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Bacillus subtilis Esterase (BS2) and its Double Mutant Have Different Selectivity in the Removal of Carboxyl Protecting Groups

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Abstract: An esterase from *Bacillus subtilis* (BS2) and its double mutant E188W/M193C quickly hydrolyze *n*-butyl, *n*-propyl, methoxyethyl and allyl esters. The wild-type BS2 preferentially removes such esters from the γ-position of glutamate diesters, while the engineered enzyme has a reversed selectivity removing esters from the α-position of glutamate diesters. Automated docking and molecular dynamic simulations were performed to understand the molecular reason for the different regioselectivity.

Keywords: *Bacillus subtilis* esterase; carboxyl protecting groups; chemoselectivity; enzymatic hydrolysis; regioselectivity

Introduction

In recent years, biocatalysis has attracted a great deal of interest because of its environmental friendliness, decreased production costs, lower consumption of resources and lower production of waste.[1] A variety of enzymes and microorganisms have found interesting applications in the pharmaceutical, agrochemical and chemical industries.[1,2] In particular, esterases (E.C. 3.1.1.1) and lipases (E.C. 3.1.1.3) are versatile biocatalysts because they are stable, may have a broad substrate spectrum and frequently exhibit interesting chemo-, regio- or stereoselectivities.[3] However, the wild-type (WT) enzymes often do not show the desired specificity and selectivity for a particular transformation. In such cases, rational protein design or directed evolution may overcome these limitations.[4] These techniques, together with the developments in analytical and assay methods,[5] promise the discovery of novel biocatalysts with improved properties.

Various esterases and lipases have found interesting applications in protecting group chemistry.[6,7] Recently, we have demonstrated that an esterase from *Bacillus subtilis* (BS2), which includes the GGG(A)X-motif in its active site region,[8] was able to cleave *tert*-butyl esters from a variety of substrates including *N*-protected amino acids.[9] In addition, we have reported that BS2 was a versatile agent for the efficient removal of a variety of carboxyl protecting groups under mild conditions that avoid side reactions.[10,11] Point mutations in BS2 had a remarkable influence on both activity and enantioselectivity.[12] Most interestingly, the double mutant E188W/M193C of BS2 presented complete inversion of enantioselectivity towards acetylated tertiary alcohols.[13] Such a modification in enantioselectivity might suggest altered selectivity for the removal of carboxyl protecting groups by the mutant. Thus, the aim of the present work was to identify the carboxyl protecting groups that may be removed efficiently by BS2 and its mutant E188W/M193C and to study the selectivity of these two enzymes for the removal of esters.

Results and Discussion

Initially, four model esters of γ-(benzyloxycarbonylamino)butanoic acid (Z-GABA) with increasing linear alcohol chains **1b–1e** (from *n*-propyl to *n*-hexyl)

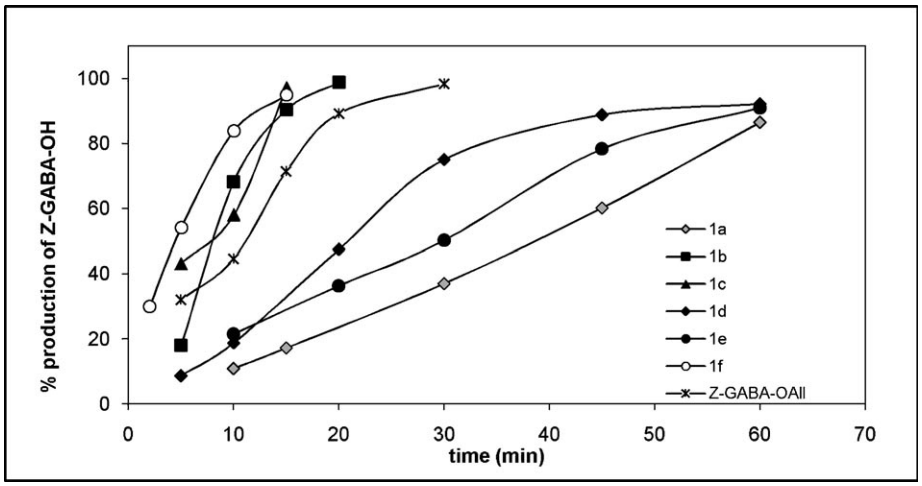


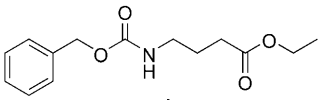
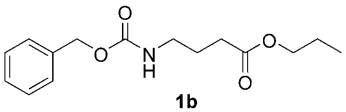
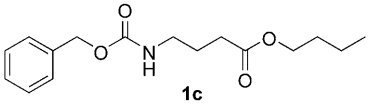
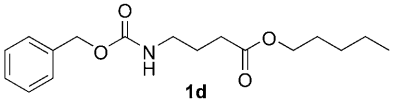
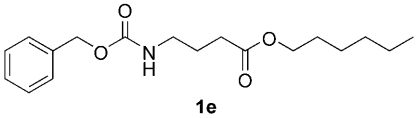
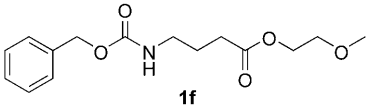
Figure 1. Time course for the hydrolysis of various esters of Z-GABA by BS2 (enzyme/substrate 1:4 w/w). *Conditions:* phosphate buffer (pH 7.4)/hexane 9:1, 37 °C.

