpentanoic acid, AHPPA) between the P2′ and P3′ residue with Gly as the P1′ residue resulted in peptides **40** and **41** in good yields (74% and 70%, respectively).

Table 2. Subtilisin BPN′ Catalyzed Coupling of Peptide Fragments Containing the Statine-Type Isostere

acyldonor	acylacceptor[a]	product[b]	Yield
Z-Phe-OBn	 (**23**)	 (**29**)[c]	83%
Z-Phe-OBn	 (**24**)	 (**30**)[c]	30%
Z-Phe-OBn	 (**25**)	 (**31**)[c]	78%
Z-Phe-OBn	 (**26**)	 (**32**)	trace
Z-Phe-OBn	 (**27**)	 (**33**)	74%
Z-Phe-OBn	 (**28**)	 (**34**)	70%

[a] The isosteres (**23**−**25**) were obtained by coupling of NH_3, $MeNH_2$, or $PheNH_2$ to vinylacetic acid, followed by epoxidation, azide opening, and reduction. Statine and AHPPA derivatives (**26**−**28**) were synthesized by isopropylidene protection of Boc-Statine and Boc-AHPPA, followed by standard peptide chemistry. [b] See Table 1.

The hydroxyketo and diketo isosteres have been applied with success in the development of HIV and FIV protease inhibitors.[13] The hydroxyketoisostere of Gly (**35**) placed between P1′ and P2′ residue gave upon coupling to Z-Phe-OBn a reasonable yield of **42** (46%), but the same isostere of Phe, alias norstatine (**36**) did not give any of the desired product (**43**) (Table 3). However, once this isostere was placed between the P2′ and P3′ residue, with Gly as P1′ residue, a moderate yield of the peptide (**44**) was obtained (36%). The hydroxyethylamine isostere, one of the most frequently used isosteres for the development of HIV protease inhibitors,[14] is an interesting nucleophile for enzymatic coupling, especially due to the presence of the various functionalities. The isostere mimicking Gly-Phe (**38**) gave a reasonable yield (Table 3), which was lowered when the Phe at P2′ was replaced with the unfavorable Pro (**39**). The yield dropped to 0% when the isostere of Gly was replaced by the isostere of Phe (**40**), again reflecting the preference of BPN′ for Gly over Phe as P1′ residue. However, the same trend as described previously was observed. By positioning this unit as P2′-P3′ with a Gly as the P1′ residue,

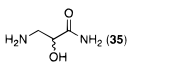
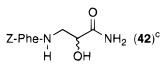
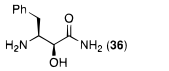
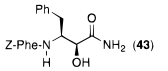
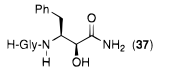
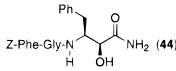
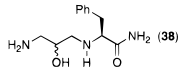
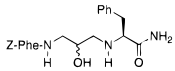
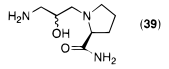
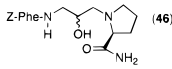
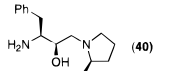
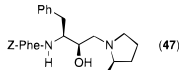
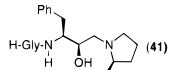
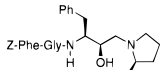
(11) *E.g.*: (a) Umezawa, H.; Aoyagi, T.; Morishima, H.; Matsuzaki, M.; Hamada, M.; Takeuchi, T. *J. Antibiotics* **1970**, *23*, 259−262. (b) Boger, J.; Lohr, N. S.; Ulm, E. H.; Poe, M.; Blaine, E. H.; Fanelli, G. M.; Lin, T.-Y.; Payne, L. S.; Schorn, T. W.; LaMont, B. I.; Vassil, T. C.; Stabilito, I. I.; Veber, D. F.; Rich, D. H.; Bopari, A. S. *Nature* **1983**, *303*, 81−84 (c) Boger, J.; Payne, L. S.; Perlow, D. S.; Lohr, N. S.; Poe, M.; Blaine, E.H.; Ulm, E. H.; Schorn, T. W.; LaMont, B. I.; Lin, T.-Y.; Kawai, M.; Rich, D. H.; Veber, D. F. *J. Med. Chem.* **1985**, *28*, 1779−1790.

