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Substituted aryl malonamates as new serine β-lactamase substrates: Structure–activity studies

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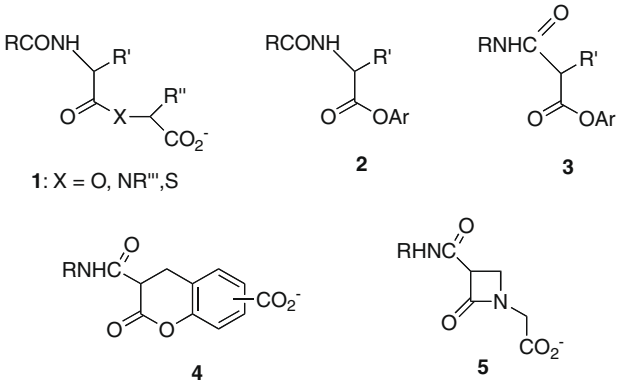
ABSTRACT

A series of substituted aryl malonamates have been prepared. These compounds are analogues of aryl phenaceturates where the amido side chain has been replaced by a retro-amide. Like the phenaceturates, these compounds are substrates of typical class A and class C β-lactamases, particularly of the latter, and of soluble DD-peptidases. The effect of substituents α to the ester carbonyl group on turnover by these enzymes is similar to that in the phenaceturates. On the other hand, N-alkylation of the side chain amide of malonamates, but not of phenaceturates, retains the susceptibility of the compounds to hydrolysis by β-lactamases. This reactivity is not enhanced, however, by bridging the amide nitrogen and Cα atoms. A phosphonate analogue of the malonamates was found to be an irreversible inhibitor of the β-lactamases. These results, therefore, provide further evidence for the covalent access of compounds bearing retro-amide side chains to the active sites of β-lactam-recognizing enzymes.

1. Introduction

The search for new substrates and inhibitors of β-lactamases continues. New substrates are of interest, of course, because, in principle, new inhibitors can be derived from them. Such inhibitors can protect β-lactams from β-lactamases and therefore amplify access of these antibiotics to their targets, the bacterial DD-peptidases. A few of these inhibitors, clavulanic acid, sulbactam and tazobactam, have been very successful in this role in medical practice,[1] and new variants continue to be developed.[2] Because of the similarity of β-lactamase and DD-peptidase active sites[3], inhibitors of the former enzymes may also be inhibitors of the latter, and thus, potentially, antibiotics in their own right. New chemical entities that interact with the β-lactamase active site may become the foci of new inhibitor development, for example diazabicyclooctanones such as NXL104[4] and aryloxycarbonyl hydroxamates.[5]

Most good β-lactamase substrates, of structure **1** (X = O, S), are characterized by an amido side chain. Crystal structures of complexes of **1** (X = O, N, S) with a variety of β-lactamases[6,7] and DD-peptidases[8,9] show that the side chain amide donates a hydrogen bond to a backbone carbonyl of the conserved β-strand adjacent to the active site, and accepts a hydrogen bond from the side chain amide of the asparagine residue of a S(Y)XN motif that is found in most of these enzymes (Fig. 1). Molecules lacking the amido side chain are usually poorer β-lactamase substrates. For example, penicillanic acid is some 10^2–10^3 times less effective as a substrate (k_{cat}/K_m) of typical class A and class C β-lactamases than is benzylpenicillin.[10] Effective transition state analogue inhibitors, for example phosphonates[11] and boronates,[12] also possess the amido side chain.



Aryl phenaceturates, **2**, have been shown to be substrates of serine β-lactamases of all three classes, A, C and D.[13–15] More recently, we have shown, rather counter intuitively, that retro-amido analogues, aryl malonamates, **3** (R′ = H), are also β-lactamase substrates with reactivities approaching those of **2**.[15] Molecular modeling suggested, not surprisingly, that hydrogen bonding of the amido side chain of **3** with the enzyme active site would be different from that of **2**, and may involve hydrogen bond acceptance