were prepared. The hydrolysis of these esters and the corresponding ethyl ester[11] **1a** by BS2 in a mixture of buffer/hexane (9:1) containing a small amount of methanol was studied. Their hydrolysis was monitored by HPLC and the results are presented in Figure 1. *n*-Propyl and *n*-butyl esters **1b** and **1c** were clearly hydrolyzed more quickly than the other esters, while with the ethyl ester **1a**, the lowest hydrolysis rate was observed. Table 1 summarizes the isolated yields of the corresponding acid obtained after BS2 hydrolysis of the above esters in 30 min. In accordance with the results of Figure 1, the yields for the hydrolysis of *n*-butyl and *n*-propyl esters (entries 2 and 3, Table 1), are higher than those of the other esters, indicating that the preferred alcohol has three or four carbon atoms length.

Figure 2 shows the curves for the hydrolysis of the Z-GABA esters by the double mutant E188W/M193C using similar units compared with the WT since the double mutant shows only 14% of the specific WT activity (towards pNPA).[13] The *n*-butyl ester **1c** was quickly hydrolyzed quantitatively in less than 20 min. However, there was no considerable difference between the rates of hydrolysis of *n*-propyl and ethyl esters **1b** and **1a**. The hydrolysis of the *n*-hexyl ester **1e** by the mutant E188W/M193C was slow and the ester was not hydrolyzed more than 60% within 60 min. The isolated yields of the acid obtained after hydrolysis of the esters **1a**–**1f** by the mutant E188W/M193C are included in Table 1.

The curves for the alkaline hydrolysis of ethyl, *n*-butyl and *n*-pentyl esters did not present remarkable differences (see Supporting Information). In contrast to chemical means, both enzymes may differentiate their preference for the five esters. BS2 clearly shows preference for the propyl and butyl esters, while the double mutant E188W/M193C mainly shows preference for the butyl ester.

Table 1. Hydrolysis of various alkyl esters of *Z*-GABA by BS2 (enzyme/substrate 1:4 w/w) and the mutant E188W/M193C (enzyme/substrate 1:1 w/w). *Conditions:* phosphate buffer (pH 7.4)/hexane 9:1, 37 °C.

Entry	Ester	Yield [%][a] BS WT	BS2 E188W/ M193C
1	 **1a**	36	66
2	 **1b**	83	81
3	 **1c**	94	95
4	 **1d**	76	64
5	 **1e**	48	39
6	 **1f**	100	99

[a] Yield of isolated product after 30 min reaction time.

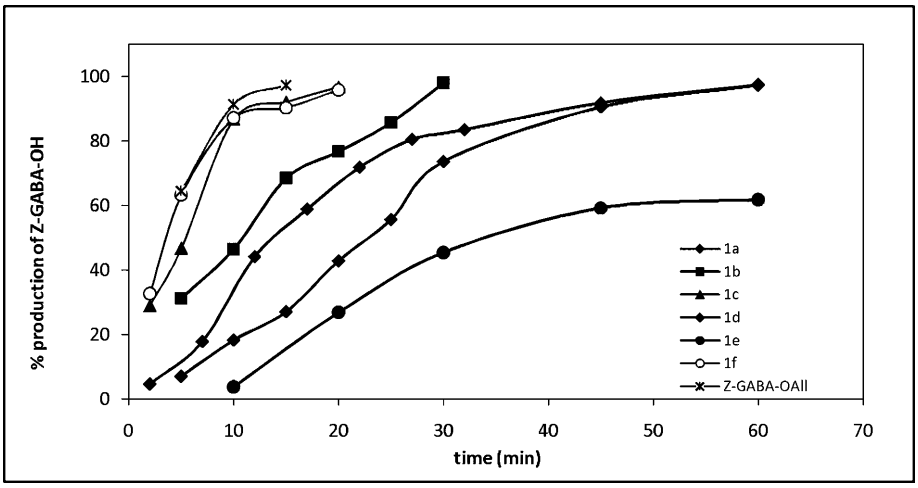


Figure 2. Time course for the hydrolysis of various esters of Z-GABA by the double mutant E188W/M193C of BS2 (enzyme/substrate 1:1 w/w). *Conditions:* phosphate buffer (pH 7.4)/hexane 10:1, 37 °C.