(12) *E.g.*: (a) Dreyer, G. B.; Metcalf, B. W.; Tomaszek, Jr., T. A.; Carr, T. J.; Chandler, A. C. III, Hyland, L.; Fakhoury, S. A.; Magaard, V. W.; Moore, M. L.; Strickler, J. E.; Debouck, C.; Meek, T. D. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 9752−9756. (b) Sakurai, M.; Sugano, M.; Handa, H.; Komai, T.; Yagi, R.; Nishigaki, T.; Yabe, Y. *Chem. Pharm. Bull.* **1993**, *41*, 1369−1377. (c) Scholz, D.; Billich, A.; Charpiot, B.; Ettmayer, P.; Lehr, P.; Rosenwirth, B.; Schreiner, E.; Gstach, H. *J. Med. Chem.* **1994**, *37*, 3079−3089. (d) Hui, K. Y.; Hermann, R. B.; Mannetta, J. V.; Gygi, T.; Angleton, E.L. *FEBS Lett.* **1993**, *327*, 355−360. (d) Fehrentz, J. A.; Chromier, B.; Bignon, E.; Venaud, S.; Chermann, J.C.; Nisato, D. *Biochem. Biophys. Res. Commun.* **1992**, *188*, 873−878.

(13) (a) Munoz, B.; Giam, C.-Z.; Wong, C.-H. *Bioorg. Med. Chem.* **1994**, *2*, 1085−1090. (b) Slee, D. H.; Laslo, K. L.; Elder, J. H.; Ollmann, I. R.; Gustchina, A.; Kervinen, J.; Zdanov, A.; Wlodawer, A.; Wong, C.-H. *J. Am. Chem. Soc.* **1995**, *117*, 11867−11878. (c) Mimoto, T.; Imai, J.; Tanaka, S.; Hattori, N.; Takahashi, O.; Kisanuki, S.; Nagano, Y.; Shintani, M.; Hayashi, H.; Sakikawa, H.; Akaji, K.; Kiso, Y. *Chem. Pharm. Bull.* **1991**, *39*, 2465−2467.

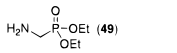
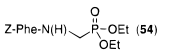
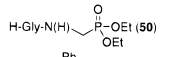
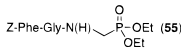
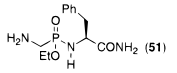
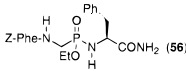
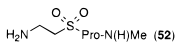
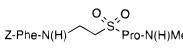
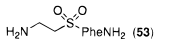
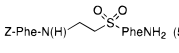
(14) See, *e.g.*: (a) Rich, D. H.; Green, J.; Toth, M. V.; Marshall, G. R.; Kent, S. B. H. *J. Med. Chem.* **1990**, *33*, 1285−1288. (b) Rich, D. H.; Sun, C.-Q.; Vara Prasad, J. V. N.; Pathiasseril, A.; Toth, M. V.; Marshall, G. R.; Clare, M.; Mueller, R. A.; Houseman, K. *J. Med. Chem.* **1991**, *34*, 1222−1225. (c) Alewood, P. F.; Brinkworth, R. I.; Dancer, R. J.; Garnham, B.; Jones, A.; Kent, S. B. H. *Tetrahedron Lett.* **1992**, *33*, 977−980. (d) Krohn, A.; Redshaw, S.; Ritchie, J. C.; Graves, B. J.; Hatada, M. H. *J. Med. Chem.* **1991**, *34*, 3340−3342.

Table 3. Subtilisin BPN′ Catalyzed Coupling of Peptide Fragments Containing Peptide Bond Isosteres and Peptide Mimetics

acyldonor	acylacceptor[a]	product[b]	Yield
Z-Phe-OBn	 (**35**)	 (**42**)[c]	46%
Z-Phe-OBn	 (**36**)	 (**43**)	trace
Z-Phe-OBn	 (**37**)	 (**44**)	36%
Z-Phe-OBn	 (**38**)	 (**45**)[c]	43%
Z-Phe-OBn	 (**39**)	 (**46**)[c]	21%
Z-Phe-OBn	 (**40**)	 (**47**)	trace
Z-Phe-OBn	 (**41**)	 (**48**)	47%

[a] The hydroxyketoisostere (**35**) was prepared by addition of TMSCN to *N*-benyzloxycarbonyl glycinaldehyde, followed by hydrolysis under basic conditions and deprotection. The hydroxyketoisosteres (**36**) and (**37**) were prepared by aminolysis of (2*R*,3*S*)-*N*-(benzyloxycarbonyl)(3-amino-2-hydroxy-4-phenylbutanoic acid) ethyl ester,[13] followed by standard peptide chemistry. The hydroxyethyleneamine isosteres (**38**) and (**39**) were obtained by opening of the corresponding Z protected epoxide with an amino acid followed by deprotection, whereas the hydroxyethyleneamine isosteres (**40**) and (**41**) were synthesized by opening of the epoxide derived from Z-Phe-OH with proline amide followed by standard peptide chemistry. [b] See Table 1. [c] No enantioselectivity was observed; the peptide was obtained as a diastereomeric mixture in a ratio of approximately 1/1.

