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Cross-Linked Crystals of Subtilisin: Versatile Catalyst for Organic Synthesis

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Cross-linked enzyme crystals (CLECs) of subtilisin exhibit excellent activity in aqueous and various organic solvents. This catalyst is more stable than the native enzyme in both aqueous and mixed aqueous/organic solutions. Subtilisin-CLEC was shown to be a versatile catalyst. It was used for the syntheses of peptides and peptidomimetics, mild hydrolysis of amino acid and peptide amides, enantio- and regioselective reactions, and transesterifications.

Introduction

The protease subtilisin is broadly used in organic synthesis for enantio- and regiospecific reactions, amide bond syntheses, and hydrolyses in both aqueous and organic solvents.[1] Two major problems—low stability in water (mostly due to autolysis) and water-miscible organics[2] and low activity in neat organic solvents—limit the synthetic utility of this catalyst in organic chemistry. Autodigestion not only reduces the life of the catalyst, but it also makes separation and purification of reaction products time consuming and expensive. This problem is especially serious in the synthesis of peptides and pharmaceuticals, where the requirements for product purity are very high. Moreover, the low activity of subtilisin in neat organic solvents makes various processes too slow and thus economically unfeasible.[3]

Recent work on cross-linked enzyme crystals (CLECs)[4] has demonstrated that lipases[5] and proteases[6] in this form acquire high stability while preserving their activity in high water mixtures.[7] We have also developed a novel drying procedure of CLEC catalysts by washing them with solutions of different surfactants in organic solvents.[8] This procedure yields dry CLEC powders that are highly active in neat organic solvents.[9] Here, we report the use of another catalyst, subtilisin-CLEC, in the synthesis of peptides and peptidomimetics, amide hydrolysis, and enantioselective and regioselective transformations.

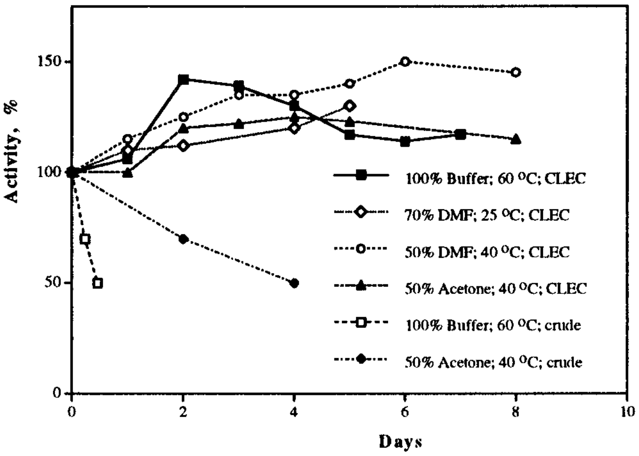

Figure 1. Stabilities of subtilisin-CLEC and crude alcalase at 60 °C and in high concentration of organic solvents. Activity was measured by TAME assay at 25 °C.

Results and Discussion

Stability. Subtilisin-CLEC demonstrated excellent stability in both aqueous and mixed aqueous/organic solutions. The catalyst retained full activity after 7 days incubation at 60 °C and in high concentration of organic solvents, including 50% acetone, 50% DMF, and 70% DMF (Figure 1). Crude subtilisin (alcalase), on the other hand, lost half of its activity after 11 h incubation at 60 °C and after 4 days incubation in 50% acetone at 40 °C. Subtilisin-CLECs are also stable against autolysis; after 5 days incubation at 40 °C, no degraded peptide fragments were detected based on SDS−PAGE analysis (data not shown). The lack of free protein in the reaction medium significantly facilitates the product purification.

Activity in Organic Solvents

Surfactant-treated dry subtilisin-CLEC shows high activity in a wide range of organic solvents. The catalytic activity in both hydrophobic (isooctane, toluene, *tert*-butyl methyl ether) and polar organic solvents (alcohols, acetone, THF, DMF, and pyridine) (Figure 2) was compared. As for enzymes in general,[10] CLECs required a small amount of water when used in neat organics, especially in polar solvents such as acetone and DMF (Figure 2). The complete absence of water resulted in

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(1) *Enzyme Catalysis in Organic Synthesis*; Drauz, K., Waldmann, H., Eds.; VCH Publishers: New York, 1995. Sears, P.; Schuster, M.; Wang, P.; Witte, K.; Wong, C. H. *J. Am. Chem. Soc.* **1994**, *116*, 6521.

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

(3) Schmitke, J. L.; Wescott, C. R.; Klibanov, A. M. *J. Am. Chem. Soc.* **1996**, *118*, 3360−3365. Khmelnitsky, Y. L.; Welch, S. H.; Clark, D. S.; Dordick, J. S. *J. Am. Chem. Soc.* **1994**, *116*, 2647−2648.

(4) St. Clair, N. L.; Navia, M. A. *J. Am. Chem. Soc.* **1992**, *114*, 7314−7316.

(5) Lalonde, J. J.; Govardhan, C. P.; Khalaf, N. K.; Martinez, O. G.; Visuri, K. J.; Margolin, A. M. *J. Am. Chem. Soc.* **1995**, *117*, 6845−6852.

(6) Persichetti, R. A.; St. Clair, N. L.; Griffith, J. P.; Navia, M. A.; Margolin, A. L. *J. Am. Chem. Soc.* **1995**, *117*, 2732−2737.

(7) For the recent review, see: Margolin, A. L. *Trends Biotechnol.* **1996**, *14*, 223.

(8) Khalaf, N.; Govardhan, C.; Lalonde, J. J.; Persichetti, R. A.; Wang, Y. F.; Margolin, A. L. *J. Am. Chem. Soc.* **1996**, *118*, 5494−5495.

(9) Persichetti, R. A.; Lalonde, J. J.; Govardhan, C.; Khalaf, N.; Margolin, A. L. *Tetrahedron Lett.*, in press.

(10) (a) Halling, P. J. *Enzyme Microb. Technol.* **1994**, *16*, 178. (b) Klibanov, A. M. *TIBS* **1989**, *14*, 141−144.

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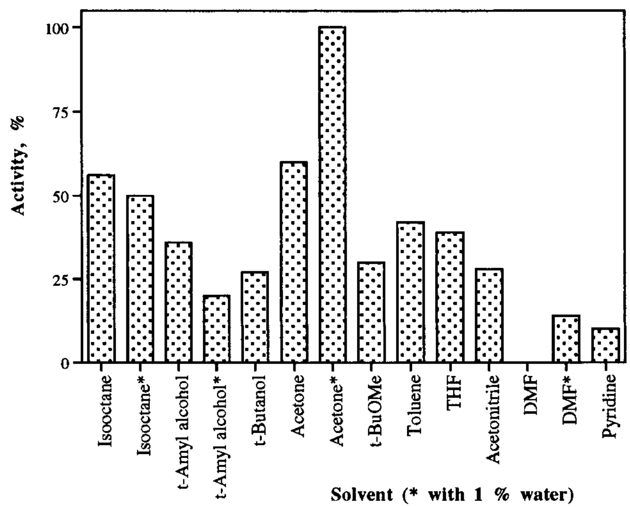

Figure 2. Relatve activity of subtilisin-CLEC in various organic solvents for the transesterification of *N*-Ac-L-Phe OEt with 1-propanol.

Table 1. Solvent Effect on the Subtilisin-CLEC Catalyzed Coupling of *N*-Cbz-Phe-OBzl with L-Leucinamide[a]

solvent	dipeptide (%)	unreacted substrate (%)	byproduct (% yield)
2-methyl-2-propanol	64	19	*N*-cbz-Phe-OH (9)
ethyl acetate	57	34	*N*-cbz-Phe-OEt (8)
3-methyl-3-pentanol	55	29	*N*-cbz-Phe-OH (16)
DMF		100	
pyridine	40	32	*N*-cbz-Phe-OH (27)
acetone	<20		many byproducts
ethyl alcohol			*N*-cbz-Phe-OEt (90)
isopropyl alcohol	16		*N*-cbz-Phe-O^{i}Pr (84)
tetrahydrofuran	40	60	
acetonitrile	84	16	

[a] The results were based on 2 h reaction at 40 °C.

loss of catalyst selectivity and, ultimately, in the complete loss of activity in solvents such as DMF. The addition of water to nonpolar organic solvents such as isooctane, toluene, or methyl *tert*-butyl ether, however, did not increase CLEC catalytic activity.

Synthetic Applications

(a) Synthesis of Peptides and Peptidomimetics. The use of polar, water-miscible organic solvents is extremely useful in enzyme-catalyzed peptide synthesis, since it improves the substrate solubility and shifts the equilibrium toward product formation.[11] Ethyl alcohol and 2-methyl-2-propanol were widely used as solvents for subtilisin-catalyzed peptide synthesis.[12] In order to find the best solvent, we screened several organic solvents including acetonitrile, 2-methyl-2-propanol, 3-methyl-3-pentanol, ethyl acetate, THF, pyridine, 2-propanol, ethanol, acetone, and DMF in the coupling of *N*-cbz-L-phenylalanine benzyl ester with L-leucinamide (Table 1). Acetonitrile was found to be the best solvent, in terms of both reaction rates and product yields. Although the use of 2-methyl-2-propanol, 3-methyl-3-pentanol, and pyridine resulted in good reaction rates, the reactions in these solvents were accompanied by the hydrolysis of the acyl donor. Ethyl acetate and tetrahydrofuran gave good product yields, but the reaction rates in these solvents were slow. The major product of the reactions in 2-propanol and ethanol was the corresponding isopropyl and ethyl esters formed from the transesterification of ester substrate with solvents. No reaction occurred when DMF was used as solvent. The water effect on the reaction was also examined. We found that the reaction rate either in acetonitrile or in 2-methyl-2-propanol increased when 1% water was added. However, the yield of product decreased due to the hydrolysis of ester substrate. The ester substrate was completely hydrolyzed to its corresponding acid when 5% of water was added in the above reactions. Thus, neat acetonitrile was chosen as optimal solvent for the subtilisin-CLEC-catalyzed syntheses of peptides and peptidomimetics.

