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Enzymatic Removal of Carboxyl Protecting Groups. 2. Cleavage of the Benzyl and Methyl Moieties

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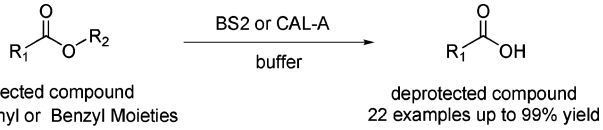

Enzymes are versatile reagents for the efficient removal of methyl and benzyl protecting groups. An esterase from *Bacillus subtilis* (BS2) and a lipase from *Candida antarctica* (CAL-A) allow a mild and selective removal of these moieties in high yields without affecting other functional groups.

Introduction

The problem of functional group incompatibility is especially acute in the design and construction of polyfunctional organic molecules such as peptides, oligosaccharides, glycopeptides, and nucleotides and in general natural products, which often require a scaffold of protecting groups.[1] Methyl and benzyl esters are the most common of the carboxylic protecting groups because of their ease of introduction. However, classical methods for the removal are limited mainly by the stability of polyfunctional substrates to basic and hydrogenation conditions. The application of biocatalysts in the protecting group chemistry may offer excellent alternatives to chemical methods, because enzymes (i) carry out highly chemo- and regioselective transformations, (ii) usually operate at neutral pH values, and (iii) combine a high selectivity for the reactions they catalyze and the structure they recognize with a broad substrate tolerance.[2] Among the biocatalysts, hydrolases have attracted special interest for their chemo-, regio-, and enantioselectivities and have found interesting applications in organic synthesis, in particular for the production of enantiopure organic molecules.[3] Indeed, some hydrolases were already found suitable for the selective removal of protecting groups.[2] Thermitase, carboxypeptidase A, α-chymotrypsin, porcine liver esterase, and a lipase from *Mucor javanicus* have been studied for the hydrolysis of peptide esters.[4,5] *Candida rugosa* lipase,[6] penicillin G acylase,[7] an immobilized form of *Candida antarctica* lipase B,[8] and *Microbacterium* sp. strain 7-1W cells[9] were demonstrated to cleave methyl esters of various substrates. Herein, we report a method for the enzymatic hydrolysis of methyl and benzyl esters from a variety of substrates under extremely mild conditions that avoid side reactions.

Very recently, we demonstrated that an esterase (BS2, EC 3.1.1.1) and a lipase (CAL-A, EC 3.1.1.3) were able to remove *tert*-butyl esters from a variety of substrates including *N*-protected amino acids.[10] Both enzymes include a GGG(A)X motif and are able to react on tertiary

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(1) (a) Greene, T. W.; Wuts, P. G. M. *Protecting Groups*; Georg Thieme Verlag: Stuttgart, 1994. (b) Kocienski, P. J. *Protecting Groups in Organic Synthesis*, 3rd ed.; Wiley and Sons: New York, 1999.
(2) (a) Kadereit, D.; Waldmann, H. *Chem. Rev.* **2001**, *101*, 3367−3396. (b) Waldmann, H.; Sebastian, D. *Chem. Rev.* **1994**, *94*, 911−937.
(3) Bornscheuer, U. T.; Kazlauskas, R. J. *Hydrolases in Organic Synthesis−Regio- and Stereoselective Biotransformations*, 2nd ed.; Wiley-VCH: Weinheim, 2005.
(4) Reissmann, S.; Greiner, G. *Int. J. Pept. Protein Res.* **1992**, *40*, 110−113.
(5) Braun, P.; Waldmann, H.; Kunz, H. *Synlett* **1992**, *1*, 39−40.
(6) Bellezza, F.; Cipiciani, A.; Cruciani, G.; Fringuelli, F. *J. Chem. Soc., Perkin Trans. 1* **2000**, 4439−4444.
(7) Pohl, T.; Waldmann, H. *Tetrahedron Lett.* **1995**, *36* (17), 2963−2966.
(8) Kurokawa, M.; Sugai, T. *Bull. Chem. Soc. Jpn.* **2004**, *77*, 1021−1025.
(9) Honda, K.; Sakamoto, K.; Kita, S.; Kataoka, M.; Shimizu, S. *Biosci. Biotechnol. Biochem.* **2003**, *67*, 192−194.