Table 4. Subtilisin BPN′ Catalyzed Coupling of Peptide Fragments Containing Peptide Bond Isosteres

acyldonor	acylacceptor[a]	product[b]	Yield
Z-Phe-OBn	 (**49**)	 (**54**)	54%
Z-Phe-OBn	 (**50**)	 (**55**)	87%
Z-Phe-OBn	 (**51**)	 (**56**)	0%
Z-Phe-OBn	 (**52**)	 (**57**)	19%
Z-Phe-OBn	 (**53**)	 (**58**)	29%

[a] The phosphonates (**49**) and (**50**) and phosphonamidate (**51**) were obtained starting from diethyl(phthaloylaminomethyl)phosphonate according to the procedures described by Jacobsen and Bartlett.[15] The taurine derivatives (**52**) and (**53**) were synthesized using the methodology described previously.[20] [b] See Table 1.

the yield increased considerably (47%). Interestingly, both isomers of the isostere substrates are accepted by the enzyme.

The dimethyl ester of amino methane phosphonic acid (**49**), an analog of Gly, can be coupled to the acyl donor in 54% yield without any hydrolysis of the phosphate ester (Table 4). The results were even better when this phosphonic ester containing mimetic was placed as the P2′ residue with Gly as the P1′ residue (87%). The phosphonamidate is one of the earliest described transition-state isosteres of the hydrolysis of an amide bond[15] and frequently applied to the development of protease inhibitors[16] and catalytic antibodies.[17] An attempt to couple phosphonamidate (**51**) enzymatically failed, with neither aminolysis nor hydrolysis of the benzyl ester taking place. It is not clear why this coupling failed, since glycine and phenylalanine (the residues mimicked by this isostere) are preferred in the S1′ and S2′ sites, respectively. The fact that no hydrolysis is observed indicates that enzymatic activity has been inhibited, so perhaps the phosphonamidate is lying at the position of the scissile bond, despite the fact that glycine is not favorable in the S1 site. In close resemblance to the phosphonamidate is the sulfonamide transition-state isostere. Taurine[18] and taurine peptides contain a sulfonamide isostere and have often been employed as potential pharmacophores for the development of protease inhibitors.[19,20] Coupling of two taurine containing peptides (**52**) and (**53**) to the acyl donor gave the desired products (**57** and **58** respectively) in moderate yields (Table 4).

Simple peptide conjugates, such as *p*-nitroanilides (*p*NA), have been used extensively as substrates for proteases in chromogenic assays, though synthesis of these base-sensitive substrates is not trivial. A previous enzymatic approach toward the synthesis of a *p*NA derivative of a laminin fragment[21] prompted us to investigate small *p*NA fragments (**66**) and (**67**) as acyl acceptors (Table 5). Good yields of the corresponding products **71** and **72** (64% and 69%, respectively) were obtained.

Protein conjugates containing glycosyl- and phosphoamino acids are involved in various specific biological processes, and in order to elucidate these processes, continued effort has been devoted to find synthetic routes for these types of compounds. Only a limited number of enzymatic methods have been described, and coupling of peptide conjugates catalyzed by subtilisin BPN′ has only been reported for *N*- and *O*-glycosylamino acids and glycopeptide fragments.[22] Tyrosine phosphorylation appears to be a major mechanism of cellular signal transduction,[23] and since there is still a lack of synthetic approaches to these phosphoconjugates, it is of considerable interest to develop enzymatic routes for them. Enzymatic phosphorylation of tyrosine-containing peptides was achieved by use of glutamine synthetase adenylyltransferase.[24] We exploited an alternative method to enzymatically couple phosphorylated Tyr containing peptides either as part of an acyl acceptor to Z-Phe-OBn or as part of an acyl donor to glycinamide (**1**). The use of the diethyl ester of phosphotyrosine amide (**69**) as a nucleophile gave a moderate yield (26%) of dipeptide upon coupling. The ethyl ester was chosen as the protecting group of the phosphate (**69** and **70**) since it is small enough to fit in the hydrophobic S2′ subsite and will not be hydrolyzed by subtilisin (*vide infra*). Surprisingly, placing the Tyr dieth-