Subtilisin-CLEC effectively catalyzed the synthesis of peptides in acetonitrile (Table 2). The yield was greater than 90% in most of the reactions. This catalyst accepted both L- and D-amino acid amides as nucleophiles and exhibited similar reaction rates for both enantiomers (Table 2, entries 1, 2, 4, and 5). When amino acid esters that possess a reactive functional group such as *N*-cbz-L-Ser-OMe and *N*-cbz-L-Glu-DiOMe were used as acyl donor, the protection of functional group was not necessary. The α-ester group selectively reacted with nucleophile to give product in high yield (Table 2, entries 13 and 14). The unnatural amino acid ester *N*-Boc-3-(2-naphthyl)alanine methyl ester (*N*-Boc-Nap OMe) was also accepted by this catalyst to give 67% of product while ester substrate was recovered in 28% yield. It is worth mentioning that both yield and reaction rate were substantially reduced when the HCl salt of amino acid amides (plus 1 equiv of triethylamine) was used as nucleophile. When *tert*-butyl esters of amino acids were used as nucleophiles, the reactions were slow and resulted in only moderate yield, presumably due to the steric hindrance originated from the *tert*-butyl group of nucleophile.

Subtilisin-CLEC enantiospecifically catalyzed the synthesis of optically active alkylamides of amino acids and peptides. As we reported before, this catalyst exhibited high enantioselectivity toward L-amino acids and L-amines.[13] The L,L-alkylamide was selectively formed regardless of the optical purity of the substrates. We found that the use of optically pure substrates was not necessary in these coupling reactions. Indeed, optically pure L,L-alkylamide products were obtained via *in situ* resolution when racemic amines **9a** and **10a** (entries 15, 17−19, 21, and 22, Table 2) or even both amines and amino acids (entry 16, Table 2) were used in the racemic form. In the latter case, the diastereomeric excess of product was greater than 98%.

(b) Mild Hydrolysis of Amino Acid and Peptide Amides. The regioselective hydrolysis of the terminal amide of peptide amides requires harsh conditions, is difficult, and usually leads to racemization. We found that subtilisin-CLEC effectively hydrolyzes the amide group of peptide and amino acid amides under mild reaction conditions. This catalyst accepted a broad range of substrates, including different amino acids (Phe, Tyr, Trp), different N-protecting groups (acetyl, Boc, Cbz, and free amine), and different peptides (Leu-Trp, Gly-Phe)

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[11] (a) Homandberg, G. A.; Mattis, J. A.; Laskovski, M., Jr. *Biochemistry* **1978**, *17*, 5220. (b) Margolin, A. L.; Klibanov, A. M. *J. Am. Chem. Soc.* **1987**, *109*, 3802. (c) Barbas, C. F.; Matos, J. R.; West, J. B.; Wong C. H. *J. Am. Chem. Soc.* **1988**, *110*, 5162. (d) Chen, S. T.; Chen, S. Y.; Wang, K. T. *J. Org. Chem.* **1992**, *57*, 6960.

[12] (a) Chen. S.-T.; Chen, S.-Y.; Wang, K.-T. *J. Org. Chem.* **1992**, *57*, 6960. (b) Chen, S.-T.; Chen, S.-Y.; Chen, H.-J.; Huang, H.-C.; Wang, K.-T. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 727. (c) Chen. S.-T.; Wu, S.-Y.; Chen, S.-Y.; Wang, K.-T. *Biotechnol. Lett.* **1993**, *15*, 373.

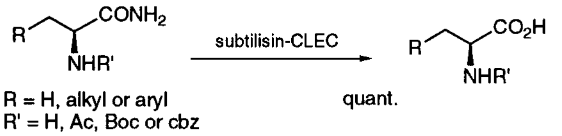
[13] Wang, Y.-F.; Yakovlevsky, K.; Margolin, A. L. *Tetrahedron Lett.* **1996**, *37*, 5317−5320.

Table 2. Use of Subtilisin-CLEC in the Synthesis of Peptides and Peptidomimetics

entry	acyl donor	nucleophile	product	time (h)	yield/de (%)
1	*N*-cbz-L-Phe-OBzl	L-Leu-NH$_2$	*N*-cbz-L-Phe-L-Leu-NH$_2$	29	93
2	*N*-cbz-L-Phe-OBzl	D-Leu-NH$_2$	*N*-cbz-L-Phe-D-Leu-NH$_2$	29	96
3	*N*-Boc-L-Tyr OMe	L-Leu-NH$_2$	*N*-Boc-L-Tyr-L-Leu-NH$_2$	120	90
4	*N*-cbz-L-Phe-OBzl	L-Phe-NH$_2$	*N*-cbz-L-Phe-L-Phe-NH$_2$	23	94
5	*N*-cbz-L-Phe-OBzl	D-Phe-NH$_2$	*N*-cbz-L-Phe-D-Phe-NH$_2$	23	95
6	*N*-Ac-L-Phe-OEt	L-Leu-NH$_2$	*N*-Ac-L-Phe-L-Leu-NH$_2$	29	94
7	*N*-cbz-L-Val-L-Phe-OMe	L-Leu-NH$_2$	*N*-cbz-L-Val-L-Phe-L-Leu-NH$_2$	40	98
8	*N*-cbz-L-Val-L-Phe-OMe	L-Phe-NH$_2$	*N*-cbz-L-Val-L-Phe-L-Phe-NH$_2$	23	92
9	*N*-cbz-L-Phe-OBzl	L-Phe-L-Leu-NH$_2$	*N*-cbz-L-Phe-L-Phe-L-Leu-NH$_2$	24	90
10	*N*-Boc-L-Nap[a]-OMe	L-Leu-NH$_2$	*N*-Boc-L-Nap-L-Leu-NH$_2$	94	67[b]
11	*N*-cbz-L-Phe-OBzl	L-Leu-O^{t}Bu	*N*-cbz-L-Phe-L-Leu-O^{t}Bu	48	32
12	*N*-cbz-L-Phe-OBzl	L-Ala-O^{t}Bu	*N*-cbz-L-Phe-L-Ala O^{t}Bu	24	46
13	*N*-cbz-L-Ser-OMe	L-Leu-NH$_2$	*N*-cbz-L-Ser-L-Leu-NH$_2$	28	94
14	*N*-cbz-L-Glu-DiOMe	L-Leu-NH$_2$	*N*-cbz-L-Glu-(r-OMe)-L-Leu-NH$_2$	32	92
15	*N*-Boc-L-Nap-OMe	(±)-**9a**	L,L-**9c**	96	91/>98
16	(±)-*N*-Boc-Nap-OMe	(±)-**9a**	L,L-**9c**	96	87/>98
17	*N*-cbz-L-Ala-L-Ser-OMe	(±)-**9a**	L,L,L-**9d**	6	98/>98
18	*N*-cbz-L-Val-L-Phe-OMe	(±)-**9a**	L,L,L-**9e**	6	98/>98
19	*N*-Ac-L-Phe-OEt	(±)-**10a**	L,L-**10e**	32	88/89
20	(±)-*N*-Ac-Phe-OMe	L-**10a**	L,L-**10e**	32	93/>98
21	*N*-Ac-L-Ala-OMe	(±)-**10a**	L,L-**10f**	24	74/90
22	*N*-Ac-L-Leu-OMe	(±)-**10a**	L,L-**10g**	24	66/91

[a] Nap = 3-(2-naphthyl)alanine. [b] *N*-Boc-Nap-OMe was recovered in 28% yield.

Scheme 1



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1a	N-Ac-Phe NH$_2$
1b	N-Ac-Trp NH$_2$
1c	N-Ac-Tyr NH$_2$
1d	Tyr NH$_2$
1e	N-Boc-Tyr NH$_2$
1f	N-cbz-Tyr NH$_2$
1g	N-cbz-Leu-Trp NH$_2$
1h	N-cbz-Gly-Phe NH$_2$

(Scheme 1). In all cases the yield was quantitative. This method may provide chemists with a mild and efficient approach for the hydrolysis of amino acid and peptide amides.

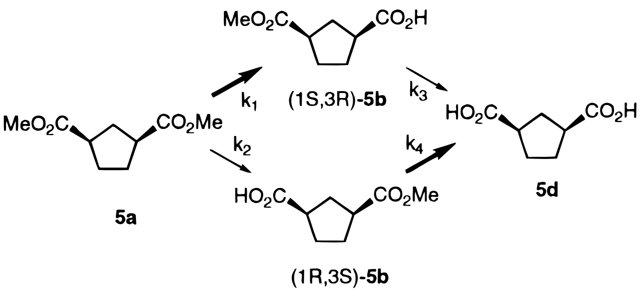
(c) Enantioselective Synthesis. Subtilisin-CLEC exhibits high enantioselectivity in the resolution of amino acid esters and many organic molecules. Both natural and unnatural amino acids were effectively resolved by this catalyst. *N*-Acetylphenylalanine methyl ester, **2a**, was hydrolyzed to give L-acid product and remaining D-ester in 96% and >99% optical purity, respectively. The unnatural amino acids, *N*-Boc-3-(2-naphthyl)alanine methyl ester, **3a**, and *threo*-*N*-acetyl-β-hydroxy-*p*-(methylsulfonyl)phenylalanine ethyl ester, **4a**, were also resolved by this catalyst with high enantioselectivity.[14] The optical purity of both hydrolyzed products **3b** and **4b** was obtained in greater than 99% yield when the conversion was less than 50%. To increase the optical purity of unreacted substrate in these two reactions, the conversion was increased to more than 50% by longer reaction times. This allowed us to obtain the unreacted substrate with optical purity greater than 99% in both reactions (Table 3, entries 3 and 5). The *meso*-diester, **5a**, was enantioselectively hydrolyzed to give monoester **5b** with 96% optical purity.[15] In this reaction, the diester **5a** was first hydrolyzed to its corresponding monoacid **5b**, which then underwent further hydrolysis by the same enzyme to form diacid **5d** (Scheme 2) The optical purity of monoacid **5a** was increased in the second step of the hydrolysis.[16] This indicates that the catalyst has the same enantiopreference toward the monoacid as the diester.