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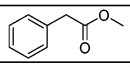
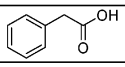
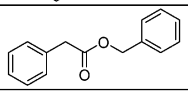
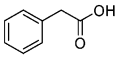
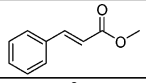
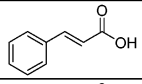
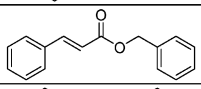
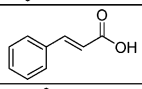
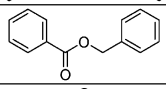
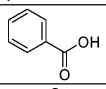
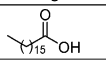
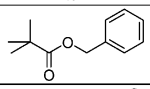
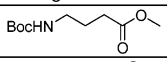
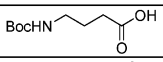
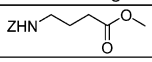
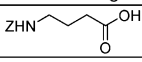
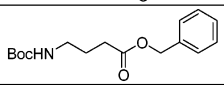
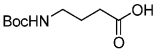
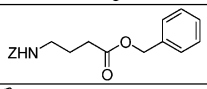
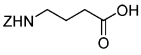
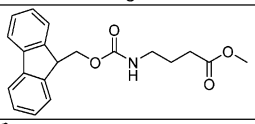
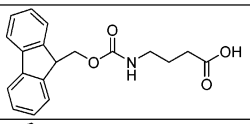
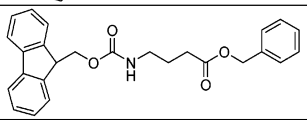
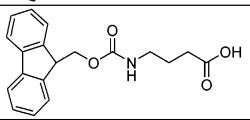
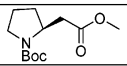
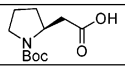
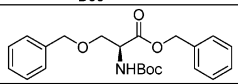
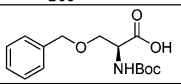
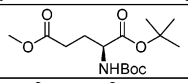
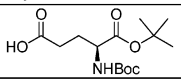
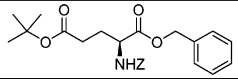
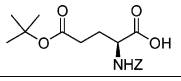
TABLE 1. Hydrolysis of Methyl and Benzyl Esters by BS2 and CAL-A

Entry	Substrate	Product	BS2		CAL-A	
			Time [h]	Yield[a] [%]	Time [h]	Yield[a] [%]
1			24	99	24	91
2			24	98	24	99
3			24	80	48	41
4			24	95	24	68
5			24	95	48	86
6			48	21	48	62
7			48	30	48	-
8			24	98	48	95
9			24	91	24	96
10			24	98	48	81
11			24	84	24	93
12			48	75	48	41
13			48	51	72	-
14			24	90	48	-
15			24	92	48	32
16			24	76	48	31[b]
17			48	60	48	39

[a] Yield of isolated product. [b] 2-*tert*-Butoxycarbonylamino-pentanedioic acid 5-methyl ester and 2-*tert*-butoxycarbonylamino-pentanedioic acid were also isolated in 19% and 20% yield, respectively.

alcohols.[11] These remarkable results prompted an investigation to determine systematically the generality and scope of the removal of ester protecting groups by BS2 and CAL-A.

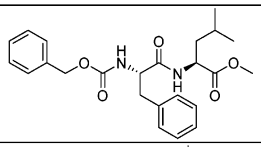
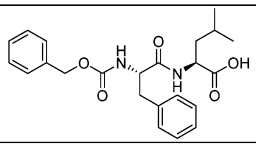
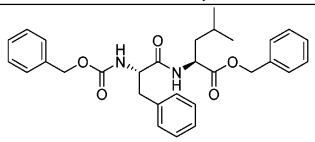
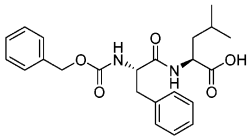
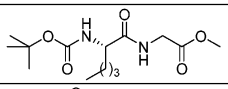
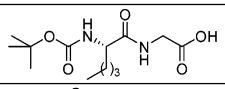
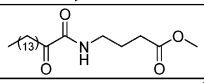
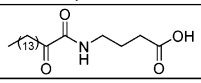
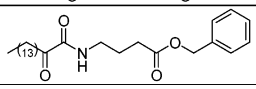
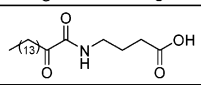
Results and Discussion