(15) Jacobsen, N. E.; Bartlett, P. A. *J. Am. Chem. Soc.* **1981**, *103*, 654−657

(16) (a) Bartlett, P. A.; Marlowe, C. K. *Science* **1987**, *235*, 569−571. (b) McLeod, D. A.; Brinkworth, R. I.; Ashley, J. A.; Janda, K. D.; Wirsching, P. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 653−658. (c) Camp, N. P.; Hawkins, P. C. D.; Hitchcock, P. B.; Gani, D. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1047−1052.

(17) Janda, K. D.; Schloeder, D.; Benkovic, S. J.; Lerner, R. A. *Science* **1988**, *241*, 1188−1191.

(18) Taurine (Tau) is the abbreviation for aminoethane sulfonic acid.

(19) (a) Sturman, J. A. *Physiol. Rev.* **1993**, *73*, 119−148. (b) Sebestvén, F.; Furka, A.; Feuer, L.; Gulvas, J.; Szokan, G. *Int. J. Peptide Protein Res.* **1980**, *16*, 245−247.

(20) (a) Moree, W. J.; van Gent, L. C.; van der Marel, G. A.; Liskamp, R. M. J. *Tetrahedron* **1993**, *49*, 1133−1150. (b) Moree, W. J.; van der Marel, G. A.; Liskamp, R. M. J. *J. Org. Chem.* **1995**, *60*, 5157−5169.

(21) Ternt'eva, E. Y.; Voyushina, T. L.; Stepanov, V. M. *Bioorg. Med. Chem.* **1995**, *5*, 2523−2526.

(22) Wong, C.-H.; Schuster, M.; Wang, P.; Sears, P. *J. Am. Chem. Soc.* **1993**, *115*, 5893−5901.

(23) (a) Boyer, P. D.; Krebs, E. G. *The Enzymes*; 1986; Vol. XVII, pp 192−237. (b) Sugimoto, Y.; Whitman, M.; Cantley, L. C.; Erikson, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 2117−2121.

(24) Gibson, B. W.; Hines, W.; Yu, Z.; Kenyon, G. L.; McNemar, L.; Villafranca, J. J. *J. Am. Chem. Soc.* **1990**, *112*, 8523−8528.

Table 5. Subtilisin BPN′ Catalyzed Coupling of Peptide Fragments Containing Peptide Conjugates

acyl donor	acyl acceptor[a]	product[b]	yield (%)
Z-Phe-OBn	HCl.H-Gly-*p*NA (**66**)	Z-Phe-Gly-*p*NA (**71**)	64
Z-Phe-OBn	HCl.H-Gly-Phe-*p*NA (**67**)	Z-Phe-Gly-Phe-*p*NA (**72**)	69
Z-Phe-OBn	TFA. H-Gly-NHNHZ (**68**)	Z-Phe-Gly-NHNHZ (**73**)	60
Z-Phe-OBn	H-Tyr(PO_3Et_2)NH_2 (**69**)	Z-Phe-Tyr(PO_3Et_2)NH_2 (**74**)	26
Z-Phe-OBn	H-Gly-Tyr(PO_3Et_2)NH_2 (**70**)	Z-Phe-Gly-Tyr(PO_3Et_2)NH_2 (**75**)	28
Z-Tyr(PO_3H_2)ONb (**59**)	HCl.H-Gly-NH_2	Z-Tyr(PO_3H_2)-Gly-NH_2 (**76**)	0
Z-Tyr(PO_3Me_2)OMe (**60**)	HCl.H-Gly-NH_2	Z-Tyr(PO_3Me_2)-Gly-NH_2 (**77**)	26
Z-Tyr(PO_3Et_2)OMe (**61**)	HCl.H-Gly-NH_2	Z-Tyr(PO_3Et_2)-Gly-NH_2 (**78**)	79
Z-Tyr(PO_3Bn_2)ONb (**62**)	HCl.H-Gly-NH_2	Z-Tyr(PO_3Bn_2)-Gly-NH_2 (**79**)	0
Z-Tyr(PO_3tBu_2)OMe (**63**)	HCl.H-Gly-NH_2	Z-Tyr(PO_3tBu_2)-Gly-NH_2 (**80**)	0
Z-Tyr(PO_3tBu_2)-Ala-OMe (**64**)	HCl.H-Gly-NH_2	Z-Tyr(PO_3tBu_2)-Ala-Gly-NH_2 (**81**)	17
Z-Tyr(PO_3Et_2)-Ala-OMe (**65**)	HCl.H-Gly-NH_2	Z-Tyr(PO_3Et_2)-Ala-Gly-NH_2 (**82**)	40