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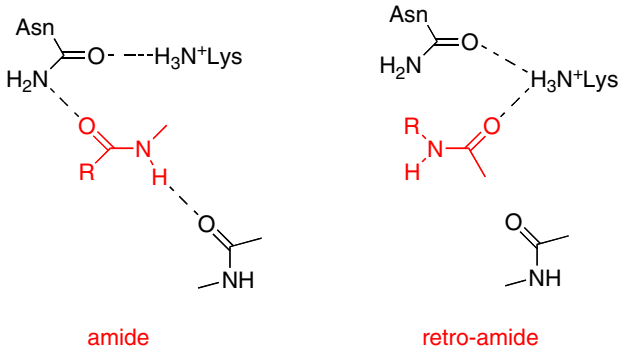
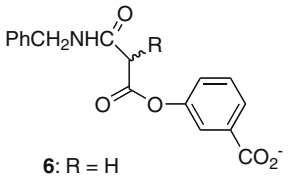


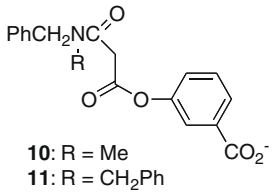
Figure 1. Possible interactions between substrate amide and retro-amide groups and active site residues.

from the protonated side chain of the lysine residue of the conserved KXYS active site motif[15] (Fig. 1). At any event, it seems that the retroamide side chain can also, under some circumstances of substrate structure at least, interact in a productive way with the β-lactamase active site. This finding has been extended to the benzopyranones, **4**, and the β-lactam **5**, which, like their normal amido-substrates, are serine β-lactamase substrates.[16,17]

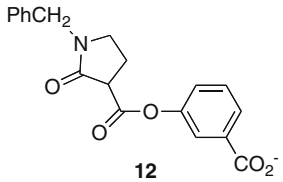
The present paper describes our ventures to further extend the theme of **3** through elaboration of the lead compound **6** (R = H). It is well known, for example, that 6α-substituents in penams (7α in cephems) confer interesting inhibitory properties on the parent molecules. The methoxy and hydroxymethyl substituents, for example, convert β-lactamase substrates into inhibitors.[18–23] In phenaceturates, these substituents (R′ in **2**) gave altered substrate activities.[24] We have therefore examined the α-substituted malonamates **7–9** and compared their reactivity with typical β-lactamases with those of the parent compound **6** (R = H) and with their normal amide analogues.



6: R = H
7: R = Me
8: R = CH_2Ph
9: R = OMe



10: R = Me
11: R = CH_2Ph



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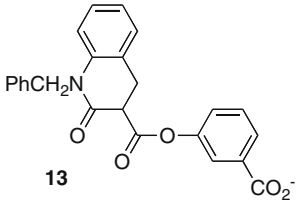

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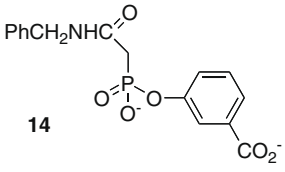

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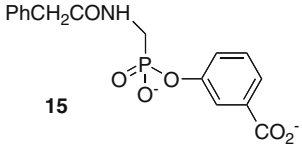


15

Inspection of the molecular model referred to above,[15] suggested that the retro- amide NH moiety may not be hydrogen-bonded to the active site and thus, unlike in **2**, N-substitution might be accepted and perhaps lead to new interactions. Compounds **10** and **11** were therefore assessed. An extension of this idea suggested that a bridge between the retro-amide nitrogen and the α-carbon position of the malonamate might also be acceptable, and even fix the molecule in a reactive conformation (see molecular modeling below). The cyclic analogues **12** and **13** were therefore prepared and assessed. Finally, the inhibitory properties of the retro-amido phosphonate **14**, an analog of the phosphonate **15**, a known transition state analogue inhibitor,[11] were determined.

2. Results and discussion

Synthesis of the linear analogues **7, 8, 10** and **11** was straightforward through selectively protected malonate derivatives (Scheme 1). The α-methoxy derivative **9** was similarly obtained (Scheme 2). The pyrrolidinone **12** was prepared by way of a similar sequence, beginning with 1-benzyl-2-pyrrolidinone (Scheme 3). A similar sequence was employed for **13** (Scheme 4). In this case, direct N-benzylation of the benzolactam **27** was not feasible. Protection of the C-3 hydrogen with a *t*-butoxycarbonyl group was therefore undertaken; N-benzylation and deprotection was then straightforward. As prepared, compounds **7–9**, **12** and **13** were racemic at the malonate central carbon atom.