Table 3. Subtilisin-CLEC-Catalyzed Enantio- and Regioselective Reactions

entry	substrate	% conversn	% ee unreacted substrate	% ee product	stereo- or regiopreference
1	**2a**	51	>99	96	*S*
2	**3a**	46	85	>98	*S*
3	**3a**	51	>99	96	*S*
4	**4a**	47	84	>98	(2*S*,3*R*)
5	**4a**	52	>99	92	(2*S*,3*R*)
6	**5a**	65[a]		96	(1*S*,3*R*)
7	**6a**	97		81	*pro-S*
8	**7a**	>98		85	*pro-S*
9	**8a**	88		76	*pro-S*
10	**9a**	50	>98	98	*S*
11	**10a**	20		94	*S*
12	**10a**	53	>98		*S*
13	**11a**	64	>98	54	*S*
14	**12a**	30	40	92	*S*
15	**13a**	65	30[b]	100[c]	6-OH
16	**14a**	75	25[b]	100[c]	3-OH
17	**15a**	78	20[b]	80[c]	17-OH
18	**16a**	97		100[c]	α-ester

[a] One ester corresponding 50% conversion. [b] Yield of recovery substrate. [c] Regioselectivity.

Scheme 2



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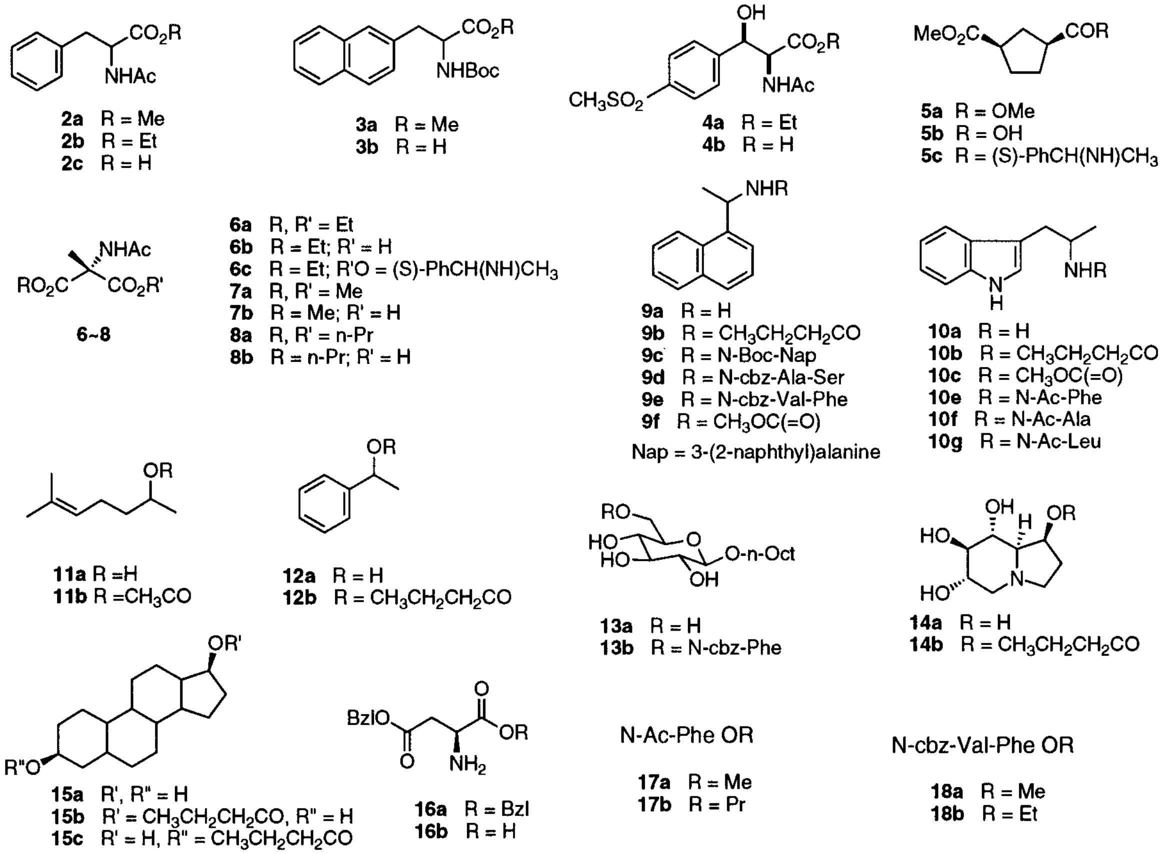
(14) Examples for the resolution of natural and unnatural amino acids with subtilisin see: (a) Wong, C. H.; Chen, S. T.; Hennen, W. J.; Bibbs, J. A.; Wang, Y.-F.; Liu, J. L.C.; Pantoliano, M. W.; Whitlow, M.; Bryan, P. N. *J. Am. Chem. Soc.* **1990**, *112*, 945. (b) Imperiali, B.; Prins, T. J.; Fisher, S. L. *J. Org. Chem.* **1993**, *58*, 1613. (c) Chenevert, R.; Letourneau, M.; Thiboutot, S. *Can. J. Chem.* **1990**, *68*, 960. (d) Leanna, M. R.; Morton, H. E. *Tetrahedron Lett.* **1993**, *34*, 4485.

(15) Chenevert, R.; Martin, R. *Tetrahedron: Asymmetry* **1992**, *3*, 199.

Scheme 3



The *pro*-chiral diethyl ester **6a** was enantioselectively hydrolyzed to give 97% of the corresponding monoacid with 81% optical purity.[17] It was well-known that the size of group near the chiral center would affect the enantioselectivity in the enzymatic resolution. Therefore, methyl ester **7a** and propyl ester **8a** were prepared from **6a** and hydrolyzed with this catalyst. Unfortunately, the enantioselectivities in both **7a** and **8a** were not significantly different from that of **6a**. The optical purity of **7b** and **8b** was obtained in 85% and 76%, respectively.

As we mentioned before, subtilisin-CLEC was active and stable in neat organic solvents. The catalyst was used to perform the resolution of alcohols and amines in neat organic solvents. α-(1-Naphthyl)ethylamine, **9a**, was resolved to give the corresponding butyramide and remaining amine with 98% and >98% ee's, respectively.[18b] α-Methyltryptamine, **10a**, was resolved by this catalyst to give R-amine with ee greater than 98% at 53% conversion. The optical purity of amide product was 94% at 20% conversion. When *sec*-phenethyl alcohol **12a** was transesterified by this catalyst, the optical purity of ester product (*S*)-**12b** and remaining alcohol (*R*)-**12a** was 92% and 40% at 30% conversion, respectively.[18] Surprisingly, the catalyst showed no enantioselectivity in the hydrolysis of the corresponding butyrate **12b** in aqueous buffer (Scheme 3).

(d) Regioselective Reactions. Subtilisin-CLEC exhibited excellent regioselectivity toward many polyfunctional molecules. The 6-hydroxyl group of *O*-*n*-octyl *β*,D-glucopyranoside, **13a**., was selectively reacted with *N*-Cbz-Phe-OBzl to give peptido-sugar **13b**. The 3-hydroxyl group of castanospermine, **14a**, and the 17-hydroxyl group of 5α-androstane-3*β*,17*β*-diol, **15a**, were selectively esterified to give their corresponding monoesters **14b** and **15b**, respectively.[19] On the other hand, the α-ester of aspartic dibenzyl ester, **16a**, was selectively hydrolyzed to give 97% corresponding monoacid **16b**.

(e) Transesterification of Amino Acid and Peptide Esters. The ester exchange of amino acid and peptide esters was also achieved by this catalyst. *N*-Acetylphenylalanine methyl ester, **17a**, and *N*-Cbz-Val-Phe methyl ester, **18a**, were smoothly converted to their corresponding propyl ester and ethyl ester, respectively, without racemization. This method is particularly useful for the transesterification of compounds that are sensitive to acid or base.

(f) Recycling. Since subtilisin-CLEC is stable, insoluble, and composed of mechanically strong particles, it can be easily recovered by filtration or centrifugation and be reused. The high stability and activity of subtilisin-CLEC, along with the ability to reuse the solid catalyst, were demonstrated in repetitive batch resolution of α-methyltryptamine, **10a**. After seven 22 h cycles in the resolution of amine **10a**, subtilisin-CLEC retained full

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(16) (a) Wang, Y.-F.; Chen, C.-S.; Girdaukas, G.; Sih, C. J. *J. Am. Chem. Soc.* **1984**, *106*, 3695. (b) Wang, Y.-F.; Lalonde, J. J.; Momongan, M.; Bergbreiter, D. E.; Wong, C.-H. *J. Am. Chem. Soc.* **1988**, *110*, 7200.

(17) Iriuchijima, S.; Hasegawa, K.; Tsuchihashi, G. *Agric. Biol. Chem.* **1982**, *46*, 1907.