[a] The *p*-nitroanilide containing fragments (**66**) and (**67**) were prepared using standard peptide chemistry; the hydrazine (**68**) was purchased from Bachem. The phosphate methyl esters and phosphate ethyl esters of the tyrosine derivatives (**69**, **70** and **60**, **61**, and **65**) were obtained by phosphorylation using the phosphite/iodine method,[35] whereas the *tert*-butyl and benzyl esters (**62**, **63**, and **64**) and the phosphate **59** were prepared using phosphonamidite followed by oxidation.[36] [b] See Table 1.

ylphosphate ester in the P2′ position, hardly improved the yield (28%) (Table 5).

Since Tyr is favorable in the P1 position, we envisioned that phosphorylated Tyr might be accepted as an acyl donor. Various phosphate esters (as shown in Table 5) were tested for coupling to glycinamide (**1**). The benzyl ester (**62**) and *tert*-butyl ester (**63**) were not accepted as substrates. These groups are probably too large for the hydrophobic pocket. The unprotected phosphate (**59**) was not a substrate either. The charge on the phosphate most likely causes unfavorable interactions with the Glu 156 residue in the S1 subsite. The methyl ester (**60**) and the ethyl ester (**61**) however, were very good substrates, giving rise to coupling yields of 26% and 79%, respectively. The low yield of the methyl ester (**77**) was due to hydrolysis of the phosphate methyl ester, which under the coupling conditions did not occur for the ethyl ester. Finally, when we placed the *tert*-butyl protected phosphorylated Tyr in the P2 position (**64**), some peptide formation took place (17%), indicating that the preference of the P2 position is more flexible. The results were slightly better with the phosphate ethyl ester (**65**, 40%), although at this position the hydrolysis of the methyl ester was very fast.

Conclusions

The results of this study, based on a limited number of noncoded amino acids, peptide isosteres, and peptide conjugates chosen as potential substrates for subtilisin BPN′ indicate that the enzyme accepts numerous unnatural units both as acyl donors and acyl acceptors. Preference was observed for small amine fragments closely related to Gly, positioned as the P1′ residue. Isosteres mimicking the Gly-XX bond were generally well accepted as the P1′-P2′ residues, giving moderate to good yields of coupled product, depending on XX. Isosteres mimicking the Phe-XX bond as the P1′-P2′ residues gave only trace amounts of products. The yields of the latter considerably improved, once placed as the P2′-P3′ residues with a Gly as P1′ residue. Furthermore it was found that phosphorylated Tyr was a good substrate both as the P1 and P2 residue if the phosphate was protected as a diethyl ester.

To gain further insights into the specificity of subtilisin BPN′ in order to provide a general guideline for the design of substrates, we consider the results of this study together with the great number of specificity studies reported previously. Figure 2 illustrates the active site complexed with potent inhibitor (chymotrypsin inhibitor 2) which interacts with the S4-S4′ region of the enzyme in a manner similar to the binding

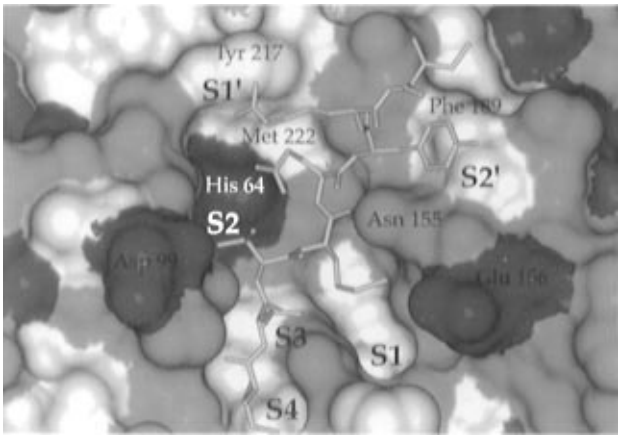


Figure 2. Connolly surface of the active site of subtilisin BPN′ containing a portion of the chymotrypsin inhibitor 2 and showing the positions of four subtsites (S1, S2, S1′, and S2′). Amino acids are color-coded by type: acids, red; bases, blue; polar residues, green; and hydrophobic residues, white. The chymotrypsin inhibitor is shown in yellow. The black arrow shows the position of the amide bond corresponding to the scissile bond of a subtilisin substrate.