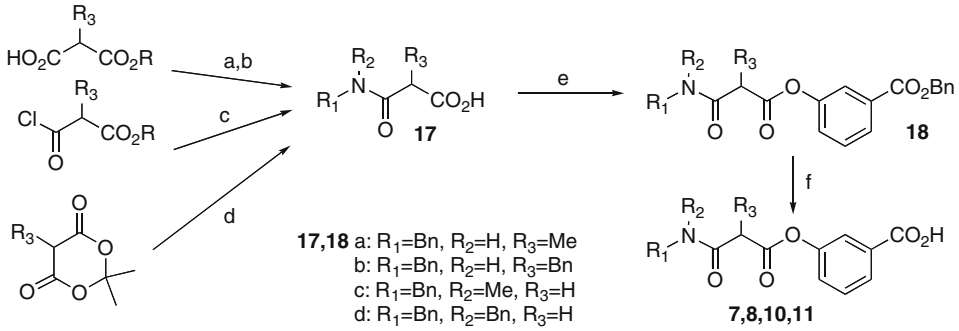
The phosphonate **14** was initially sought from direct coupling of the relevant phosphonic acid with benzyl 3-hydroxybenzoate using either dicyclohexylcarbodiimide or trichloracetonitrile as the condensation agent. After this route was found to be unsuccessful, the Arbusov reaction (Scheme 5) was then successfully applied.

The malonamates **6–13** slowly hydrolyze in aqueous buffer. Under the conditions of the enzyme kinetics experiments, pseudo-first order rate constants of hydrolysis of **6–13** were 5.4×10^{-6} s^{-1},[15] 5.7×10^{-6} s^{-1}, 1.4×10^{-6} s^{-1}, 28×10^{-6} s^{-1}, 2.4×10^{-6} s^{-1}, 5.6×10^{-6} s^{-1}, 6.4×10^{-6} s^{-1}, and 22×10^{-6} s^{-1}, respectively. The phosphonate **14** was stable to hydrolysis under these conditions.

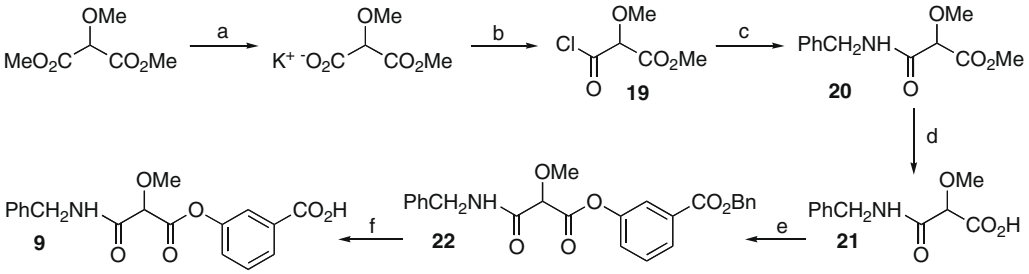
The α-substituted phenaceturates **7–9** are, in general, like the parent compound **6**,[15] substrates of the class C P99 β-lactamase (Table 1). The methyl, benzyl and methoxy substituents do not, however, yield a better substrate. This result resembles that for classical amido-depsipeptides, **2**, where, in general, simple α-substitution does not, in general, significantly enhance reactivity.[24,25] In the present case, it is noticeable that the larger benzyl substituent does induce tighter apparent binding and markedly slower turnover. Further development of this lead could result in quite effective inhibitory substrates. It should be noted that all of the new compounds, like the original depsipeptides, **1**, are poorer β-lactamase substrates than optimally-structured β-lactams.

Successive application of the P99 β-lactamase and the R61 DD-peptidase to **7**, a stratagem employed previously,[24] shows that the former enzyme prefers the enantiomer of **7** not favored by the latter enzyme, although both enantiomers are substrates of the former. If it is assumed that the DD-peptidase is specific for the *S*-enantiomer (corresponding to its D-amino acid preference in amide substrates[24,26]) then the *R*-enantiomer of **7** must be preferred by the P99 β-lactamase. This result also resembles that for the classical phenaceturate **2** where the L-alanyl substrate is preferred, although, as here, both enantiomers are substrates. The structural basis for the latter preference has been discussed.[24]

