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Enzymatic Removal of Carboxyl Protecting Groups. III. Fast Removal of Allyl and Chloroethyl Esters by *Bacillus subtilis* Esterase (BS2)

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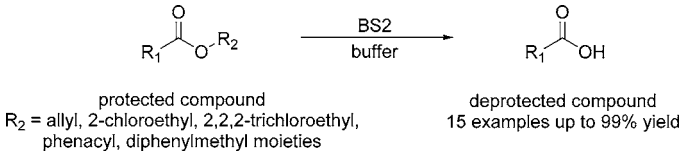

An esterase from *Bacillus subtilis* (BS2) allows the fast and selective removal of allyl, 2-chloroethyl, and 2,2,2-chloroethyl esters under mild conditions in high yields. In addition, BS2 easily hydrolyzes phenacyl esters, while the hydrolysis of sterically hindered diphenylmethyl esters is slow, requiring longer reaction time and higher enzyme/substrate ratio.

Introduction

In recent years, biocatalysis has become an established technology not only for small-scale synthetic applications but also for the industrial production of fine chemicals.[1] Enzymes are increasingly applied for transformations leading to complex target molecules, mainly because of their high selectivity and mild operational conditions. Novel enzymes with improved properties are now accessible as a result of advances in high-throughput screening methods, genomics, and rational protein design.[2]

Among the biocatalysts, hydrolases have attracted special attention for their chemo-, regio-, and enantioselectivities and have found wide application in organic synthesis, in particular for the production of enantiopure organic molecules[3] but also in protecting group chemistry.[4] Several esters have been examined primarily for the preparation of peptides and glycopeptides. Heptyl, 2-*N*-(morpholino)ethyl, choline, (methoxyethoxy)ethyl, and methoxyethyl esters are examples of enzymatically removable protecting groups, and their applications are summarized in the book of Greene and Wuts.[5]

Recently, we demonstrated that an esterase from *Bacillus subtilis* (BS2, EC 3.1.1.1) and lipase A from *Candida antarctica* (CAL-A, EC 3.1.1.3) were able to cleave *tert*-butyl esters from a variety of substrates including N-protected amino acids.[6] Both enzymes include a GGG(A)X motif in their oxyanion binding pocket, and as has been reported, such enzymes are able to react on esters of tertiary alcohols.[7] Moreover, we demonstrated that BS2 and CAL-A are versatile reagents for the efficient removal of methyl and benzyl esters under mild conditions that avoid side reactions.[8] These remarkable results prompted an investiga-

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(1) Panke, S.; Held, M.; Wubbolts, M. *Curr. Opin. Biotechnol.* **2004**, *15*, 272−279.

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(3) Bornscheuer, U. T.; Kazlauskas, R. J. *Hydrolases in Organic Synthesis: Regio- and Stereoselective Biotransformations*, 2nd ed.; Wiley-VCH: Weinheim, Germany, 2005.

(4) (a) Kadereit, D.; Waldmann, H. *Chem. Rev.* **2001**, *101*, 3367−3396. (b) Waldmann, H.; Sebastian, D. *Chem. Rev.* **1994**, *94*, 911−937.

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(7) (a) Henke, E.; Pleiss, J.; Bornscheuer, U. T. *Angew. Chem., Int. Ed.* **2002**, *41*, 3211−3213. (b) Henke, E.; Bornscheuer, U. T.; Schmid, R. D.; Pleiss, J. *ChemBioChem* **2003**, *4*, 485−493.

(8) Barbayianni, E.; Fotakopoulou, I.; Schmidt, M.; Constantinou-Kokotou, V.; Bornscheuer, U. T.; Kokotos, G. *J. Org. Chem.* **2005**, *70*, 8730−8733.