A variety of methyl and benzyl esters of simple carboxylic acids and protected amino acids were tested as substrates of BS2 and CAL-A, and the results are summarized in Table 1. Both enzymes hydrolyzed methyl and benzyl ester of phenylacetic acid in excellent to quantitative yields (entries 1 and 2, Table 1). BS2 easily hydrolyzed both esters of cinnamic acid, whereas CAL-A hydrolyzed the benzyl ester in lower yield and the methyl ester in low yield, even after longer reaction time (entries

(10) Schmidt, M.; Barbayianni, E.; Fotakopoulou, I.; Höhne, M.; Constantinou-Kokotou, V.; Bornscheuer, U. T.; Kokotos, G. *J. Org. Chem.* **2005**, *70*, 3737−3740. BsubpNBE reported therein, is identical to BS2 used in the present work.

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

TABLE 2. Enzymatic Removal of Methyl and Benzyl Esters from Peptides and Phospholipase A_2 Inhibitors

Entry	Substrate	Product	BS2		CAL-A	
			Time [h]	Yield[a] [%]	Time [h]	Yield[a] [%]
1			24	78	48	69
2			24	84	48	36
3			48	87	72	74
4			72	61	72	75
5			72	60	72	66

[a] Yield of isolated product.

3 and 4, Table 1). Both enzymes hydrolyzed the benzyl ester of benzoic acid in high yields (entry 5, Table 1). Using methyl palmitate as a substrate (entry 6, Table 1), BS2 produced the corresponding acid in very low yield, and hydrolysis by CAL-A led to 62% isolated yield. CAL-A, as a lipase, accepts long chain substrates, while BS2, as an esterase, fails to efficiently hydrolyze such substrates. Benzyl pivalate (entry 7, Table 1) was a poor substrate for BS2 and remained intact by treatment with CAL-A.

BS2 and CAL-A hydrolyzed methyl and benzyl esters of Boc- and Z-protected amino acids in excellent to quantitative yields (entries 8−11, Table 1). Fmoc-GABA-OMe (entry 12, Table 1) was hydrolyzed by BS2 in high yields but in low yields by CAL-A. However, hydrolysis of Fmoc-GABA-OBn (entry 13, Table 1) by BS2 produced the corresponding acid in moderate yield, whereas CAL-A failed to hydrolyze the substrate even after 72 h. The removal of the methyl ester group from Fmoc-protected derivatives is impossible by classical chemical methods, apart from the one using trimethyltin hydroxide, which was recently introduced by Nicolaou.[12] BS2 cleaved the methyl ester of Boc-homoproline (entry 14, Table 1) in high yield, but CAL-A was unable to hydrolyze this substrate. It should be noticed that the three urethane-type N-protective groups, Boc-, Z-, and Fmoc-, widely used in peptide chemistry, remained intact throughout the enzymatic hydrolysis. Thus, we investigated the effects of our enzymatic protocols on trifunctional substrates (entries 15−17, Table 1). BS2 selectively removes benzyl ester in the presence of benzyl ether (entry 15, Table 1), an achievement not possible by hydrogenation. Although BS2 is able to cleave *tert*-butyl esters,[10] methyl and benzyl esters may be selectively removed from particular substrates in the presence of *tert*-butyl ester (entries 16 and 17, Table 1). CAL-A was unable to deprotect efficiently the trifunctional substrates leading to low yields and mixture of products (entries 15−17, Table 1).

The scope of the enzymatic protocols was further expanded by testing a variety of peptides and other sensitive molecules (Table 2). BS2 easily cleaved methyl and benzyl esters from dipeptides in high yields (entries 1−3, Table 2). CAL-A required longer reaction times and in general led to lower yields (entries 1 and 3, Table 2) or very low yield (entry 2, Table 2). Although we have found that BS2 is active toward a range of simple amides,[13] it seems that it cannot hydrolyze the amide bonds of dipeptides.