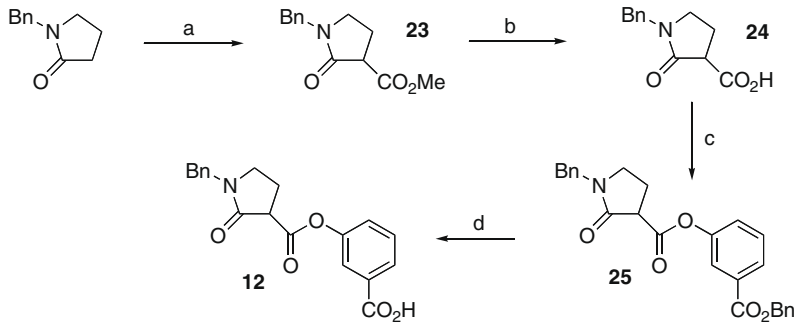
In contrast to the above result, successive application of the P99 and R61 enzymes to **9** shows that both enzymes have the same enantiomeric preference, presumably *S*. As for **7**, both enantiomers of **9** are P99 β-lactamase substrates. Such a change in stereospecificity for the P99 enzyme has been observed previously[27,28] and probably reflects the general lack of structural specificity for either



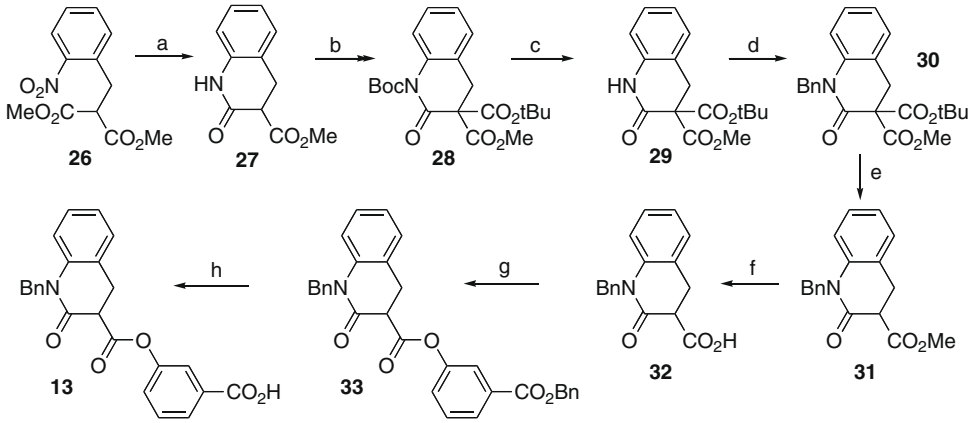
Scheme 1. Reagents and conditions: (a) $R^1R^2NH/DCC/CH_2Cl_2/12$ h; (b) KOH, 1 equiv/ROH/25 °C/1–12 h, then 20% HCl/25 °C; (c) $R^1R^2NH/CH_2Cl_2/25$ °C/3 h, then 10% HCl/25 °C; (d) R^1R^2NH, toluene/105 °C/16 h; (e) 3-$HOC_6H_4CO_2Bn$/DCC or DCC/DMAP/THF or $CH_2Cl_2/25$ °C/12 h; (f) H_2/10% Pd/C/AcOEt/25 °C/2–12 h.



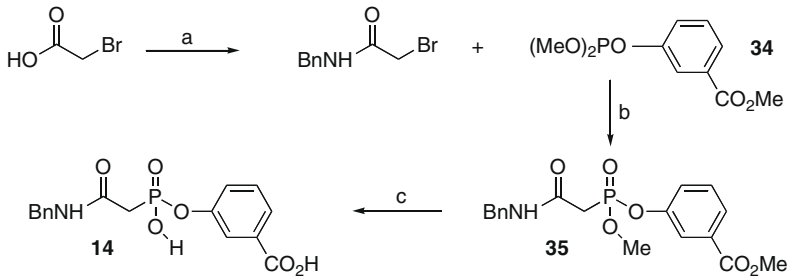
Scheme 2. Reagents and conditions: (a) aq KOH; (b) $SOCl_2$; (c) $PhCH_2NH_2/CH_2Cl_2/1$ h; (d) 1 M aq NaOH/MeOH, 1/1, 25 °C/16 h; (e) $HOC_6H_4CO_2Bn$/DCC/$CH_2Cl_2/25$ °C/16 h; (f) H_2/10% Pd/C/AcOEt/25 °C/16 h.



Scheme 3. Reagents and conditions: (a) LDA (1.3 equiv)/THF/−78 °C/30 min, then $ClCO_2Me$/30 min; (b) 1 M NaOH/MeOH/25 °C/12 h, then aq HCl; (c) 3-$HOC_6H_4CO_2Bn$/DCC/$CH_2Cl_2/25$ °C/12 h; (d) H_2/10% Pd/C/AcOEt/25 °C/2 h.