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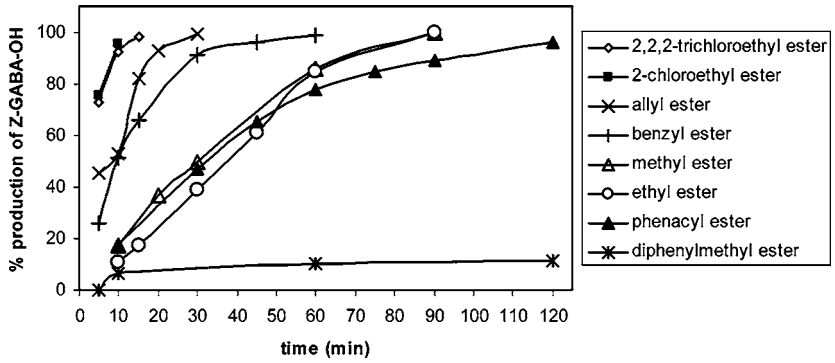
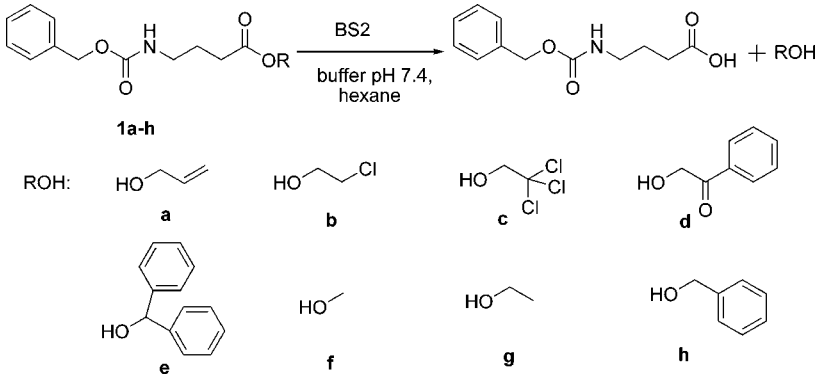


FIGURE 1. Time course for the hydrolysis of various esters of Z-GABA by BS2 (enzyme/substrate 1:4 w/w). Conditions: phosphate buffer (pH 7.4)/hexane 7:1, 37 °C. The hydrolysis of phenacyl ester was monitored by the production of the corresponding alcohol.

SCHEME 1. Hydrolysis of Various Esters of γ-(Benzyloxycarbonylamino)butanoate by BS2



tion to determine systematically the generality and scope of the enzymatic removal of carboxyl protecting groups. Trying to improve our protocol for the enzymatic removal of carboxyl protecting groups from sensitive esters, in terms of velocity and selectivity, we report here our studies on the removal of various carboxyl protecting groups, such as allyl, chloroethyl, phenacyl, and diphenylmethyl esters, by BS2 and CAL-A.

Results and Discussion

In the present work, we have chosen to study the enzymatic hydrolysis of five common carboxyl protecting groups, namely, allyl esters, 2-chloroethyl esters, 2,2,2-trichloroethyl esters, phenacyl esters, and diphenylmethyl esters. Methods for their introduction and removal are summarized in two books.[5,9] The most common cleavage method for allyl esters is the use of Pd^0.[10] Papain has also reported to be able to cleave allyl esters.[11] 2-Chloroethyl esters have been cleaved under a variety of conditions involving nucleophilic addition,[12] while 2,2,2-trichloroethyl esters may be removed using Zn in AcOH or Zn in THF buffered at pH 4.2−7.2.[13] Phenacyl esters may be cleaved under various conditions, including Zn in AcOH or hydrogenolysis.[14] Recently, it has been reported that phenacyl esters are cleaved by photolysis[15] or using Mg/AcOH.[16] Diphenylmethyl esters can be removed under various acidic conditions, as well as by hydrogenolysis.[14b,17]

Allyl (**1a**), 2-chloroethyl (**1b**), 2,2,2-trichloroethyl (**1c**), phenacyl (**1d**), and diphenylmethyl (**1e**) esters of γ-(benzyloxycarbonylamino)butanoate were prepared, and their hydrolysis by BS2 in a mixture of buffer/hexane (7:1) containing a small amount of methanol was studied (Scheme 1). Their hydrolysis was monitored by HPLC, and the results are presented in Figure 1. For comparison, the hydrolysis of methyl (**1f**), ethyl (**1g**), and benzyl (**1h**) esters of γ-(benzyloxycarbonylamino)butanoate was also studied. As shown in Figure 1, 2-chloroethyl and 2,2,2-trichloroethyl esters were quickly hydrolyzed in less than 15 min, while the allyl ester was hydrolyzed quantitatively within 30 min. The hydrolysis of benzyl ester was slightly slower than that of the allyl ester, and it went to completion in 1 h. Methyl, ethyl, as well as phenacyl esters showed similar curves, and their hydrolysis took from 1.5 to 2 h. The corresponding diphenylmethyl ester was not hydrolyzed more than 10% within 2 h.