(18) For examples for the resolution of organic molecules with subtilisin see: (a) Mazdiyasni, H.; Konopacki, D. B.; Dickman, D. A.; Zydowsky, T. M. *Tetrahedron Lett.* **1993**, *34*, 435. (b) Kitaguchi, H.; Fitzpatrick, P. A.; Huber, J. E.; Klibanov, A. M. *J. Am. Chem. Soc.* **1989**, *111*, 3094. (c) Fitzpatrick, P. A.; Klibanov, A. M. *J. Am. Chem. Soc.* **1991**, *113*, 3166.

(19) For examples for the regioselective reactions with subtilisin see: (a) Wu, S.-H.; Lo, L.-C.; Chen, S.-T.; Wang, K.-T. *J. Org. Chem.* **1989**, *54*, 4220. (b) Margolin, A. L.; Delinck, D. L.; Whalon, M. R. *J. Am. Chem. Soc.* **1990**, *112*, 2849. (c) Singh, H. K.; Cote, G. L.; Sikorski, R. S. *Tetrahedron Lett.* **1993**, *34*, 5201. (d) Riva, S.; Chopineau, J.; Kieboom, A. P. G.; Klibanov, A. M. *J. Am. Chem. Soc.* **1988**, *110*, 584. (e) Riva, S.; Klibanov, A. M. *J. Am. Chem. Soc.* **1988**, *110*, 3291.

activity and was without loss of enantioselectivity.[20] In contrast, crude alcalase[21] lost more than 50% of its original activity after two cycles.

Conclusions

Subtilisin-CLEC is a versatile catalyst that combines high activity and stability in different reaction media. It is useful for the syntheses of peptides and peptidomimetics, mild hydrolyses of amino acid and peptide amides, enantio- and regioselective reactions, and transesterifications. The combination of these features makes subtilisin-CLEC a useful catalyst for organic synthesis both in the laboratory and in large-scale applications.

Experimental Section

NMR spectra were recorded on a Bruker 500 spectrometer. ^1H-chemical shifts are reported relative to TMS at δ 0. HPLC analyses were conducted with a Hewlett-Packard 1050 chromatograph. GC analyses were conducted with a Hewlett-Packard 5890 chromatograph. CLEC-subtilisin and crude subtilisin activities were assayed by hydrolysis of the substrate *N*-α-*p*-tosyl-L-arginine methyl ester. Unit definition: 1 unit converts 1 μmol of tosyl-L-arginine methyl ester per minute at 25 °C, pH 8.0.

Preparation of Subtilisin-CLEC. Alcalase (Novo Nordisk) from *Bacillus licheniformis* was crystallized and cross-linked with glutaraldehyde according to the procedure reported for subtilisin Carlsberg by Tüchsen and Ottesen[22] with slight modifications. Cross-linked enzyme crystals of subtilisin are sold under the trade names Chir°CLEC-BL (for chiral resolutions) and PeptiCLEC-BL (for peptide synthesis), respectively, and are commercial products of Altus Biologics, Inc. (Cambridge, MA).

Activity Assay. Subtilisin-CLEC activity was assayed by hydrolysis of the substrate *N*-α-*p*-tosyl-L-arginine methyl ester (TAME). Assay conditions: 2 mmol of TAME (80 mM) in 25 mL of 0.3 M phosphate buffer (pH 7.5) was incubated with 2 mg of subtilisin-CLEC at room temperature. One unit was definited as hydrolysis of 1 μmol substrate per minute per mg of CLEC at room temperature. The activity of subtilisin-CLEC used in here was 33−36 U. This catalyst was very stable in storage. It did not lose activity after 6 months of storage at room temperature.

Preparation of Surfactant-Treated Subtilisin-CLEC. Subtilisin-CLEC (20 g protein) suspended in 10 mM Tris, 10 mM $CaCl_2$, pH 7.0 was transferred to a sintered glass funnel (porosity ~5 μm). The buffer above the CLECs was decanted or removed by suction. The equal volume of 2-butanone containing 30 g of the detergent Tergitol Type 15-S-3 (poly(glycol ether)) was added to the CLECs cake. The solvent and surfactant were removed by gentle suction. The mixture was transferred to a fritted pressure filter funnel after breaking up any lumps and dried in a stream of nitrogen to a water content of about 2−3% as determined by Karl Fisher titration.

General Procedures for the Synthesis of Peptides. To a solution of 0.3 mmol of N-protected amino acid ester (acyl donor) and 0.9 mmol of amino acid amide (or other nucleophiles) in 15 mL of acetonitrile was added 100 mg of dry subtilisin-CLECs. The resulting mixture was incubated on a rotary shaker at 40 °C. The reaction was monitored by HPLC. After the acyl donor had disappeared, the catalyst was removed by filtration. The excess nucleophile was removed by washing with 1 N HCl, and the products were then separated by silica gel column chromatography or by recrystallization.

HPLC Conditions. (a) Microsorb C_{18} column (4.6 × 50 mm, 5 μm, 300A); mobile phase: gradient, ratio (v/v) of water (with 0.1% trifluoroacetic acid) and acetonitrile (with 0.1% trifluoroacetic acid) was 90:10 at 0 min, 50:50 at 8 min, 90:10 at 10 min; flow rate: 1 mL/min; UV detection at 254 nm; Retention times: *N*-cbz-L-Phe-OBzl, 8.61 min; *N*-cbz-L-Phe-L-Leu-NH_2, 5.75 min; *N*-Boc-L-Tyr-OMe, 6.89 min; *N*-Boc-L-Tyr-L-Leu-NH_2, 6.36 min; *N*-Boc-3-(2-naphthyl)alanine methyl ester, 7.77 min; *N*-Boc-L-Nap-L-Leu-NH_2, 6.49 min; *N*-cbz-L-Val-L-Phe-OMe, 9.70 min; *N*-cbz-L-Val-L-Phe-L-Leu-NH_2, 8.95 min; *N*-cbz-L-Val-L-Phe-L-Phe-NH_2, 9.25 min. (b) Microsorb C_{18} column (4.6 × 150 mm, 5 μm, 100A); mobile phase: gradient, ratio (v/v) of water (with 0.1% trifluoroacetic acid) and acetonitrile (with 0.1% trifluoroacetic acid) was 90:10 at 0 min, 20:80 at 10 min, 20:80 at 12 min, 90:10 at 15 min; flow rate: 1 mL/min; UV detection at 254 nm; retention times: *N*-cbz-L-Phe-OBzl, 12.59 min; Phe-NH_2, 3.84 min; *N*-cbz-L-Phe-L-Phe-NH_2, 9.81 min; *N*-cbz-L-Phe-L-Leu-O^tBu, 12.99 min; *N*-cbz-L-Phe-L-Ala-O^tBu, 11.68 min; *N*-cbz-L-Ser-L-Leu-NH_2, 7.45 min; *N*-cbz-L-Glu-DiOMe, 7.99 min; *N*-cbz-L-Glu(OMe)-L-Leu-NH_2, 7.55 min.

***N*-cbz-L-Phe-L-Leu-NH_2** (CD_3OD/TMS): 0.88 (3H, d, J = 6.5 Hz), 0.92 (3H, d, J = 6.5 Hz), 1.57 (2H, dd, J = 6.5, 7.5 Hz), 1.60 (1H, m), 2.89 (1H, dd, J = 9.0, 13.5 Hz), 3.11 (1H, dd, J = 6.0, 13.5 Hz), 4.36 (2H, m), 5.03 (2H, s), 7.15−7.35 (10H, m).

***N*-cbz-L-Phe-D-Leu-NH_2** (CD_3OD/TMS): 0.69 (3H, d, J = 6.5 Hz), 0.76 (3H, d, J = 6.5 Hz), 0.82−0.92 (1H, m), 1.36 (1H, ddd, J = 4.0, 11.5, 14.0 Hz), 1.51 (1H, ddd, J = 4.0, 10.5, 14.0 Hz), 2.96 (2H, d, J = 8.0 Hz), 4.17 (1H, dd, J = 3.5, 11.5 Hz), 4.30 (1H, t, J = 8.0 Hz), 5.06 (2H, s), 7.15−7.35 (10H, m).

***N*-Boc-L-Tyr-L-Leu-NH_2** (CD_3OD/TMS): 0.89 (3H, d, J = 6.0 Hz), 0.93 (3H, d, J = 6.0 Hz), 1.39 (9H, s), 1.58 (2H, dd, J = 6.5, 7.2 Hz), 1.61 (1H, m), 2.77 (1H, dd, J = 8.5, 14.0 Hz), 2.98 (1H, dd, J = 6.0, 14.0 Hz), 4.22 (1H, dd, J = 6.0, 8.5 Hz), 4.36 (1H, t, J = 7.2 Hz), 6.70 (2H, d, J = 8.5 Hz), 7.06 (2H, d, J = 8.5 Hz).

***N*-cbz-L-Phe-L-Phe-NH_2** (CD_3OD/TMS): 2.76 (1H, dd, J = 9.2, 13.8 Hz), 2.91 (1H, dd, J = 8.6, 13.8 Hz), 2.99 (1H, dd, J = 5.8, 13.8 Hz), 3.14 (1H, dd, J = 5.6, 13.8 Hz), 4.31 (1H, dd, J = 5.8, 9.2 Hz), 4.58 (1H, dd, J = 5.6, 8.6 Hz), 4.99 (1H, d, J = 12.5 Hz), 5.03 (1H, d, J = 12.5 Hz), 7.15−7.35 (15H, m).

***N*-cbz-L-Phe-D-Phe-NH_2** (CD_3OD/TMS): 2.68 (1H, dd, J = 9.0, 13.8 Hz), 2.84 (1H, dd, J = 8.8, 13.8 Hz), 2.85 (1H, dd, J = 5.6, 14.0 Hz), 3.12 (1H, dd, J = 5.1, 14.0 Hz), 4.27 (1H, dd, J = 5.6, 8.8 Hz), 4.60 (1H, dd, J = 5.1, 9.0 Hz), 4.99 (1H, d, J = 12.6 Hz), 5.02 (1H, d, J = 12.6 Hz), 7.15−7.35 (15H, m).