Since both enzymes seem to prefer a four-carbon alcohol chain (*n*-butyl ester), we decided to synthesize and study the hydrolysis of the methoxyethyl (MOE) ester of Z-GABA (**1f**). This ester seems to have the optimum length, but in addition, the presence of the oxygen atom in replacement of one carbon atom, may facilitate the hydrolysis due to electronic reasons. MOE esters have been studied in the past as protecting groups in peptide, glycopeptide and nucleopeptide chemistry and have been subjected to lipase-catalyzed hydrolysis.[14,15] Indeed, both BS2 and its double mutant quickly hydrolyzed the ester **1f** and the curves for the hydrolysis are demonstrated in Figure 1 and Figure 2. As shown in Table 1 (entry 6), the product of the hydrolysis of **1f** by BS2 and the mutant E188W/M193C was isolated in 100% and 99% yield, respectively. We have previously demonstrated the quick hydrolysis of the allyl ester of Z-GABA (Z-GABA-OAll) by BS2.[11] The double mutant E188W/M193C quickly hydrolyzed this ester within 15 min. The curves for their hydrolysis are included in Figure 1 and Figure 2.

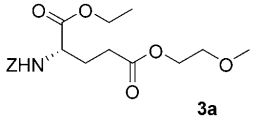
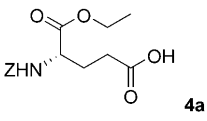
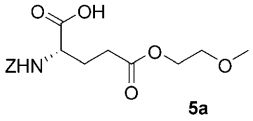
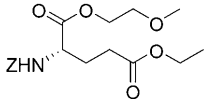
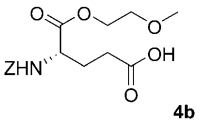
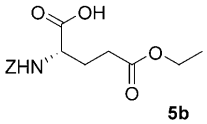
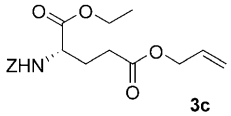
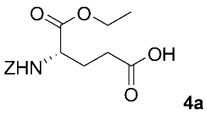
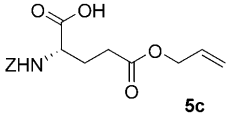
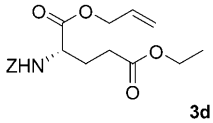
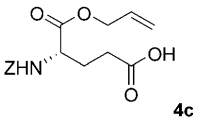
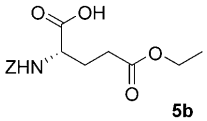
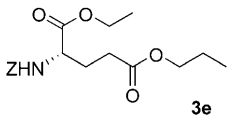
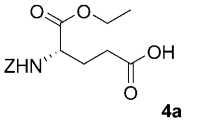
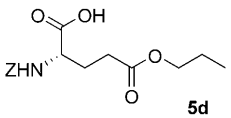
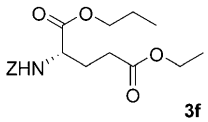
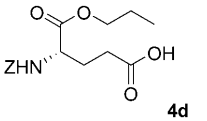
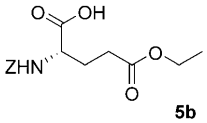
The different rates of hydrolysis for various monoesters prompted us to prepare and study in detail the selective deprotection of various glutamate diesters.

First, we studied the hydrolysis of α-methoxyethyl γ-*tert*-butyl Z-glutamate (**2**) by BS2. The MOE group was selectively removed in the presence of the *tert*-butyl ester in 84% yield after 90 min (data not shown in Tables). This result is in accordance with previous studies, where we showed that the removal of *tert*-butyl ester took 2 days using an enzyme/substrate ratio 1:1.[9] Then, we studied the selectivity for the hydrolysis of MOE, allyl and propyl esters in the presence of ethyl ester by both BS2 and its double mutant. The results of the diesters **3a**–**f** hydrolysis are summarized in Table 2.

The MOE group was selectively removed from the γ-position of substrate **3a** in the presence of ethyl ester at the α-position. In 30 min, partial hydrolysis was observed and the corresponding free γ-carboxyl product **4a** was isolated only in 31% yield (entry 1, Table 2). However, the product **4a** was isolated in 89% yield within 120 min (entry 2, Table 2). In both cases hydrolysis of the ethyl ester was not observed at all, supporting the already reported activation of the MOE ester group.[16] We previously showed that BS2 was able to remove selectively allyl ester from the γ-position of substrate **3c** in the presence of α-ethyl ester affording product **4a** in 80% yield (entry 4, Table 2).[10] When an MOE or allyl group were at the α-position in the presence of a γ-ethyl ester, a mixture of products was isolated from the hydrolysis of either substrate **3b** or substrate **3d** (entries 3 and 5, Table 2), indicating that apart from the nature of the group (chemoselectivity), the position (α- or γ-) (regioselectivity) determines the products of the hydrolysis. Very interestingly, the *n*-propyl ester was preferentially cleaved either from the γ-position of substrate **3e** or from the α-position of substrate **3 f** in the presence of an α-ethyl or γ-ethyl ester, respectively (entries 6 and 8, Table 2). When a lower enzyme/substrate ratio (1:10 w/w) was used and after a longer reaction time, the selectivity was slightly improved (entry 7, Table 2). In previous studies, we had already observed low conversions of esters with very short chain lengths using BS2. Due to the very big active site, completely open to the solvent, we propose only weak interactions between the substrate and the enzyme, if the ester chain is very small, matching the reported results.