It has been recently demonstrated that long chain 2-oxoamides containing a free carboxyl group are potent inhibitors of human GIVA phospholipase A_2, exhibiting very interesting antiinflammatory and analgesic properties.[14] Methyl and benzyl esters of such derivatives (entries 4 and 5, Table 2) cannot be deprotected by chemical methods because both alkaline conditions and hydrogenation destroy the reactive 2-oxoamide functionality, and thus they are challenging substrates for the enzymatic procedures. It was very intriguing to find that both enzymes cleaved methyl and benzyl esters of long chain 2-oxoamides after 72 h incubation, producing the free carboxylic acid in satisfactory isolated yield. CAL-A hydrolyzed both long chain substrates in higher yields than with BS2, in agreement with the results obtained with methyl palmitate (entry 6, Table 1).

(12) Nicolaou, K. C.; Estrada, A. A.; Zak, M.; Hyup Lee, S.; Safina, B. S. *Angew. Chem.* **2005**, *117*, 1402−1406; *Angew. Chem., Int. Ed.* **2005**, *44*, 1378−1382.

(13) Henke, E.; Bornscheuer, U. T. *Anal. Chem.* **2003**, *75*, 255−260.

(14) (a) Kokotos, G.; Six, D. A.; Loukas, V.; Smith, T.; Constantinou-Kokotou, V.; Hadjipavlou-Litina, D.; Kotsovolou, S.; Chiou, A.; Beltzner, C. C.; Dennis, E. A. *J. Med. Chem.* **2004**, *47*, 3615−3628. (b) Kokotos, G.; Kotsovolou, S.; Six, D. A.; Constantinou-Kokotou, V.; Beltzner, C. C.; Dennis, E. A. *J. Med. Chem.* **2002**, *45*, 2891−2893.

Although it appears that our methodology requires relatively large amounts of enzyme, it must be noted that these are crude preparations. In case of BS2 the protein content of the lyophilized *E. coli* extract is approximately 40% and the overall esterase content is estimated to be approximately 15%. Still, these values are higher than those reported for most commercial biocatalyst preparations.[3] In addition, the preparation does not contain any competing hydrolase activity, which allows the use of the cheap crude preparation.

In conclusion, using a wide variety of substrates it has been demonstrated that methyl and benzyl ester protecting groups can be cleaved by BS2 and CAL-A under extremely mild conditions, avoiding side reactions and leading to high isolated yields of the corresponding carboxylic acid. In general, BS2 provides products in higher yields than CAL-A, exhibiting broader substrate tolerance. CAL-A may be used in cases where BS2 is not effective, for example, in the hydrolysis of long chain substrates. Most importantly, for several examples no alternative chemical method exists. Thus, the presently introduced enzymatic protocols present general applicability, may serve as tools in cases where chemical methods are incompatible, and are anticipated to find wide use in organic synthesis.

Experimental Section

Synthesis of Substrates. The synthesis of methyl and benzyl esters of carboxylic acids and protected amino acids is described in Supporting Information.

Benzyl 4-(2-Hydroxyhexadecanamido)butanoate. To a stirred solution of 2-hydroxy-hexadecanoic acid (0.27 g, 1 mmol) and HCl·H-GABA-OBn (0.23 g, 1 mmol) in CH_2Cl_2 (10 mL) were added Et_3N (0.3 mL, 2.2 mL) and subsequently 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (WSCI) (0.21 g, 1.1 mmol), and 1-hydroxybenzotriazole (HOBt) (0.14 g, 1 mmol) at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and overnight at room temperature. The solvent was evaporated under reduced pressure, and EtOAc (10 mL) was added. The organic layer was washed consecutively with brine, 1 N HCl, brine, 5% $NaHCO_3$, and brine, dried over Na_2SO_4, and evaporated under reduced pressure. The residue was purified by column chromatography using $CHCl_3/CH_3OH$ (95:5) as eluent. Yield 0.27 g (61%); white solid; mp 89−91 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.36 (m, 5H), 6.72 (t, 1H, J = 5.8 Hz), 5.13 (s, 2H), 4.06 (m, 1H), 3.32 (m, 2H), 2.85 (b, 1H), 2.42 (t, 2H, J = 7 Hz), 1.95−1.60 (m, 4H), 1.26 (m, 24H), 0.88 (t, 3H, J = 6.6). ^{13}C NMR (50 MHz, $CDCl_3$): δ 174.1, 173.1, 135.7, 128.6, 128.3, 128.2, 72.1, 66.4, 38.4, 34.9, 31.9, 31.6, 29.7, 29.62, 29.58, 29.5, 29.4, 29.3, 25.0, 24.6, 22.7, 14.1. Anal. Calcd for $C_{27}H_{45}NO_4$: C, 72.44; H, 10.13; N, 3.13. Found: C, 72.23; H, 10.35; N, 3.25.