Since the hydrolysis of 2-chloroethyl, 2,2,2-trichloroethyl, allyl, and benzyl esters was quick under the conditions used, the hydrolysis of these esters using a lower ratio of enzyme/substrate (1:10) was studied. It is clear that both 2-chloroethyl and 2,2,2-trichloroethyl esters were readily hydrolyzed (Figure

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(10) Kunz, H.; Waldmann, H. *Angew. Chem., Int. Ed. Engl.* **1984**, *23*, 71−72.
(11) Xaus, N.; Clapes, P.; Bardaji, E.; Torres, J. L.; Jorba, X.; Mata, J.; Valencia, G. *Tetrahedron* **1989**, *45*, 7421−7426.
(12) Eckert, H.; Ugi, I. *Angew. Chem., Int. Ed. Engl.* **1976**, *15*, 681.
(13) (a) Woodward, R. B.; Heusler, K.; Gosteli, J.; Naegeli, P.; Oppolzer, W.; Ramage, R.; Ranganathan, S.; Vorbruggen, H. *J. Am. Chem. Soc.* **1966**, *88*, 852−853. (b) Just, G.; Grozinger, K. *Synthesis* **1976**, 457−458.
(14) (a) Hendrickson, J. B.; Kandall, C. *Tetrahedron Lett.* **1970**, *11*, 343−344. (b) Stelakatos, G. C.; Paganou, A.; Zervas, L. *J. Chem. Soc. C* **1966**, 1191−1199.
(15) Banerjee, A.; Falvey, D. E. *J. Org. Chem.* **1997**, *62*, 6245−6251.
(16) Kokinaki, S.; Leondiadis, L.; Ferderigos, N. *Org. Lett.* **2005**, *7*, 1723−1724.
(17) (a) Haslam, E.; Haworth, R. D.; Makinson, G. K. *J. Chem. Soc.* **1961**, 5153−5156. (b) Hiskey, R. G.; Smithwick, E. L. *J. Am. Chem. Soc.* **1967**, *89*, 437−441.

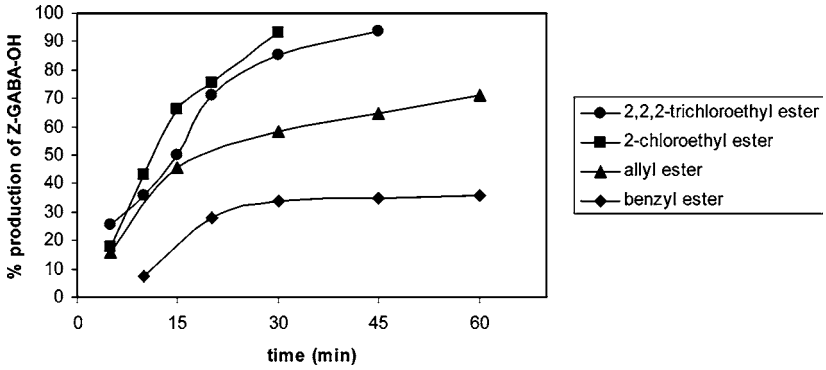
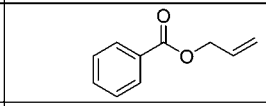
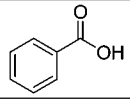
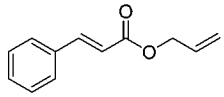
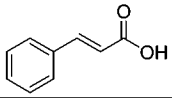
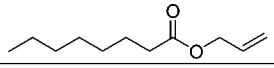
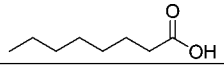
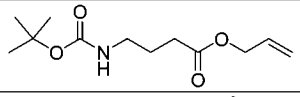
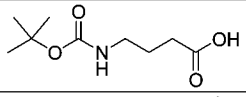
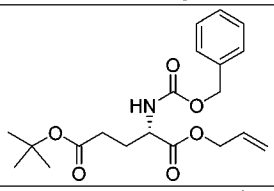
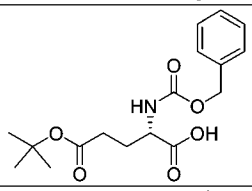
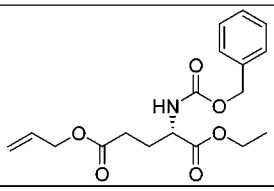
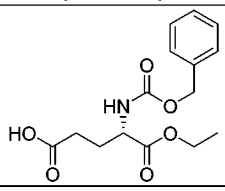
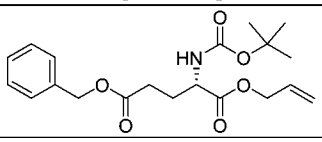
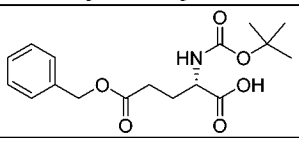