***N*-cbz-L-Val-L-Phe-L-Leu-NH_2** (CD_3OD/TMS): 0.85 (6H, d, J = 7.0 Hz), 0.87 (3H, d, J = 6.0 Hz), 0.91 (3H, d, J = 6.0 Hz), 1.57 (2H, dd, J = 6.0, 7.5 Hz), 1.61 (1H, m), 1.96 (1H, m), 2.97 (1H, dd, J = 8.0, 13.5 Hz), 3.13 (1H, dd, J = 6.5, 13.5 Hz), 3.87 (1H, d, J = 7.0 Hz), 4.33 (1H, t, J = 7.5 Hz), 4.63 (1H, dd, J = 6.5, 8.0 Hz), 5.04 (1H, d, J = 12.0 Hz), 5.10 (1H, d, J = 12.0 Hz), 7.15−7.35 (10H, m).

***N*-cbz-L-Val-L-Phe-L-Phe-NH_2** (CD_3OD/TMS): 0.85 (6H, d, J = 7.0 Hz), 1.95 (1H, m), 2.75 (1H, dd, J = 9.2, 13.8 Hz), 2.91 (1H, dd, J = 8.6, 13.8 Hz), 2.99 (1H, dd, J = 5.8, 13.8 Hz), 3.14 (1H, dd, J = 5.6, 13.8 Hz), 3.87 (1H, d, J = 7.0 Hz), 4.31 (1H, dd, J = 5.8, 9.2 Hz), 4.58 (1H, dd, J = 5.6, 8.6 Hz), 5.04 (1H, d, J = 12.0 Hz), 5.10 (1H, d, J = 12.0 Hz), 7.15−7.35 (15H, m).

***N*-cbz-L-Phe-L-Phe-L-Leu-NH_2** (CD_3OD/TMS): 0.88 (3H, d, J = 6.5 Hz), 0.92 (3H, d, J = 6.5 Hz), 1.57 (2H, dd, J = 6.0, 7.5 Hz), 1.60 (1H, m), 2.75 (1H, dd, J = 9.2, 13.8 Hz), 2.90 (1H, dd, J = 8.8, 13.8 Hz), 2.99 (1H, dd, J = 5.8, 13.8 Hz), 3.12 (1H, dd, J = 5.8, 13.8 Hz), 4.33 (1H, t, J = 7.5 Hz), 4.36 (1H, dd, J = 5.8, 9.2 Hz), 4.63 (1H, dd, J = 5.8, 8.8 Hz), 4.99 (1H, d, J = 12.5 Hz), 5.03 (1H, d, J = 12.5 Hz), 7.15−7.35 (15H, m).

***N*-Boc-L-Nap-L-Leu-NH_2** (CD_3OD/TMS): 0.87 (3H, d, J = 5.2 Hz), 0.89 (3H, d, J = 5.2 Hz), 1.32 (9H, s), 1.58 (3H, m), 3.03 (1H, dd, J = 9.0, 13.5 Hz), 3.28 (1H, dd, J = 5.5, 13.5 Hz), 4.39 (1H, m), 4.41 (1H, dd, J = 5.5, 9.0 Hz), 7.38−7.48 (3H, m), 7.71 (1H, s), 7.76−7.83 (3H, m).

***N*-cbz-L-Phe-L-Leu-O^tBu** (CD_3OD/TMS): 0.89 (3H, d, J = 6.5 Hz), 0.93 (3H, d, J = 6.5 Hz), 1.40 (9H, s), 1.58−1.62 (3H,

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[20] After each reaction cycle, the catalysts were filtered off and washed with 3-methyl-3-pentanol. The washed catalysts were directly used in the next cycle.

[21] The dry crude alcalase was prepared according to the procedure described in ref 12b.

[22] Tüchsen, E.; Ottesen, M. *Carlsberg Res. Commun.* **1977**, *42*, 407.

m), 2.89 (1H, dd, $J = 9.0, 13.5$ Hz), 3.11 (1H, dd, $J = 6.0, 13.5$ Hz), 4.36 (2H, m), 5.03 (2H, s), 7.15−7.35 (10H, m).

N-cbz-L-Phe-L-Ala-$\mathrm{O^tBu}$ (CD_3OD/TMS): 1.16 (3H, d, $J = 6.8$ Hz), 1.40 (9H, s), 2.90 (1H, dd, $J = 9.0, 13.5$ Hz), 3.12 (1H, dd, $J = 6.0, 13.5$ Hz), 4.36 (1H, dd, $J = 6.0, 9.0$ Hz), 4.41 (1H, q, $J = 6.8$ Hz), 5.03 (2H, s), 7.15−7.35 (10H, m).

General Procedures for the Coupling of N-Protected Amino Acid and Peptide Esters with α-(Naphthyl)-ethylamine.[9a] To a solution of 0.5 mmol (1 mmol for racemic ester) of N-protected amino acid or peptide ester and 2 mmol of (±)-amine in 25 mL of acetonitrile was added 165 mg of dry subtilisin-CLEC. The resulting mixture was incubated on a rotary shaker at 40 °C. The conversion was analyzed by HPLC. The catalyst was filtered off and washed with acetonitrile. Ethyl acetate was added, and the excess amine was removed by extraction with 0.5 N aqueous HCl. After drying over sodium sulfate, the organic solvents were evaporated off to give a residue that was purified by silica gel column chromatography to give the product.

HPLC Conditions. Microsorb C_{18} column (4.6 × 50 mm, 5 μm, 300A); mobile phase: gradient, ratio (v/v) of water (with 0.1% trifluoroacetic acid) and acetonitrile (with 0.1% trifluoroacetic acid) was 90:10 at 0 min, 20:80 at 10 min, 20:80 at 12 min, and 90:10 at 15 min; flow rate: 1 mL/min; UV detection at 254 nm; retention times: *N*-Boc-L-Nap-OMe, 8.69 min; L,L-**9c**, 9.87 min; L,D-**9c**, 9.94 min; *N*-cbz-L-Ala-L-Ser-OMe, 4.10 min; L,L,L-**9d**, 6.70 min; *N*-cbz-L-Val-L-Phe-OMe, 7.84 min; L,L,L-**9e**, 9.41 min.

L,L-9c: 1.33 (9H, s), 1.55 (3H, d, $J = 7.0$ Hz), 3.0 (1H, dd, $J = 8.0,13.5$ Hz), 3.14 (1H, dd, $J = 7.0, 13.5$ Hz), 4.48 (1H, dd, $J = 7.0, 8.0$ Hz), 5.80 (1H, q, $J = 7.0$ Hz), 6.9−8.1 (14H, m).

L,L,L-9d: 1.35 (3H, d, $J = 7.0$ Hz), 1.58 (3H, d, $J = 7.0$ Hz), 3.76 (1H, dd, $J = 4.8,11.0$ Hz), 3.86 (1H, dd, $J = 5.3, 11.0$ Hz), 4.13 (1H, q, $J = 7.0$ Hz), 4.41 (1H, dd, $J = 4.8, 5.3$ Hz), 5.12 (2H, s), 5.80 (1H, q, $J = 7.0$ Hz), 7.2−7.6 (9H, m), 7.76 (1H, d, 8.0 Hz), 7.86 (1H, d, $J = 8.0$ Hz), 8.09 (1H, d, 8.5 Hz).

L,L,L-9e: 0.85 (6H, d, $J = 7.0$ Hz), 1.58 (3H, d, $J = 7.0$ Hz), 1.96 (1H, m), 2.97 (1H, dd, $J = 8.0,13.5$ Hz), 3.13 (1H, dd, $J = 6.5, 13.5$ Hz), 3.87 (1H, d, $J = 7.0$ Hz), 4.63 (1H, dd, $J = 6.5, 8.0$ Hz), 5.04 (1H, d, $J = 12.0$ Hz), 5.10 (1H, d, $J = 12.0$ Hz), 5.80 (1H, q, $J = 7.0$ Hz), 7.0−8.1 (17H, m).

General Procedures for the Coupling of N-Protected Amino Acid Ester with α-Methyltryptamine, 10a. To a solution of 0.3 mmol of N-protected amino acid ester and 0.3 mmol of amine in 1.5 mL of 3-methyl-3-pentanol was added 7.5 mg of dry subtilisin-CLEC. The resulting mixture was incubated on a rotary shaker at 40 °C. The conversion was analyzed by HPLC. The catalyst was then removed by filtration, and the products were separated by silica gel column chromatography.

HPLC Conditions. Microsorb C_{18} column (4.6 × 50 mm, 5 μm, 300A); mobile phase for *N*-Ac-L-Phe-OEt and *N*-Ac-L-Leu-OMe: 70% water (with 0.1% trifluoroacetic acid) and 30% acetonitrile (with 0.1% trifluoroacetic acid). For *N*-Ac-L-Ala-OMe: 80% water (with 0.1% trifluoroacetic acid) and 20% acetonitrile (with 0.1% trifluoroacetic acid). Flow rate: 1 mL/min; UV detection at 254 nm. Retention times: L,L-**10e**, 3.89 min; L,D-**10e**, 4.53 min; *N*-Ac-L-Phe-OEt, 1.89 min; L,L-**10f**, 3.09 min; L,L-**10g**, 2.80 min; L,D-**10g**, 3.28 min.

L,L-10e: 1.11 (3H, d, $J = 6.5$ Hz), 1.86 (3H, s), 2.67 (1H, dd, $J = 7.5, 14.2$ Hz), 2.73 (1H, dd, $J = 8.0, 13.5$ Hz), 2.80 (1H, dd, $J = 6.5, 14.2$ Hz), 2.88 (1H, dd, $J = 6.5, 13.5$ Hz), 4.17 (1H, ddq, $J = 6.5, 6.5, 7.5$ Hz), 4.51 (1H, dd, $J = 6.5, 8.0$ Hz), 6.9−7.6 (10H, m).