of the substrate.[25] Only a portion of the inhibitor is shown, in yellow. Both steric and electrostatic factors are indicated to provide a general picture about how substrates are positioned in the active site. Figure 3 indicates the active site binding model based on the results of this and previous studies,[26] and Table 6 tabulates the trends compiled from the available kinetic data regarding the subsite preferences of subtilisin BPN′. When planning a kinetically controlled synthesis in large concentrations of cosolvent, however, one should treat the data in Table 6 with care. The trends in the table, other than those directly related to this work, were taken from studies performed in buffer (with no cosolvent), since these were most amenable to comparison: actual kinetic constants were available and typically were obtained under similar conditions of ionic strength and pH. The crystal structure of subtilisin 8397/Lys256→Tyr (a stabilized BPN′ variant, which shows only minor changes in structure when compared to BPN′ [27a]) in 50% DMF[27b] demonstrates that addition of solvent to 50% has little change on the structure of the subsites; one should bear in mind, though, that the addition of cosolvent may have some effects on substrate partitioning

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(26) The model is an extension of the model proposed by J. Bryan Jones et al. outlined in the following: Bonneau, P. R.; Graycar, T. P.; Estell, D. A.; Jones, J. B. *J. Am. Chem. Soc.* **1991**, *113*, 1026.
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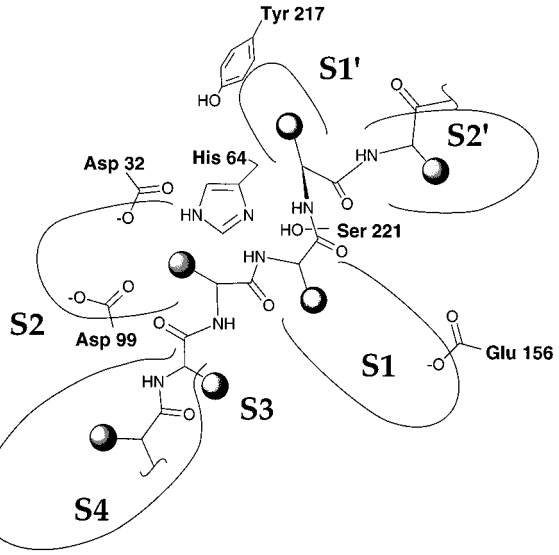

Figure 3. Schematic drawing of the general features of the subtilisin active site. The large S1 pocket is primarily hydrophobic but contains a carboxylic acid (Glu 156) at the bottom, which explains its affinity for basic and polar residues but its poor affinity for acids, including unprotected phosphotyrosine. The S2 site is a somewhat smaller cleft lying behind Asp 99 (the red bulge in the lower left corner of Figure 1). It is lined with hydrophobic and acidic residues, explaining the poorness of subtilisin at cleaving peptides with acids in the P2 position (unprotected phosphotyrosine is not accepted). It accepts proline quite well, presumably since the substrate naturally "kinks" at this site anyway, and though it prefers small hydrophobes, it can also accept very large residues, such as butyl-protected phosphotyrosine, to a certain extent. The S3 site is not much of a pocket; the substrate side chain projects out of the active site. As a result, the specificity is broad. The narrow "neck" of the subsite probably precludes bulky groups at the β position and may explain the L-stereoselectivity at this site. The S4 site is an extremely large hydrophobic pocket. The S1′ site is narrow and hydrophobic, backed by Met222, and capped by Tyr217. Small residues are preferred, and α-branched amino acids are accepted poorly. Isosteres are accepted in this site as long as the mimicked residue is small. (Note that in Figures 1 and 2, the P3′ residue of the chymotrypsin inhibitor 2 has bent around to sit primarily in the S1′ pocket, and the P1′ residue, a glutamic acid, has bent out of the plane of the page.) There is strong stereoselectivity at this site, and α-branched amino acids are poorly acccepted, as are bulky side chains. Although subtilisin can acetylate sugars and nucleotides, it is not clear how they are binding. The S1′ site clearly cannot hold them entirely. The S2′ site is not so much a cleft as a hydrophobic surface, flanked by phenylalanine. It prefers large hydrophobes and can accept a variety of isosteres, as long as the P1′ residue is small (esp. glycine).