The selectivity studies for the double mutant E188W/M193C are also shown in Table 2. MOE and allyl esters were selectively cleaved from the α-posi-

Table 2. Hydrolysis of various *Z*-glutamates by BS2 (enzyme/substrate 1:4 w/w) and by the double mutant E188W/M193C of BS2 (enzyme/substrate 1:1 w/w). *Conditions:* phosphate buffer (pH 7.4)/hexane 7:1, 37 °C.

Entry	Substrate	Products		BS2 WT			BS2 E188W/M193C		
		4	**5**	Time [min]	Yield [%][a]	Ratio **4:5**	Time [min]	Yield [%][a]	Ratio **4:5**
1	 **3a**	 **4a**	 **5a**	30	31	only **4a**	25	57	1:2[b]
2				120	89	only **4a**			
3	 **3b**	 **4b**	 **5b**	30	71	1:5[b]	15	69	only **5b**
4	 **3c**	 **4a**	 **5c**	60[c]	80	only **4a**[c]	25	70	1:3[b]
5	 **3d**	 **4c**	 **5b**	45	78	1:4[b]	20	73	only **5b**
6	 **3e**	 **4a**	 **5d**	15	77	5:1[b]	25	70	1:3[b]
7				210[d]	89	5.5:1[b]			
8	 **3f**	 **4d**	 **5b**	25	66	1:9[b]	25	89	1:18[b]

[a] Yield of isolated product.
[b] Ratio **4**:**5** was determined by NMR spectroscopy.
[c] Data taken from ref.[11]
[d] Enzyme/substrate 1:10 w/w.

tion of either substrate **3b** or substrate **3d**, respectively, in the presence of a γ-ethyl ester, producing only the product **5b** in 69% and 73% yields within 15 and 20 min, respectively (entries 3 and 5, Table 2). Even the *n*-propyl ester was almost exclusively removed from the α-position of substrate **3f** in the presence of a γ-ethyl ester (entry 8, Table 2). Contrary to the results observed with the WT, the double mutant of BS2 preferentially cleaved in all cases the ester at the α-position, irrespectively of the chemical nature of the ester. This preference leads to a reversed regioselectivity in comparison to the WT BS2 using the substrates **3a**, **3c** and **3e** (entries 1, 4 and 6, Table 2). Thus, in the case of the mutant E188W/M193C, its preference for the regioselective hydrolysis at the α-position of the substrate is the critical factor determining the product of the catalytic hydrolysis. However, for the WT BS2 the chemoselectivity seems a more important factor than the regioselectivity.

To get an idea for the different selectivity of the BS2 double mutant E188W/M193C and the WT, automated docking and molecular dynamic (MD) simulations were performed using substrate **3e** as an example. For both enzymes, one conformation of the substrate was found after docking which could yield the first tetrahedral intermediate (TI1). The TI1 was built and MD simulations showed for both enzymes that all relevant hydrogen bonds for the catalysis (Figure 3 d) stayed stable during the 500 ps MD simulation. The resulting conformation of the substrate in both enzymes was very different indicating the molecular reason for the different regioselectivities (Figure 3). In the case of BS2 WT, attack at the γ-*n*-propyl ester was favored and the aromatic ring pointed out of the active site, while in the case of the double mutant, the aromatic ring was located deep inside the active site next to the M193C mutation which facilitated an attack at the α-ethyl ester (Figure 3 a/b). By the M193C mutation a previously described channel[13] is