L,L-10f: 1.11 (3H, d, $J = 6.5$ Hz), 1.35 (3H, d, $J = 7.0$ Hz), 1.86 (3H, s), 2.67 (1H, dd, $J = 7.5, 14.2$ Hz), 2.80 (1H, dd, $J = 6.5, 14.2$ Hz), 4.13 (1H, q, $J = 7.0$ Hz), 4.17 (1H, ddq, $J = 6.5, 6.5, 7.5$ Hz), 6.9−7.6 (5H, m).

L,L-10g: 0.88 (3H, d, $J = 6.5$ Hz), 0.92 (3H, d, $J = 6.5$ Hz), 1.11 (3H, d, $J = 6.5$ Hz), 1.5−1.7 (3H, m), 1.86 (3H, s), 2.67 (1H, dd, $J = 7.5, 14.2$ Hz), 2.80 (1H, dd, $J = 6.5, 14.2$ Hz), 4.17 (1H, ddq, $J = 6.5, 6.5, 7.5$ Hz), 4.36 (1H, t, $J = 7.0$ Hz), 6.9−7.6 (5H, m).

General Procedures for the Hydrolysis of Amino Acid and Peptides Amides. To a solution of 1 mmol of amino acid or peptide amide in 9 mL of 0.2 M, pH 7.5, phosphate buffer was added 40−80 mg of subtilisin-CLEC. The resulting mixture was incubated on a rotary shaker at 40 °C. The reaction was monitored by HPLC analysis. After the substrate disappeared (1−2 days), the catalyst was separated by centrifugation or filtration. The pH of supernatant or filtrate was adjusted to 4 with 1 N HCl. The product was then extracted with ethyl acetate. The combined organic extracts were washed with saturated sodium chloride, dried over sodium sulfate, and evaporated in vacuo to give a residue that was then purified by short silica gel column chromatography.

HPLC Conditions. (a) Microsorb-MV C_{18} column (4.6 × 150 mm, 5 μm, 100A)l mobile phase for **1a**: 80% water (with 0.1% trifluoroacetic acid) and 20% acetonitrile (with 0.1 % trifluoroacetic acid). For **1g** and **1h**: 40% water (with 0.1% trifluoroacetic acid) and 60% acetonitrile (with 0.1 % trifluoroacetic acid). Flow rate: 1 mL/min; UV detection at 254 nm. Retention times: **1a**, 3.60 min; *N*-Ac-L-Phe-OH, 6.04 min; **1g**, 5.59 min; *N*-cbz-L-Leu-L-Trp-OH, 7.9 min; **1h**, 4.78 min; *N*-cbz-L-Gly-L-Phe-OH, 6.63 min. (b) Microsorb-MV C_{18} column (4.6 × 50 mm, 5 μm, 300A), mobile phase for **1c**: 90% water (with 0.1% trifluoroacetic acid) and 10% acetonitrile (with 0.1 % trifluoroacetic acid). For **1d**: 95% water (with 0.1% trifluoroacetic acid) and 5% acetonitrile (with 0.1% trifluoroacetic acid). For **1b**, **1e**, and **1f**: 80% water (with 0.1% trifluoroacetic acid) and 20% acetonitrile (with 0.1 % trifluoroacetic acid). Flow rate: 1 mL/min; UV detection at 254 nm. Retention times: **1b**, 2.43 min; *N*-Ac-L-Trp-OH, 4.05 min; **1c**, 1.04 min; *N*-Ac-L-Tyr-OH, 1.39 min; **1e**, 1.86 min; *N*-Boc-L-Tyr-OH, 3.27 min; **1f**, 3.02 min; *N*-cbz-L-Tyr-OH, 5.58 min; **1d**, 1.16 min; L-Tyr-OH, 1.62 min.

Resolution of *N*-Acetylphenylalanine Methyl Ester, 2a. To a solution of 442 mg of racemic *N*-acetylphenylalanine methyl ester in 4 mL of acetone was added 16 mL of 0.2 M, pH 7.0 phosphate buffer. Subtilisin-CLEC (0.2 mL, 4 mg) suspension was added, and the resulting mixture was incubated on a rotary shaker for 0.5 h at 40 °C. The catalyst was then removed by centrifugation. The pH of the supernatant was adjusted to 4 with 1 N HCl, and the products were extracted with ethyl acetate (15 mL × 3). The combined ethyl acetate extracts were washed with saturated sodium chloride, dried over sodium sulfate, and evaporated in vacuo. The residue was then purified by silica gel column chromatography to give *N*-acetyl-L-phenylalanine and *N*-acetyl-D-phenylalanine methyl ester. The optical purity of the unreacted *N*-acetyl-D-phenylalanine methyl ester was directly analyzed using HPLC with a chiral column. *N*-Acetyl-L-phenylalanine product was converted to its methyl ester with (trimethylsilyl)diazomethane, which was then analyzed for optical purity with the same method as above. Results: ee of remaining ester >99%; ee of acid product 96%, conversion 51%.

Chiral HPLC Conditions. Chiracel OD 25 cm column (Daicel Chemical Inc.), mobile phase = 85% hexane, 15% 2-propanol, flow rate = 1 mL/min, UV detection at 254 nm. Retention times: *N*-acetyl-D-phenylalanine methyl ester, 8.99 min, and *N*-acetyl-L-phenylalanine methyl ester, 11.06 min.

Resolution of *N*-Boc-3-(2-naphthyl)alanine Methyl Ester, 3a. To a solution of 99 mg of *N*-Boc-*p*-chlorophenylalanine methyl ester in 1.2 mL of acetone was added 1.2 mL of 0.2 M, pH 7.5, phosphate buffer. A 0.15 mL (3.3 mg) portion of subtilisin-CLEC was added. The resulting mixture was incubated on a rotary shaker for 21 h at 40 °C. After the reaction mixture was acidified with 1 N HCl, the products were extracted with a mixture of ethyl acetate/hexanes (4:1). The combined organic extracts were dried over sodium sulfate and evaporated in vacuo. The residue was resolved in 10 mL of ethyl acetate/hexanes (2:1), which was then extracted with 0.2 N sodium hydroxide. The organic layer was dried over sodium sulfate and then evaporated to give 54 mg of remaining ester. The aqueous layer was reextracted with ethyl acetate/hexanes and then acidified with 1 N HCl. The acid product was then extracted with ethyl acetate/hexanes. The organic layer was dried over sodium sulfate and then evaporated to afford 39 mg of L-acid product. The optical purity of the remaining *N*-Boc-D-3-(2-naphthyl)alanine methyl ester was directly analyzed using HPLC with a chiral column. The acid product was first converted to its corresponding methyl ester with (trimethylsilyl)diazomethane, and then the optical purity was analyzed by the same method as above. Results: ee of remaining ester = 85%; ee of acid product >98%; conversion 46%.

Chiral HPLC Conditions. Chiracel OD 25 cm column (Daicel Chemical Inc.), mobile phase = 98% of 0.05% trifluoroacetic acid in hexanes, 2% 2-propanol, flow rate = 1 mL/min, UV detection at 254 nm. Retention times: *N*-Boc-D-3-(2-naphthyl)alanine methyl ester, 11.4 min, and *N*-Boc-L-3-(2-naphthyl)alanine methyl ester, 12.5 min.

Resolution of *threo-N*-Acetyl-*β*-hydroxy-*p*-(methylsulfonyl)phenylalanine Ethyl Ester, 4a. To a solution of 240 mg of **4a** in 3 mL of DMF was added 12 mL of 0.1 M, pH 7.0, phosphate buffer. Fifteen mg of subtilisin-CLEC was added. The resulting mixture was incubated on a rotary shaker for 7 h at 40 °C. After 1 mL of 2 N HCl was added, the unreacted substrate was extracted with tetrahydrofuran. The combined organic extracts were dried over sodium sulfate and evaporated in vacuo. The residue was then purified by short silica gel column to give 110 mg of ester. The aqueous layer was acidified with 2 mL of 2 N HCl, which was then extracted with a mixture of tetrahydrofuran/ethyl acetate (2:1). The organic layer was dried over sodium sulfate and evaporated in vacuo to give the acid product. To determine the optical purity, the remaining ester was converted to its MTPA ester by reaction of it with 1.1 equiv of MTPA chloride in pyridine. The optical purity of the resulting MTPA ester was then analyzed by NMR spectrophotometry. The acid product was first converted to the methyl ester by treatment with (trimethylsilyl)diazomethane, which was then transformed to its MTPA ester and the optical purity analyzed by the same procedures as above. The resonances assigned to the methoxy groups in MTPA of the diastereomers at δ 3.45 and δ 3.51 were used for the determination of optical purity. Results: ee of remaining ester = 84%; ee of acid product >98%, conversion = 47%.

4a ($CDCl_3$/TMS): 1.20 (3H, t, J = 7.0 Hz), 1.82 (3H, s), 2.99 (3H, s), 4.14 (2H, q, J = 7.0 Hz), 4.77 (1H, d, J = 4.0 Hz), 5.25 (1H, d, J = 4.0 Hz), 7.53 (2H, d, J = 8.0 Hz), 7.80 (2H, d, J = 8.0 Hz).

MTPA derivative of (2*R*,3*R*)-4a ($CDCl_3$/TMS): 1.26 (3H, t, J = 7.0 Hz), 1.90 (3H, s), 3.05 (3H, s), 3.51 (3H, s), 4.18 (2H, q, J = 7.0 Hz), 5.15 (1H, dd, J = 3.3, 9.5 Hz), 5.99 (1H, d, J = 9.5 Hz), 6.51 (1H, d, J = 3.3 Hz), 7.33 (2H, d, J = 8.0 Hz), 7.35−7.60 (5H, m), 7.85 (2H, d, J = 8.0 Hz).