FIGURE 2. Time course for the hydrolysis of various esters of Z-GABA by BS2 (enzyme/substrate 1:10 w/w). Conditions: phosphate buffer (pH 7.4)/ hexane 7:1, 37 °C.

2). Under the present conditions, the allyl ester was hydrolyzed easier than the benzyl ester.

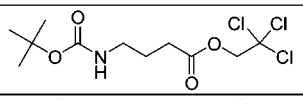
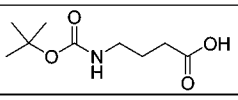
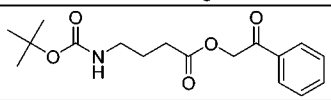
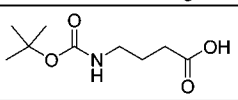
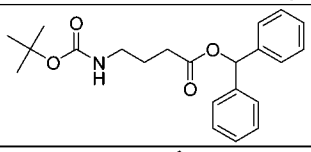
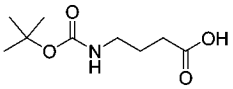
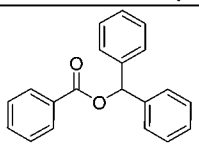
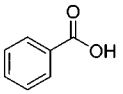
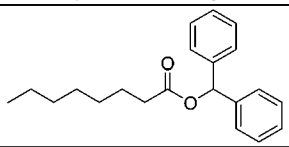
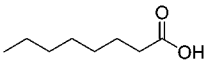
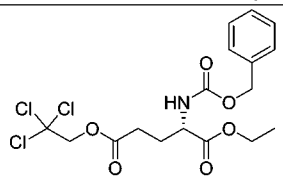
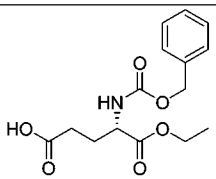
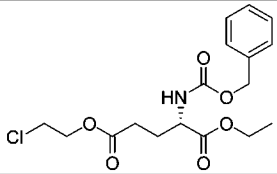
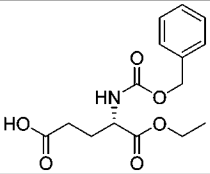
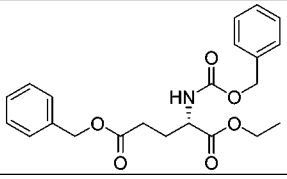
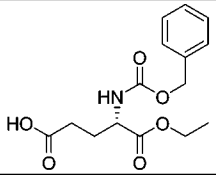
The observation that the allyl ester of Z-GABA was quickly hydrolyzed by BS2 prompted us to prepare a variety of allyl esters of simple carboxylic acids, N-protected amino acids, and trifunctional substrates. The results of their hydrolysis by BS2 and CAL-A are summarized in Table 1. Allyl esters of benzoic and cinnamic acid were hydrolyzed by BS2 in high yields within 3 h (entries 1 and 2, Table 1). However, the hydrolysis of allyl octanoate (entry 3, Table 1) was slow, and the product was isolated in moderate yield (62%) even after 24 h using a higher enzyme/substrate ratio (1:2). The allyl ester of Boc-GABA was quickly hydrolyzed quantitatively (entry 4, Table 1). Selective removal of the allyl group in the presence of *tert*-butyl, ethyl,