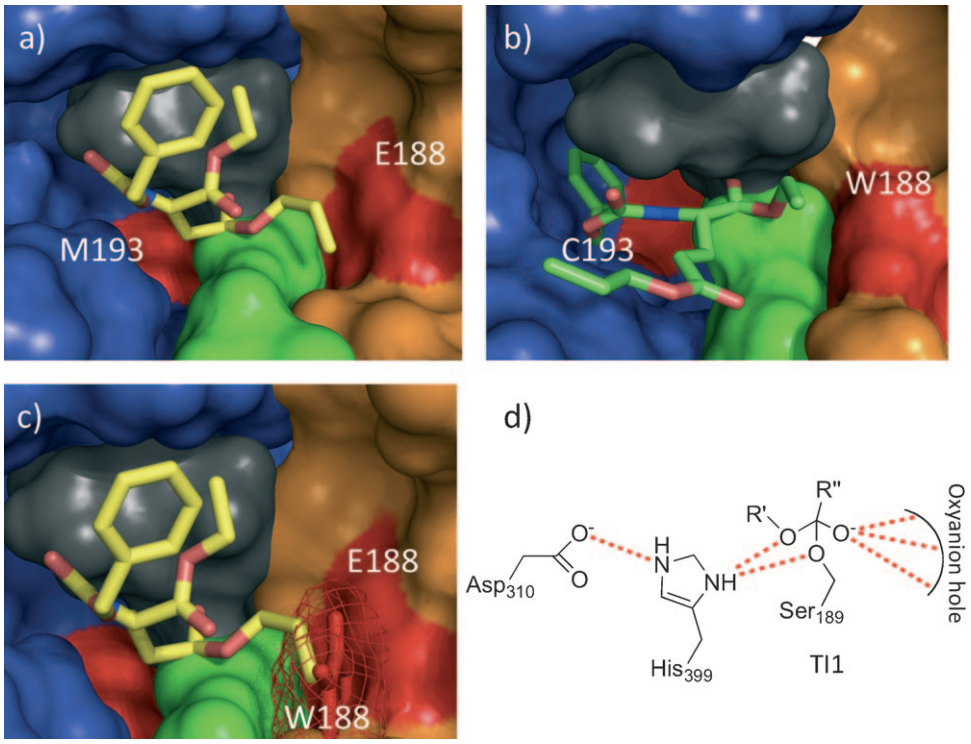


Figure 3. Docked substrate **3e** after 500 ps simulation in the active site of (**a**) the BS2-WT and (**b**) the double mutant E188W/M193C. The surface of the two binding-pockets are shown in blue and orange, the residues 188 and 193 are shown in red, the catalytic triad surface is shown green and the oxyanion hole in black. (**c**) Difference between the two active sites with substrate **3e** docked into the BS2-WT. The E188W mutation is shown as grid and sticks, the WT-E188 as surface. (**d**) Catalytic triad in the first tetrahedral intermediate. All catalytic relevant hydrogen bonds are indicated in red.

opened making way for the protecting group which is not possible in the WT enzyme. Furthermore, the E188W mutation interferes with larger ester groups due to a significantly decreased size of the binding pocket.

Conclusions

In the present work, we have demonstrated that both wild-type BS2 and the double mutant E188W/M193C quickly hydrolyze *n*-butyl, *n*-propyl, methoxyethyl and allyl esters. We have found that both WT BS2 and the double mutant E188W/M193C may preferentially cleave an *n*-propyl ester in the presence of an ethyl ester. Such a differentiation for the hydrolysis of esters varying in just one carbon atom is impossible by conventional chemical means. In addition, the hydrolysis data of the present study indicate that WT BS2 and the double mutant E188W/M193C present remarkable differences in regioselectivity. The mutant E188W/M193C has a clear preference for the regioselective hydrolysis at the α-position of glutamate diesters, while for the WT BS2 the chemoselectivity seems a more important factor than the regioselectivity. Thus, by protein engineering we have created an enzyme which presents complementary regioselectivities for the removal of carboxyl protecting groups. To the best of our knowledge, this is the first example of an engineered variant enzyme that presents reversed regioselectivity in comparison to the wild-type enzyme.

Experimental Section

General Remarks

Melting points were determined on a Büchi 530 hot-stage apparatus. Specific rotations were measured on a Perkin–Elmer 343 polarimeter at 25 °C using a 10 cm cell. NMR spectra were recorded on a 200 MHz Varian spectrometer. TLC plates (silica gel 60 F254) and silica gel 60 (70–230 or 230–400 mesh) were used for column chromatography. Visualization of spots was effected with UV light and/or phosphomolybdic acid and/or ninhydrin, both in EtOH stain. Electron spray ionization (ESI) mass spectra were recorded on a Finnigan, Surveyor MSQ Plus spectrometer. Details for recombinant enzymes are given in text and previous work.[9–11]

General Procedure for the Synthesis of Substrates

To a stirring solution of the appropriate acid (1 mmol) and the appropriate alcohol (3 mmol) in CH_2Cl_2 (2 mL), 4-(dimethylamino)pyridine (0.01 g, 0.1 mmol) and subsequently *N,N′*-dicyclohexylcarbodiimide (0.25 g, 1.2 mmol) were added at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and overnight at room temperature. After filtration, the solvent was evaporated under reduced pressure and EtOAc (20 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 eluting with $CHCl_3$ or various mixtures of petroleum ether (40–60 °C)/EtOAc.

4-Benzyloxycarbonylamino-butyric acid propyl ester (1b): Oil; yield: 76%; ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.38–7.21 (m, 5H), 5.08–4.94 (m, 3H), 4.00 (t, J = 6.6 Hz, 2H), 3.21 (m, 2H), 2.33 (t, J = 7.0 Hz, 2H), 1.88–1.73 (m, 2H), 1.71–1.52 (m, 2H), 0.90 (t, J = 7.2 Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 173.5, 156.7, 136.8, 128.7, 128.6, 128.3, 66.8, 66.4, 40.7, 31.7, 25.4, 22.1, 10.6; MS (ESI): m/z (%) = 302 (100) $[M+Na]^+$; anal. calcd. for $C_{15}H_{21}NO_4$: C 64.50, H 7.58, N 5.01; found: C 64.28, H 7.71, N 4.89.