MTPA derivative of (2*S*,3*S*)-4b methyl ester ($CDCl_3$/TMS): 1.87 (3H, s), 3.06 (3H, s), 3.45 (3H, s), 3.72 (3H, s), 5.10 (1H, dd, J = 3.3, 9.5 Hz), 5.88 (1H, d, J = 9.5 Hz), 6.55 (1H, d, J = 3.3 Hz), 7.33 (2H, d, J = 8.0 Hz), 7.35−7.60 (5H, m), 7.91 (2H, d, J = 8.0 Hz).

Resolution of Dimethyl *cis*-1,3-Cyclopentanedicarboxylate, 5a. To a solution of 186 mg of dimethyl *cis*-1,3-cyclopentanedicarboxylate, **5a**, in 9 mL of 0.2 M, pH 7.5 phosphate buffer was added 1 mL (40 mg) of subtilisin-CLEC. The resulting mixture was incubated on a rotary shaker for 48 h at 40 °C. The extent of hydrolysis was monitored by GC analysis. The reaction was terminated when 1.29 ester equivalents had been hydrolyzed; i.e., the yield of monoacid **5b** was 71% and diacid was 29% on the basis of GC analysis. (GC conditions: DB 1701 15 m × 0.25 mm capillary GC column 25 μm film thickness, J, W Scientific, Folsom, CA; helium flow at 25 cm/s; temperature program: initial = 120 °C for 3 min, gradient rate 15 °C, final 200 °C for 3 min, retention time: diester 4.96 min, monoester 6.03 min, diacid 6.74 min.) After the pH of the supernatant was adjusted to 4 with 1 N HCl, the products were extracted with ethyl acetate (15 mL × 3). The combined ethyl acetate extracts were washed with saturated sodium chloride, dried over sodium sulfate, and evaporated in vacuo. The residue was then purified by silica gel column chromatography to give (1*S*,3*R*)-monoester **5b**. To determine the optical purity, **5b** was converted to amide **5c** via the treatment of it with methyl chloroformate followed by reaction with (*S*)-α-methylbenzylamine. The optical purity of amide **5c** was determined to be 96% on the basis of GC analysis. (GC conditions same as above except temperature program: Initial 120 °C for 3 min, gradient rate 3 °C/min, final 220 °C for 3 min; retention time: 32.87 and 33.04 min).

5b ($CDCl_3$/TMS): 1.91−2.02 (4H, m), 2.14 (1H, ddd, J = 9.0, 9.0, 13.0 Hz), 2.27 (1H, ddd, J = 8.0, 8.0, 13.0 Hz), 2.76−2.84 (2H, m), 3.69 (3H, s).

Resolution of Diethyl (Acetylamino)(methyl)malonate, 6a. To a solution of 231 mg of diethyl (acetylamino)(methyl)malonate, **6a**, in 9 mL of 0.2 M, pH 7.5 phosphate buffer was added 1 mL (40 mg) of subtilisin-CLEC. The resulting mixture was incubated on a rotary shaker for 27 h at 40 °C. The reaction was halted by filtration of the catalyst when the conversion reached 97% on the basis of HPLC analysis. The pH of supernatant was adjusted to 4 with 1 N HCl, and the products were extracted with ethyl acetate (15 mL × 3). The combined ethyl acetate extracts were washed with saturated sodium chloride, dried over sodium sulfate, and evaporated in vacuo. The residue was then purified by silica gel column chromatography to give monoester **6b**. To determine the optical purity, **6b** was converted to amide **6c** via the treatment of it with methyl chloroformate followed by reaction with (*S*)-α-methylbenzylamine. The optical purity of amide **6c** was determined to be 81% on the basis of its NMR spectroscopy analysis. The resonances assigned to the methyl groups of the diastereomers at δ 1.80 and 1.75 were used for the determination of optical purity.

(*R*,*S*)-4c ($CDCl_3$/TMS): 1.25 (3H, t, J = 7.0 Hz), 1.46 (3H, d, J = 7.0 Hz), 1.75 (3H, s), 2.02 (3H, s), 4.24 (2H, q, J = 7.0 Hz), 5.05 (1H, dq, J = 7.0, 7.5 Hz), 6.58 (1H, d, J = 7.5 Hz), 7.2−7.5 (5H, m).

(*S*,*S*)-4c ($CDCl_3$/TMS): 1.15 (3H, t, J = 7.0 Hz), 1.50 (3H, d, J = 7.0 Hz), 1.80 (3H, s), 2.00 (3H, s), 4.1−4.2 (2H, m), 5.06 (1H, dq, J = 7.0, 7.5 Hz), 6.50 (1H, d, J = 7.5 Hz), 7.2−7.5 (5H, m).

General Procedures for the Resolution of Amines 9a and 10a. A solution of 0.5 mmol of amine and 1 mmol of 2,2,2-trifluoroethyl butyrate in 3 mL of 3-methyl-3-pentanol was incubated with 30 mg of subtilisin-CLEC on a rotary shaker at 40 °C. The reaction was monitored by HPLC analysis. When the desired level of conversion was reached, the catalyst was removed by centrifugation and washed with ethyl acetate. The combined organic mixtures were evaporated in vacuo to give a residue that was then purified by silica gel column chromatography to give the remaining (*R*)-amine and (*S*)-butyramide product. The optical purity of (*S*)-butyramide was directly analyzed by HPLC with a chiral column. The remaining (*R*)-amine was converted to its methoxyformamide derivative (**9f** or **10c**) by treatment of amine with methyl chloroformate. The optical purity of the methoxyformamide derivative was then analyzed by the same method as that for butyramide. Results: (*S*)-**10b**: ee = 94% at 20% conversion; (*R*)-**10a**: ee > 98% at 53% conversion; (*S*)-**9b**: ee = 98%; (*R*)-**9a**: ee > 98% at 50% conversion.

HPLC Conditions: Chiracel OJ 25 cm column (Daicel Chemical Inc.), mobile phase = 80% hexane (with 0.1% trifluoacetic acid), 20% 2-propanol (with 0.1% trifluoroacetic acid), flow rate = 1 mL/min, UV detection at 254 nm. Retention times: (*R*)-**10b**, 9.3 min, and (*S*)-**10b**, 11.2 min, (*R*)-**10c**, 24.8 min, and (*S*)-**10c**, 27.6 min.

10b ($CDCl_3$/TMS): 0.89 (3H, t, J = 7.5 Hz), 1.16 (3H, d, J = 7.0 Hz), 1.61 (2H, tq, J = 7.5, 7.5 Hz), 2.07 (2H, t, J = 7.5 Hz), 2.91 (1H, ddd, J = 0.75, 7.0, 14.5 Hz), 2.97 (1H, ddd, J = 0.75, 5.5, 14.5 Hz), 4.40 (1H, dddq, J = 5.5, 7.0 , 7.0, 7.5 Hz), 5.34 (1H, d, J = 7.5 Hz), 7.03 (1H, d, J = 2.0 Hz), 7.12 (1H, ddd, J = 1.0, 7.0, 8.0 Hz), 7.19 (1H, ddd, J = 1.5, 7.0, 8.0 Hz), 7.36 (1H, ddd, J = 1.0, 1.0, 8.0 Hz), 7.65 (1H, ddd, J = 1.0, 1.5, 8.0 Hz), 8.15 (1H, bs).

9f ($CDCl_3$/TMS): 0.90 (3H, t, J = 7.5 Hz), 1.57 (3H, d, J = 7.0 Hz), 1.63 (2H, tq, J = 7.5, 7.5 Hz), 2.08 (2H, t, J = 7.5 Hz), 5.50 (1H, d, J = 7.5 Hz), 5.79 (1H, dq, J = 7.0, 7.5 Hz), 7.2−8.2 (7H, m).

Resolution of (±)-Sulcatol, 11a. To a solution of 102 mg of (±)-sulcatol, **11a**, and 112 μL of vinyl acetate in 2 mL of 3-methyl-3-pentanol was added 34 mg of dry subtilisin-CLEC. The mixture was incubated at 40 °C for 40 h. The optical purity of product and remaining substrate were directly analyzed by GC with chiral column. Results: (*R*)-**11a**: ee >98%; (*S*)-**11b**: ee = 54%; conversion = 64%. The catalyst was then removed by filtration and washed with ethyl acetate. The combined organic mixtures were evaporated in vacuo to give a residue that was then purified by silica gel column chromatography to give the remaining alcohol (*R*)-**11a** and ester product (*S*)-**11b**.

GC Conditions. Cyclodex B capillary GC 25 m column, 25 μm i.d. (J & W Scientific, Folsom, CA); helium flow at 1 mL/s; temperature program: initial = 90 °C for 10 min, gradient rate: 5 °C/min from 90 to 130 °C, final, 130 °C for 0 min; retention time: (*S*)-sulcatol, 12.25 min; (*R*)-sulcatol, 12.49 min; (*S*)-sulcatol acetate, 14.32 min; (*R*)-sulcatol acetate, 14.98 min.

Resolution of *sec*-Phenethyl Alcohol, 12a. A solution of 244 mg of alcohol **12a** and 456 mg of vinyl butyrate in 10 mL of dioxane was incubated with 96 mg of subtilisin-CLEC on a rotary shaker for 48 h at 40 °C. The catalyst was removed by centrifugation and washed with ethyl acetate. The combined organic mixtures were evaporated in vacuo to give a residue that was then purified by silica gel column chromatography to give the remaining alcohol (*R*)-**12a** and ester product (*S*)-**12b**. The optical purity of both (*R*)-**12a** and (*S*)-**12b** was directly determined by GC analysis. Results: (*R*)-**12a**: ee = 40%; (*S*)-**12b**: ee = 92%; conversion = 30%.