TABLE 1. Hydrolysis of Various Allyl Esters by BS2 and CAL-A

entry	substrate	product	BS2			CAL-A		
			time [h]	enzyme\ substrate	yield[a] [%]	time [h]	enzyme: substrate	yield[a] [%]
1			3	1/4	92	48	1/1	35
2			3	1/4	83	48	1/1	60
3			24	1/2	62	24	1/1	87
4			1	1/4	96	48 48	1/4 1/1	66 85
5			1 3	1/4 1/4	74 87	72	1/1	28
6			1	1/4	80	nd[b]	nd[b]	nd[b]
7			1	1/4	[c]	4	1/1	50[d]

[a] Yield of isolated product. [b] Not determined. [c] A mixture of benzyl *N*-*tert*-butoxycarbonyl-γ-glutamate and allyl *N*-*tert*-butoxycarbonyl-α-glutamate was obtained. [d] 50% starting material was recovered.

TABLE 2. Hydrolysis of 2-Chloroethyl, 2,2,2-Trichloroethyl, Phenacyl, and Diphenylmethyl Esters by BS2

entry	substrate	product	BS2		
			time [h]	enzyme\substrate	yield[a] [%]
1			1	1/4	98
2			1.5	1/4	92
3			48	1/1	99
4			48	1/1	97
5			48	1/1	48
6			0.5	1/4	99
7			0.5	1/4	65
8			1	1/4	73

[a] Yield of isolated product.

and benzyl groups was studied in the case of glutamic acid derivatives. As shown in Table 1 (entry 5), the product was obtained in 74 and 87% yields after 1 and 3 h, respectively. Similarly, the product of the selective allyl ester removal was obtained in 80% yield (entry 6, Table 1), while the ethyl ester remained intact. BS2 was unable to differentiate allyl and benzyl groups. A mixture of products, namely, benzyl *N*-*tert*-butoxycarbonyl-γ-glutamate and allyl *N*-*tert*-butoxycarbonyl-α-glutamate, was obtained. The similar rates of hydrolysis for the removal of allyl and benzyl groups do not allow selective cleavage. *tert*-Butyl esters are slowly hydrolyzed by BS2 requiring higher enzyme/substrate ratio (1:1),[6] thus allyl esters may be selectively deprotected in the presence of *tert*-butyl esters at an appropriate choice of reaction conditions (see entry 5, Table 1).

The hydrolysis of allyl esters by CAL-A was slow, requiring 24−72 h and a 1:1 ratio of substrate/enzyme (Table 1). It is important to note that, using allyl octanoate as substrate, CAL-A produced the corresponding acid in higher yield (87%) than that obtained by BS2 (62%) (entry 3, Table 1). Boc-GABA was isolated in 66% yield after 48 h when a 1:4 enzyme/substrate ratio was employed, while the yield was increased to 85% using a higher enzyme/substrate ratio (1:1). Contrary to the results obtained with BS2, using CAL-A, the allyl ester was removed selectively in the presence of the benzyl ester, although in moderate yield (50%) (entry 7, Table 1).

To explore the utility of other carboxyl protecting groups for synthetic purposes and their cleavage selectivity, we prepared a series of 2,2,2-trichloroethyl, 2-chloroethyl, phenacyl, and

diphenylmethyl esters of various acids. The results of their hydrolysis by BS2 are summarized in Table 2. 2,2,2-Trichloroethyl and phenacyl esters were removed from Boc-GABA within 1 and 1.5 h, respectively, in quantitative yields (entries 1 and 2, Table 2). Diphenylmethyl esters require longer reaction time and higher enzyme/substrate ratios (entries 3−5, Table 2). However, benzoic acid and Boc-GABA were isolated in almost quantitative yields (entries 3 and 4, Table 2). 2,2,2-Trichloroethyl and 2-chloroethyl esters were selectively removed in the presence of ethyl ester, and the products were isolated in 99 and 65% yields, respectively, after 30 min (entries 6 and 7, Table 2). In addition, the benzyl ester was selectively removed leading to the isolation of ethyl *N*-benzyloxycarbonyl-α-glutamate in 73% (entry 8, Table 2).