4-Benzyloxycarbonylaminobutyric acid butyl ester (1c): Oil; yield: 29%; ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.37–7.18 (m, 5H), 5.11–4.91 (m, 3H), 4.03 (t, J = 6.6 Hz, 2H), 3.19 (m, 2H), 2.31 (t, J = 7.4 Hz, 2H), 1.86–1.72 (m, 2H), 1.64–1.50 (m, 2H), 1.42–1.24 (m, 2H), 0.90 (t, J = 7.0 Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 173.5, 156.7, 136.8, 128.6, 128.5, 128.2, 66.8, 64.6, 40.7, 31.7, 30.8, 25.4, 19.3, 13.9; MS (ESI): m/z (%) = 316 (100) $[M+Na]^+$; anal. calcd. for $C_{16}H_{23}NO_4$: C 65.51, H 7.90, N 4.77; found: C 65.34, H 8.03, N 4.65.

4-Benzyloxycarbonylaminobutyric acid pentyl ester (1d): Oil; yield: 50%; ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.38–7.21 (m, 5H), 5.17–4.97 (m, 3H), 4.02 (t, J = 6.6 Hz, 2H), 3.20 (m, 2H), 2.31 (t, J = 7.2 Hz, 2H), 1.83–1.71 (m, 2H), 1.61–1.42 (m, 2H), 1.32–1.24 (m, 4H), 0.88 (t, J = 6.6 Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 173.2, 156.3, 136.8, 128.3, 128.0, 127.9, 66.4, 64.6, 40.3, 31.4, 28.1, 27.9, 25.0, 22.2, 13.8; MS (ESI): m/z (%) = 330 (100) $[M+Na]^+$; anal. calcd. for $C_{17}H_{25}NO_4$: C 66.43, H 8.20, N 4.56; found: C 66.21, H 8.37, N 4.39.

4-Benzyloxycarbonylaminobutyric acid hexyl ester (1e): Oil; yield: 49%; ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.38–7.19 (m, 5H), 5.22 (m, 1H), 5.06 (s, 2H), 4.00 (t, J = 6.6 Hz, 2H), 3.18 (m, 2H), 2.29 (t, J = 7.4 Hz, 2H), 1.84–1.70 (m, 2H), 1.55–1.43 (m, 2H), 1.40–1.16 (m, 6H), 0.84 (t, J = 6.0 Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 173.7, 156.8, 136.8, 128.6, 128.5, 128.2, 66.8, 64.9, 40.6, 31.7, 31.6, 28.7, 25.7, 25.4, 22.8, 14.1; MS (ESI) m/z (%) = 344 (100) $[M+Na]^+$; anal. calcd. for $C_{18}H_{27}NO_4$: C 67.26, H 8.47, N 4.36; found: C 66.98, H 8.62, N 4.18.

4-Benzyloxycarbonylaminobutyric acid 2-methoxyethyl ester (1f): White low melting point solid-syrup; yield: 73%; ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.38–7.08 (m, 5H), 5.12–4.98 (m, 3H), 4.19 (t, J = 4.8 Hz, 2H), 3.54 (t, J = 4.8 Hz, 2H), 3.34 (s, 3H), 3.21 [q (ap), J = 6.6 Hz, 2H], 2.37 (t, J = 7.2 Hz, 2H), 1.89–1.71 (m, 2H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 173.4, 156.7, 136.8, 128.7, 128.6, 128.3, 70.6, 66.8, 63.7, 59.1, 40.5, 31.6, 25.3; anal. calcd. for $C_{15}H_{21}NO_5$: C 61.00, H 7.17, N 4.74; found: C 60.78, H 7.33, N 4.62.

(*S*)-2-Benzyloxycarbonylaminopentanedioic acid 5-*tert*-butyl ester 1-(2-methoxyethyl ester (2): White solid; yield: 75%; mp 44–47 °C; $[\alpha]_D$: +3.3 (c 1.0, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.37–7.29 (m, 5H), 5.49 (d, J = 8.2 Hz, 1H), 5.10 (s, 2H), 4.48–4.36 (m, 1H), 4.34–4.20 (t, J = 4.4 Hz, 2H), 3.58 (t, J = 4.4 Hz, 2H), 3.36 (s, 3H), 2.39–2.27 (m, 2H), 2.23–2.08 (m, 1H), 2.05–1.85 (m, 1H), 1.43 (s, 9H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 172.1, 171.9, 155.8, 136.1, 128.4, 128.1, 128.0, 80.7, 70.1, 66.9, 64.3, 58.9, 53.4, 31.3, 28.0, 27.5; anal. calcd. for $C_{20}H_{29}NO_7$: C 60.74, H 7.39, N 3.54; found: C 60.53, H 7.51, N 3.45.