GC Conditions. Cyclodex B capillary GC 25 m column, 25 μm i.d. (J, W Scientific, Folsom, CA); helium flow at 1 mL/s; temperature program: initial = 100 °C for 10 min, gradient rate 5 °C/min, final 150 °C for 5 min; retention time: (*R*)-**12a**, 16.40 min; (*S*)-**12a**, 16.83 min; (*S*)-**12b**, 21.76 min; (*R*)-**12b**, 21.87 min.

Regioselective Reaction of *O*-*n*-Octyl *β*,D-glucopyranoside with N-Cbz Phenylalanine Benzyl Ester. To a solution of 0.5 mmol of *N*-Cbz-phenylalanine benzyl ester and 1 mmol of *O*-*n*-octyl *β*,D-glucopyranoside in 25 mL of acetonitrile was added 165 mg of dry subtilisin-CLEC. The resulting mixture was incubated on a rotary shaker at 40 °C. The reaction was monitored by HPLC. After 6 days, the reaction was terminated by filtration of the catalyst. The solvent was evaporated in vacuo to give a residue that was then purified by silica gel column chromatography (ethyl acetate) to give 222 mg of **13b**. The yield of product was 65%, and ester substrate was recovered in 30%.

HPLC Conditions. Microsorb C_{18} column (4.6 × 150 mm, 5 μm, 300A), mobile phase: gradient, ratio (v/v) of water (with 0.1% trifluoroacetic acid) and acetonitrile (with 0.1% trifluoroacetic acid) was 90:10 at 0 min, 20:80 at 10 min, 20:80 at 12 min, and 90:10 at 15 min; flow rate: 1 mL/ min; UV detection at 254 nm; retention times: **13b**, 11.62 min; *N*-Cbz-Phe-OBzl, 11.98 min.

13b ($CDCl_3$−D_2O/TMS): 0.85 (3H, t, *J* = 7.0 Hz), 1.20−1.27 (10H, m), 1.55−1.58 (2H, m), 3.06 (1H, dd, *J* = 7.0, 14.0 Hz), 3.15 (1H, dd, *J* = 5.5, 14.0 Hz), 3.35 (1H, t, *J* = 8.0 Hz), 3.37 (1H, t, *J* = 8.0 Hz), 3.42−3.55 (2H, m), 3.77−3.85 (1H, m), 4.25 (1H, d, *J* = 8.0 Hz), 4.34 (1H, dd, *J* = 5.5, 12.0 Hz), 4.42 (1H, dd, *J* = 2.0, 12.0 Hz), 4.67 (1H, d, *J* = 5.5, 7.0 Hz), 5.02 (1H, d, *J* = 12.0 Hz), 5.10 (1H, d, *J* = 12.0 Hz), 5.39 (1H, d, *J* = 8.0 Hz), 7.10−7.35 (10 H, m).

Regioselective Esterification of Castanospermine, 14a. To a solution of 56 mg of castanospermine and 150 μL of 2,2,2-trifluoroethyl butyrate in 4 mL of pyridine was added 4 mg of subtilisin-CLEC. The resulting mixture was incubated on a rotary shaker for 72 h at 40 °C. The catalyst was then removed by filtration, and the solvent was evaporated in vacuo. The residue was dissolved in 1 mL of methanol, 10 mL of ethyl acetate was added, and the resulting mixture was cooled in refrigerator overnight to give 14 mg of recovered substrate. The mother liquid was evaporated to give a crude solid that was then purified by a short silica gel column chromatography to afford 48 mg of 1-*O*-butanoylcastanospermine. The structure of product was confirmed by comparison of the NMR spectra with that of reported in the literature.[7]

14b ($CDCl_3$−CD_3OD/TMS): 0.95 (3H, t, *J* = 7.5 Hz), 1.66 (2H, tq, *J* = 7.5, 7.5 Hz), 1.81 (1H, ddt, *J* =1.0, 9.5, 14.5 Hz), 2.00 (1H, t, *J* = 10.5 Hz), 2.08 (1H, dd, *J* = 4.5, 9.5 Hz), 2.21 (1H, dt, *J* = 9.0, 9.5 Hz), 2.36 (2H, t, *J* = 7.5 Hz), 2.30−2.45 (1H, m), 3.15 (1H, dt, *J* = 2.3, 9.0 Hz), 3.19 (1H, dd, *J* = 5.0, 11.0 Hz), 3.42 (1H, t, *J* = 9.0 Hz), 3.50 (1H, t, *J* = 9.0 Hz), 3.62 (1H, ddd, *J* = 5.0, 10.0, 10.0 Hz), 5.30 (1H, ddd, *J* = 1.0, 5.0, 5.0 Hz), 5.10 (1H, d, *J* = 12.0 Hz), 5.39 (1H, d, *J* = 8.0 Hz), 7.10−7.35 (10 H, m).

Regioselective Esterification of 5α-Androstane-3*β*,17*β*-diol, 15a. To a solution of 44 mg of **15a** and 112 μL of 2,2,2-trifluoroethyl butyrate in 10 mL of acetone was added 160 mg of subtilisin-CLEC. The resulting mixture was incubated on a rotary shaker for at 40 °C. The reaction was monitored by GC analysis. After 20 h, the reaction was terminated by filtration of the catalyst. The solvent was evaporated in vacuo to give a residue that was then purified by silica gel column chromatography to afford 39 mg of 17-butyrate **15b** and 7 mg of 3-butyrate **15c**. The structure of the product was identified by comparison with that reported in the literature (Riva, S.; Klibanov, A. M. *J. Am. Chem. Soc.* **1988**, *110*, 3291.)

GC Conditions. DB 1701 15 m × 0.25 mm capillary GC column, 25 μm film thickness (J & W Scientific, Folsom, CA); helium flow at 25 cm/s; temperature program: initial = 220 °C, gradient rate 15 °C/min, final 250 °C for 25 min; retention time: **15a**, 11.67 min; **15b**, 21.85 min; **15c**, 22.51 min.

Regioselective Hydrolysis of Aspartic Acid Dibenzyl Ester, 16a. To a solution of 157 mg of aspartic acid dibenzyl ester, **16a**, in 12 mL of 0.2 M, pH 7.5 phosphate buffer was added 0.3 mL (6 mg) of subtilisin-CLEC. The resulting mixture was incubated on a rotary shaker for 2 h at 25 °C. The yield of monoacid **16b** was 97% on the basis of HPLC analysis. The catalyst was then removed by centrifugation, and the pH of supernatant was adjusted to 6.2 with 1 N HCl and then cooled in a refrigerator overnight to complete the precipitation of the product, which was collected and dried in vacuo. The structure of product was confirmed by ^{1}H NMR. The resonance attributed to the benzylic methylene group of the *β*-ester is a singlet peak at δ 5.05. The corresponding methylene group of α-ester appears as two doublets at δ 5.10 and 5.14.

16b: 2.86 (1H, dd, *J* = 6.5, 18.0 Hz), 2.90 (1H, dd, *J* = 5.0, 18.0 Hz), 3.88 (1H, dd, *J* = 5.0, 6.5 Hz), 5.05 (2H, s), 7.26 (5H, m).

Asp α-Bzl ester: 2.65 (1H, dd, *J* = 4.0, 17.5 Hz), 2.74 (1H, dd, *J* = 6.0, 17.5 Hz), 4.14 (1H, dd, *J* = 4.0, 6.0 Hz), 5.10 (1H, d, *J* = 12.0 Hz), 5.14 (1H, d, *J* = 12.0 Hz), 7.26 (5H, m).

Transesterification of *N*-Acetyl-L-phenylalanine Methyl Ester. To a solution of 221 mg of *N*-acetyl-L-phenylalanine methyl ester in 2 mL of 1-propanol was added 2 mg of subtilisin-CLEC. The resulting mixture was incubated on a rotary shaker for 5 h at 40 °C. The reaction was terminated by filtration of the catalyst when HPLC analysis indicated that the ratio of methyl ester to propyl ester was 2:98 (HPLC conditions:LiChrospher 100 RP 8, 5 μm, 125 × 4 mm; mobile phase = 40% (0.1% trifluoroacetic acid/acetonitrile), 60% (0.1% trifluoroacetic acid/water), flow rate = 1 mL/min, UV detection at 254 nm. Retention times: *N*-acetyl-L-phenylalanine methyl ester, 2.76 min, and *N*-acetyl-L-phenylalanine propyl ester, 5.18 min). The catalyst was washed with ethyl acetate, and the combined organic solvents were evaporated in vacuo to give a residue that was then purified by silica gel column chromatography to give *N*-acetyl-L-phenylalanine propyl ester.

Transesterification of *N*-Cbz-Val-Phe-OMe. To a solution of 82.5 mg of *N*-Cbz-Val-Phe-OMe in 10 mL of ethanol was added 66 mg of subtilisin-CLEC. The resulting mixture was incubated on a rotary shaker for 8 h at 40 °C. The yield was 95% on the basis of HPLC analysis. The catalyst was filtered off and washed with ethanol. The combined organic solvents were evaporated in vacuo to give a residue that was then purified by recrystallization.

HPLC Conditions. Microsorb C_{18} column (4.6 × 50 mm, 5 μm, 300A), mobile phase: gradient, ratio (v/v) of water (with 0.1% trifluoroacetic acid) and acetonitrile (with 0.1% trifluoroacetic acid) was 90:10 at 0 min, 50:50 at 8 min, 90:10 at 10 min; flow rate: 1 mL/ minute; UV detection at 254 nm; retention times: *N*-Cbz-Val-Phe-OMe, 9.70 min; *N*-Cbz-Val-Phe-OEt, 10.46 min.

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