into the active site. In general, the longer the peptide chain that is used as a substrate, the better. Addition of an extra glycine to AcGlyTyrOMe to form the tripeptide, AcGlyGlyTyrOMe, for example, improves the rate of hydrolysis by 50−100-fold,[28] and similar results have been obtained with other amino acids at nearly every position.[29,30] The specificity of subtilisin can often be broadened by using activated esters, such as *p*-nitrophenyl esters, which do not appear to require both base and then acid catalysis (only base catalysis) and thus can have "floppier" transition states.[31] Finally, substrates are accepted best when protected both at the N- and C-termini. This is true from the S4 site to at least the S3′[32] site.

In general, the results suggest that subtilisin BPN′ is useful for the synthesis of peptide isosteres and analogs if the non-natural components are properly positioned. Further improvement perhaps can be achieved with the use of subtilisin variants designed to prevent hydrolysis, to perform reactions in DMF, or to have altered substrate specificity.

Experimental Section

General Methods. Subtilisin BPN′ was obtained from Sigma (Protease Type XXVII, 7.8 units/mg solid). All protected amino acids as well as (3*S*,4*S*)-Boc-4-amino-3-hydroxy-6-methylheptanoic acid (Boc-Statine or Boc-AHMHA) were obtained from Bachem. (3*S*,4*S*)-4-Amino-3-hydroxy-5-phenylpentanoic acid (AHPPA) was purchased form Novabiochem. Isobutylchloroformate was distilled under Ar atmosphere. ^{1}H NMR spectra were recorded on a Bruker AC 250 (250 MHz) or AMX 400 (400 MHz) spectrometer, and chemical shifts are given in ppm (δ) relative to TMS as internal standard. ^{13}C NMR spectra were measured with a Bruker AC 250 (62 MHz) or AMX 400 (100 MHz) spectrometer and chemical shifts are given relative to $CDCl_3$ or MeOD as internal standard. ^{31}P NMR spectra were obtained with a Brucker AMX 400 spectrometer using 85% H_3PO_4 as an external standard.

Thin layer chromatography analysis was performed on precoated Merck Silica gel (60 F-254) plates (0.25 mm); the solvent system used CH_2Cl_2/MeOH (9/1, v/v) unless indicated otherwise. Spots were visualized with UV light or ninhydrin (after treatment with HCl). Column chromatography was carried out on Merck Kieselgel 60 (230−400 Mesh, ASTM). High resolution mass spectra (HRMS) were recorded on a VG ZAB-ZSE instrument under fast atom bombardment (FAB) conditions.

General Procedure for Enzymatic Peptide Coupling. To a solution of Z-Phe-OBn (40 mg, 0.10 mmol) in DMF (0.4 mL) and water (0.15 mL), a free amine (0.30 mmol) or the hydrochloride of an amine (0.30 mmol) in combination with Et_3N (0.30 mmol) were added, followed by subtilisin BPN′ (5 mg). The mixture was stirred at room temperature until the benzyl ester disappeared (as indicated by TLC). The mixture was concentrated *in vacuo*, taken up in EtOAC, and washed with 1 N $KHSO_4$, 1 M Na_2CO_3 (3×), and brine. The organic layers were dried ($MgSO_4$), filtered, and concentrated *in vacuo*. Silica gel column chromatography afforded the coupling product.

General Procedure for Aminolysis of a Methyl Ester. The methyl ester was dissolved in anhydrous MeOH and cooled to 0 °C. $NH_{3(g)}$ was bubbled through until the solution was saturated. The mixture was stirred at room temperature until TLC indicated disappearance of the methyl ester.

General Procedure for Removal of the Z Group via Hydrogenation. The Z protected compound was dissolved in a mixture of *t*BuOH/water (4/1, v/v) and a catalytic amount of 10% Pd on C was added. The mixture was stirred at 1 atm of H_2 until the Z group was cleaved, filtered over Celite, and concentrated *in vacuo* to afford the free amine.