From the above results, it is clear that the chloroethyl group, containing either one or three chlorine atoms, is easily hydrolyzed by BS2. The increased carbonyl reactivity of the ester group due to the inductive effect of chlorine atoms is in accordance with the ease hydrolysis of such esters. The high reactivity of BS2 against allyl esters may be attributed to the presence of the double bond. A similar increase in activity and enantioselectivity was reported by us several years ago, in the kinetic resolution of chiral carboxylic acids. In that case, the use of vinyl esters was significantly better compared to the ethyl ester, despite the fact that the ethyl and the vinyl ester have the same carbon length and differed only in the absence or presence of the double bond.[18] The steric hindrance of diphenylmethyl esters may explain their slow hydrolysis by BS2. However, these esters are indeed hydrolyzed by BS2, indicating that esterases bearing a GGG(A)X motif in their oxyanion binding pocket are able to cleave esters of sterically hindered alcohols, not only esters of tertiary alcohols.

To our knowledge, there is no report on the removal of phenacyl and diphenylmethyl esters by any enzyme. Although allyl esters have been reported to be cleaved by papain,[11] no esterases or lipases have been studied up to now for applications in deprotection. A few examples of employment of chloroethyl esters for enzymatic resolution are known.[19] The results of the present work show that esterases and lipases, such as BS2 and CAL-A, may find applications in deprotection chemistry of various esters. Up to now, the use of CAL-A for synthetic purposes, despite its unique properties, has attracted less interest, in comparison to CAL-B, which is one of the mostly employed hydrolases in biocatalysis.[20] This may be attributed to the fact that a relatively high CAL-A loading seems to be required, in particular when compared with CAL-B. However, during the last years, many remarkable synthetic applications of CAL-A have been presented.[20] BS2 is an enzyme very similar to a *p*-nitrobenzyl esterase from *Bacillus subtilis* (BsubpNBE) differing only in 11 amino acid residues.[21] According to our results presented here and our previous results,[6,8] it is clear that BS2 is a very attractive enzyme for applications in protecting group chemistry.

TABLE 3. Recommendations for the Hydrolysis of Various Esters by BS2

entry	ester	enzyme/substrate	time (h)
1	2-chloroethyl	1:4	0.25−1
2	2,2,2-trichloroethyl	1:4	0.25−1
3	allyl	1:4	0.5−3
4	benzyl	1:4	1−24
5	methyl	1:4	1.5−24
6	phenacyl	1:4	1.5−24
7	ethyl	1:4	1.5−24
8	diphenylmethyl	1:1	≥48
9	*tert*-butyl	1:1	≥48

The rate of the hydrolysis of esters depends on the nature of both the alcohol and the carboxylic acid. In general, the recommended conditions for the ratio BS2/substrate, as well as the expected reaction times, are summarized in Table 3. A mixture of phosphate buffer/hexane/methanol (7:1:0.1) may be used as a solvent at 37 °C. To improve the solubility, the ratio of phosphate buffer/hexane may be changed to 1:1 or a double volume of methanol may be used. Toluene may be alternatively used instead of hexane. For substrates containing long chains, higher yields of products may be achieved employing CAL-A instead of BS2.

In conclusion, we have demonstrated that allyl, 2-chloroethyl, 2,2,2-trichloroethyl, phenacyl, and diphenylmethyl protecting groups can be efficiently cleaved by an esterase from *Bacillus subtilis* (BS2). In particular, the enzymatic quick and selective removal of allyl, 2-chloroethyl, and 2,2,2-trichloroethyl esters under mild conditions in high yields makes them attractive protecting groups for applications in organic synthesis of sensitive molecules, where conventional chemical methods cannot be applied because of incompatibility.

Experimental Section

General Method for Enzymatic Hydrolysis. To a stirred solution of the substrate (0.15−0.20 mmol) in *n*-hexane (1 mL) and CH_3OH (100 μL) was added a solution of the enzyme (12−50 mg, as indicated in Tables 1 and 2) in phosphate buffer (7 mL, 100 mM, pH 7.4). The reaction mixture was stirred at 37 °C. After acidification until pH 6 and extraction with EtOAc (3 × 5 mL), the organic layers were combined and washed with 5% $NaHCO_3$ (3 × 5 mL). The aqueous layer was acidified until pH 6 and extracted with EtOAc (3 × 10 mL). The combined organic layers were dried over Na_2SO_4, and the organic solvent was removed under reduced pressure to give the product.

All products of the enzymatic hydrolysis were identified by their analytical data in comparison with authentic samples.

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Supporting Information Available: Experimental procedures for the synthesis of esters and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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