(*S*)-2-Benzyloxycarbonylaminopentanedioic acid 1-ethyl ester 5-(2-methoxyethyl) ester (3a): Oil; yield: 54%; $[\alpha]_D$: +8.0 (c 2.0, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.38–7.06 (m, 5H), 5.55 (d, J = 8.0 Hz, 1H), 5.06 (s, 2H), 4.40–4.29 (m, 1H), 4.20–4.05 (m, 4H), 3.52 (d, J = 4.6 Hz, 2H), 3.33 (s, 3H), 2.46–1.84 (m, 4H), 1.22 (t, J = 7.0 Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 172.9, 172.1, 156.2, 136.4, 128.7, 128.4, 128.3, 70.5, 67.2, 63.9, 61.9, 59.2, 53.6, 30.3, 27.8, 14.3; anal. calcd. for $C_{18}H_{25}NO_7$: C 58.84, H 6.86, N 3.81; found: C 58.60, H 6.98, N 3.68.

(*S*)-2-Benzyloxycarbonylaminopentanedioic acid 5-ethyl ester 1-(2-methoxyethyl) ester (3b): Oil; yield: 68%; $[\alpha]_D$: +5.4 (c 1.0, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.39–7.24 (m, 5H), 5.60 (d, J = 8.0 Hz, 1H), 5.09 (s, 2H), 4.48–4.34 (m, 1H), 4.32–4.19 (t, J = 4.8 Hz, 2H), 4.09 (q, J = 7.4 Hz, 2H), 3.56 (t, J = 4.8 Hz, 2H), 3.34 (s, 3H), 2.45–2.30 (m, 2H), 2.28–2.10 (m, 1H), 2.08–1.87 (m, 1H), 1.22 (t, J = 7.0 Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 172.6, 171.7, 155.8, 136.1, 128.4, 128.0, 127.9, 70.0, 66.8, 64.2, 60.5, 58.7, 53.3, 30.0, 27.4, 14.0; anal. calcd. for $C_{18}H_{25}NO_7$: C 58.84, H 6.86, N 3.81; found: C 58.63, H 7.04, N 3.69.

(*S*)-2-Benzyloxycarbonylaminopentanedioic acid 1-ethyl ester 5-propyl ester (3e): Oil; yield: 64%; $[\alpha]_D$: +7.8 (c 1.0, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.39–7.29 (m, 5H), 5.46 (d, J = 7.8 Hz, 1H), 5.11 (s, 2H), 4.45–4.31 (m, 1H), 4.20 (q, J = 7.2 Hz, 2H), 4.02 (t, J = 7.0 Hz, 2H), 2.47–2.33 (m, 2H), 2.31–2.11 (m, 1H), 2.09–1.89 (m, 1H), 1.74–1.55 (m, 2H), 1.28 (t, J = 7.0 Hz, 3H), 0.93 (t, J = 7.2 Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 172.6, 171.8, 155.8, 136.1, 128.4, 128.0, 127.9, 66.8, 66.2, 61.5, 53.3, 30.1, 27.5, 21.8, 14.0, 10.2; anal. calcd. for $C_{18}H_{25}NO_6$: C 61.52, H 7.17, N 3.99; found: C 61.28, H 7.34, N 3.81.

(*S*)-2-Benzyloxycarbonylaminopentanedioic acid 5-ethyl ester 1-propyl ester (3f): Oil; yield: 71%; $[\alpha]_D$: +6.7 (c 1.0, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.37–7.28 (m, 5H), 5.55 (d, J = 8.0 Hz, 1H), 5.10 (s, 2H), 4.46–4.31 (m, 1H), 4.17–4.02 (m, 4H), 2.48–2.34 (m, 2H), 2.31–2.10 (m, 1H), 2.08–1.85 (m, 1H), 1.77–1.58 (m, 2H), 1.23 (t, J = 7.4 Hz, 3H), 0.93 (t, J = 7.4 Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 172.6, 171.9, 155.9, 136.1, 128.4, 128.1, 128.0, 67.1, 66.9, 60.6, 53.3, 30.1, 27.6, 21.7, 14.1, 10.2; anal. calcd. for $C_{18}H_{25}NO_6$: C 61.52, H 7.17, N 3.99; found: C 61.35, H 7.31, N 3.85.