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Table 6. Subsite Preferences of Subtilisin BPN′

subsite	preference
S4	•broad specificity; generally prefers hydrophobic residues[a]
	•has a strong preference for L- vs D-amino acids[b,c]
	•prefers Bz over CBz[d]
S3	•broad range of accepted residues[a]
	•D- and L-leucine not differentiated very well[d] but L-alanine preferred over D-Ala[b,e]
	•order of preference: Phe > Ala > Gly > D-Ala[b,c]
	•Asn(β-GlcNAc) and even some disaccharide moieties accepted[f]
S2	•accepts ethyl-protected phosphotyrosine and even butyl-protected but less efficiently (Table 5)
	•prefers small hydrophobes
	•L-Ala strongly preferred over D-Ala[g]
	•acetyl > CBz > benzoyl[e,h]
	•Ala > Pro, Val > Leu > Gly > His, Tyr > D-Ala[c,d,e]
	•Ala > Pro ≫ Lys > Arg > Asp[i]
	•Gly > Leu with Phe in P1 site[j]
	•both unprotected and peracetylated, O- and N-linked glycosyl amino acids accepted[f]
S1	•accepts ethyl-, methyl-protected phosphotyrosine but not butyl- or benzyl-(Table 5)
	•prefers large hydrophobes with polar or positively charged group at the end[k]
	•Small amino acids such as glycine are much poorer than the aromatics. One should avoid Gly, Pro, Thr, Val, Ile.[a]
	•Very large hydrophobic residues can be accepted, including dansylated aminobenzene derivatives,[l] γ-diphenyl residues,[m] and various polycyclic aromatics.[n]
	•Tyr > Phe, Trp, Met > Lys > Arg[i,j,o]
	•Proline not accepted,[a] but other α-branched (locked) substrates are accepted.[m]
	•His > Glu[p]
	•vinyl acetate accepted; used for acetylating a variety of substrates, including 6′-hydroxyls of sugars[q] and 5′-hydroxyls of nucleosides[r]
	•can accept peracetylated or nonprotected O-linked xylosyl amino acids but not Asn(β-GlcNAc)[f]
S1′	•A variety of peptide isosteres are accepted (statine-type, hydroxyketo, hydroxyethylamine, phosphonate, and sulfonamide) if the residue mimicked is small (glycine), not large and hydrophobic (leucine, phenylalanine).
	•very small, narrow, restricted site; avoid α-branching (Table 1), though paradoxically will accept (ethyl) protected phosphotyrosine to a moderate degree (Table 5)
	•avoid Ile, Pro, Asp, Glu[a]
	•Gly, Ala ≫ Leu ≫ Pro[s]
	•Gly > Ser, Lys, Ala > Arg > Gln, Thr, Val, Met, Asn > His > Ile, Trp > Glu > Asp, Leu ≫ Pro[u]
	•can accept D-Leu, D-Arg to some extent[v]
	•does not accept glycosyl amino acids[f]
S2′	•methylated N not favorable between P1′ and P2′ positions (Table 2, **24**)
	•accepts (ethyl) protected phosphotyrosine to a moderate extent (Table 5)
	•most peptide isosteres accepted, if residue in P1′ position is small (glycine)
	•Leu > Gly, D-Leu > Pro[s]
	•poor differentiation of D-Ala from L-Ala[e]
	•Phe > Gly, Ala > D-Ala[c]
	•likes large hydrophobics; avoid Pro, Asp, Glu, Gly[a]
	•deprotected N- and O-linked glycosyl amino acids accepted[f]
S3′	•minimal differentiation of D- and L-Ala[c]

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General Procedure for EDCI/HOBt Peptide Coupling. The carboxylic acid (1 equiv) and the amine (1.05 equiv) were dissolved in dry CH_2Cl_2 (if necessary in combination with DMF), and HOBt (1.05 equiv) was added. The mixture was cooled to 0 °C, EDCI (1.1 equiv) was added, and the apparent pH (as indicated by wet pH paper) of the mixture was adjusted to 8 with NMM. After stirring for 0.5 h to 1 h at 0 °C, stirring was continued at room temperature until starting materials were gone. The mixture was diluted with CH_2Cl_2, washed with 1 N $KHSO_4$ (1×), 1 M Na_2CO_3 (1−3×), brine, dried (MgSO4), filtered, and concentrated *in vacuo*. The coupling product was purified by silica gel column chromatography.

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Supporting Information Available: ^{1}H NMR spectra for compounds including the substrates and the products and detailed experimental on the synthesis of the individual compounds (49 pages). See any current masthead page for ordering and Internet access instructions.

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