Benzyl 4-(2-Oxohexadecanamido)butanoate (5, Table 2). To a stirred solution of 2-hydroxy-amide (0.17 g, 0.4 mmol) in CH_2Cl_2 (10 mL) was added Dess−Martin periodinane (0.21 g, 0.5 mmol). The reaction mixture was stirred for 1 h at room temperature. The organic layer was washed with 5% $NaHCO_3$ (10 mL) and then the organic solvent was dried over Na_2SO_4 and evaporated under reduced pressure. The residue was purified by column chromatography using petroleum ether (60−80 °C)/EtOAc (8:2) as eluent. Yield 0.15 g (91%); white solid; mp 68−70 °C. ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.36 (m, 5H), 7.11 (t, 1H, J = 5.8 Hz), 5.13 (s, 2H), 3.35 (m, 2H), 2.90 (t, 2H, J = 7.4 Hz), 2.24 (t, 2H, J = 7.4 Hz), 1.91 (m,2H), 1.60 (m, 2H), 1.26 (m, 22H), 0.89 (t, 3H, J = 6.2 Hz). ^{13}C NMR (50 MHz, $CDCl_3$): δ 199.2, 172.7, 160.3, 135.7, 128.6, 128.31, 128.25, 66.5, 38.6, 36.7, 31.9, 31.5, 29.6, 29.5, 29.4, 29.3, 29.0, 24.4, 23.1, 22.7, 14.1. Anal. Calcd for $C_{27}H_{43}NO_4$: C, 72.77; H, 9.73; N, 3.14. Found: C, 72.86; H, 9.57; N, 3.23.

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 (50 mg) in phosphate buffer (9 mL, 50 mM pH 7.4). The reaction mixture was stirred for 24−72 h 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.

(*S*)-2-(2-(*tert*-Butoxycarbonyl)hexanamido)acetic Acid. Oil; $[\alpha]_D$ = +3.0 (*c* 1.6, $CHCl_3$). ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.85 (br s, 1H), 7.18 (m, 1H), 5.43 (d, 1H, J = 9.2 Hz), 4.33 (m, 1H), 4.07 (m, 2H), 1.69 (m, 2H), 1.43 (s, 9H), 1.32 (m, 4H), 0.89 (t, 1H, J = 6.6 Hz). ^{13}C NMR (50 MHz, $CDCl_3$): δ 173.1, 172.5, 156.1, 80.7, 54.2, 41.3, 32.5, 28.2, 27.6, 22.3, 13.9. Anal. Calcd for $C_{13}H_{24}N_2O_5$: C, 54.15; H, 8.39; N, 9.72. Found: C, 54.38; H, 8.44; N, 9.59.

The data for the other products of enzymatic hydrolysis are summarized in Supporting Information.

Expression of Recombinant Esterase BS2 in *Escherichia coli.* Clones containing the expression vector encoding the gene for esterase BS2 were used to inoculate 5 mL of an overnight culture (LB media supplemented with 100 μg/mL ampicillin). Then, 500 μL of the overnight culture was used to inoculate 500 mL of LB-Amp. The culture was incubated at 37 °C and 200 rpm to a cell density of OD_{600} 0.4−0.6, and enzyme expression was induced by addition of L-rhamnose solution (end concentration 0.2% w/v). After 4 h of further incubation at 37 °C, cells were harvested by centrifugation (15 min, 4 °C, 8000 *g*) and washed twice with 50 mL of sodium phosphate buffer (50 mM, pH 7.5). Cells were resuspended in 20 mL of phosphate buffer and disrupted by sonification with cooling on ice. Cell debris was removed by centrifugation (15 min, 4 °C, 8000 *g*), and the supernatant was frozen at −80 °C and then lyophilized. Specific activities of crude extracts were determined spectrophotometrically using *p*-nitrophenyl acetate for activity and the Bradford reagent for protein content.

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

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