(*S*)-2-Benzyloxycarbonylaminopentanedioic Acid 1-Allyl Ester 5-Ethyl Ester (3d)

A mixture of Z-Glu(OEt)-OH (0.31 g, 1.0 mmol) and Cs_2CO_3 (0.16 g, 0.5 mmol) was dissolved in DMF (5 mL) and a few drops of water were added. The mixture was distilled under reduced pressure to dryness and the residue re-

distilled twice from DMF (10 mL), until all the water has been removed. The solid cesium salt was stirred with allyl bromide (0.1 mL, 1.15 mmol) in DMF (0.6 mL) overnight at room temperature. After removal of DMF, EtOAc (20 mL) was added, the organic layer was washed consecutively with a saturated solution of $NaHCO_3$, water and brine, dried over Na_2SO_4, and the solvent was evaporated under reduced pressure. The residue was purified by column chromatography eluting with $CHCl_3$; yield: 0.25 g (73%); white solid; mp 41–43 °C; $[\alpha]_D$: +35.4 (*c* 1.0, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.49–7.17 (m, 5H), 6.04–5.72 (m, 1H), 5.52–5.17 (m, 3H), 5.11 (s, 2H), 4.71–4.54 (m, 2H), 4.49–4.31 (m, 1H), 4.13 (q, J = 7.2 Hz, 2H), 2.56–2.29 (m, 2H), 2.28–2.11 (m, 1H), 2.09–1.85 (m, 1H), 1.25 (t, J = 7.2 Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 172.6, 171.5, 155.9, 136.1, 131.4, 128.5, 128.2, 128.1, 119.0, 67.0, 66.1, 60.7, 53.4, 30.2, 27.6, 14.1; anal. calcd. for $C_{18}H_{23}NO_6$: C 61.88, H 6.64, N 4.01; found: C 61.51, H 6.79, N 3.91.

The syntheses of substrates **1a** and **3c** were described previously.[11]

General Procedure for the Enzymatic Hydrolysis

To a stirred solution of the substrate (0.15–0.40 mmol) in *n*-hexane (0.4–1.0 mL) and CH_3OH (20–80 μL) was added a solution of the enzyme (12.5–50 mg, as indicated in Tables) in phosphate buffer (7–10 mL, 100 mM, pH 7.4). The reaction mixture was stirred at 37 °C. After acidification until pH 6 and extraction with EtOAc (3×10 mL), the organic layers were combined, dried over Na_2SO_4, and the organic solvent was removed under reduced pressure. The residue was purified by column chromatography using $CHCl_3$/CH_3OH 95:5 as eluent. All products of the enzymatic hydrolysis were identified by their analytical data in comparison with authentic samples, which were also prepared by chemical methods.

(*S*)-2-Benzyloxycarbonylaminopentanedioic acid 1-propyl ester (4d): Oil; ^{1}H NMR (200 MHz, $CDCl_3$): δ = 11.02 (br s, 1H), 7.41–7.26 (m, 5H), 5.73 (d, J = 8.0 Hz, 1H), 5.12 (s, 2H), 4.49–4.32 (m, 1H), 4.12 (t, J = 6.6 Hz, 2H) 2.58–2.40 (m, 2H), 2.38–2.10 (m, 1H), 2.08–1.85 (m, 1H), 1.79–1.55 (m, 2H), 0.94 (t, J = 7.4 Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 178.3, 172.0, 156.5, 135.6, 128.5, 128.3, 128.0, 68.2, 67.5, 53.2, 29.8, 27.3, 21.7, 10.1; anal. calcd. for $C_{16}H_{21}NO_6$: C 59.43, H 6.55, N 4.33; found: C 59.21, H 6.68, N 4.12.

(*S*)-2-Benzyloxycarbonylaminopentanedioic acid 1-(2-methoxyethyl) ester (4b). Oil; $[\alpha]_D$: +4.4 (*c* 0.5, $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$): δ = 7.41–7.25 (m, 5H), 5.60 (d, J = 8.2 Hz, 1H), 5.11 (s, 2H), 4.52–4.39 (m, 1H), 4.37–4.19 (t, J = 4.4 Hz, 2H), 3.60 (t, J = 4.4 Hz, 2H), 3.37 (s, 3H), 2.58–2.39 (m, 2H), 2.37–2.11 (m, 1H), 2.07–1.88 (m, 1H); ^{13}C NMR (50 MHz, $CDCl_3$): δ = 177.3, 171.8, 156.0, 136.0, 128.5, 128.2, 128.1, 70.0, 67.1, 64.3, 58.8, 53.2, 29.7, 27.4; anal. calcd. for $C_{16}H_{21}NO_7$: C 56.63, H 6.24, N 4.13; found: C 56.42, H 6.37, N 4.02.

Molecular Modeling

The homology model of the BS2 WT was generated as described previously.[17] The double mutant of BS2 E188W/M193C was generated and refined using YASARA structure (version 9.2.9). The substrate was docked in the active site by Autodock4 and the Lamarckian genetic algorithm using max. 100 million energy evaluations and max. 150,000 generations with default parameters.[18] The grid-box was centered on the catalytic serine 189 with an edge length of 35 Å. The molecular dynamic simulations were performed with YASARA structure in a periodic water box at pH 7.5 using the AMBER99-force field with default settings.[19] The force field for the substrate and the TI1 were assigned by AutoSMILES.[20]

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