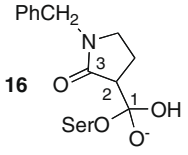
Scheme 4. Reagents and conditions: (a) H_2/10% Pd/C/AcOEt/25 °C/3 h; (b) Boc_2O (2 equiv)/DMAP/MeCN/25 °C/12 h; (c) TFA/CH_2Cl_2/0 °C/1 h; (d) BnCl/K_2CO_3/$BnEt_3N^+Cl^-$/MeCN/60 °C/4 h; (e) TFA/$CH_2Cl_2/25$ °C/1 h; (f) 10% NaOH/MeOH/25 °C/1 h, then aq HCl; (g) 3-$HOC_6H_4CO_2Bn$/DCC/$CH_2Cl_2/25$ °C/4 h; (h) H_2/10% Pd/C/AcOEt/25 °C/2 h.



Scheme 5. Reagents and conditions: (a) $PhCH_2NH_2$/DCC/HOBT/CH_2Cl_2/16 h; (b) 110 °C/16 h; (c) Me_3SiI/50 °C/16 h.

enantiomer.[24] The stereochemical assignments for **7** and **9**, achieved as discussed above, are indicated with the relevant rate constants in Table 1.

Molecular modeling[15] suggested that the N-alkylated amide species **10** and **11** may be substrates of the P99 β-lactamase because the N–H of **6** did not appear to hydrogen-bond with an enzyme functional group (Fig. 1). This is now found to be true (Table 1), although the substrates generated are poorer than **6**. Nonetheless, these substrates are more reactive than N-alkylated analogs of **2**, where the amide N–H is believed to strongly interact with a protein backbone carbonyl oxygen. The present result, therefore, does support the structural model. Modeling (Fig. 2) also suggested that extension of N-alkylation to cyclic compounds such as **12** and **13** would also be acceptable to the active site. As shown in Table 1, however, this extension did not produce better substrates and does not suggest that the freezing out of a reactive conformer was achieved. Although the molecular modeling (Fig. 2) did suggest that a deacylation tetrahedral intermediate, **16**, derived from **12** would fit well into the active site, it may be that flexibility around the C_2–C_3 bond, restricted in the cyclic compound, is needed to achieve this structure. A comparison of the conformation of **16** in Figure 2 with that of the acyclic analogue **6**,[15] shows $C_1C_2C_3N$ dihedral angles of 115° and 128°, respectively. Flexibility about this bond may be required in the acylation step.



The modeled structure includes the interesting hydrogen bond between the substrate amido-carbonyl group and the terminal ammonium ion of Lys 67. This interaction was first seen as a possibility in modeling of complexes of **6** (R = H).[15] It is not found in complexes of normal amido-substrates and may enforce a Tyr 150 general base mechanism of acylation.[29–31] All of the new depsipeptides **6–13** are considerably poorer substrates of the class A TEM β-lactamase than of the P99 enzyme. This response is found in the classical phenaceturates also[13] but the difference seems heightened by α-substitution.

On the basis of their stereospecificity towards the classical depsipeptides and to peptide substrates, one would expect specific (S) substitution to enhance the reactivity (k_{cat}/K_m) of DD-peptidases towards **3**. This result is certainly seen in **7** and **9** with both the R61 and R39 DD-peptidases (Table 1). With both enzymes, one enantiomer of **7** and **9**, which can be assumed to be *S*, is considerably more reactive than the other (*R*), a preference, as noted above, presumably rooted in the strong specificity of DD-peptidases for natural D-alanyl peptide substrates. The N-alkylation of **6** seems to yield molecules that only poorly interact with the DD-peptidases (Table 1).

The phosphonate **14** was found to be an inhibitor of the P99 β-lactamase ($k_i = 45\ s^{-1}\ M^{-1}$) but is considerably less effective against the TEM enzyme ($k_i < 1\ s^{-1}\ M^{-1}$), again reflecting the relative reactivities with **15**. The effectiveness of **14** against the P99 enzyme, however, is only around 10-fold less than that of **15**,[11] and may be, as in the latter case, enhanced to a considerable degree with suitable substituents.[32] Neither **14** nor **15** inhibited the DD-peptidases.

3. Conclusions

The results described above affirm the viability of the retro-amide side chain platform as a source of covalent access to the active sites of β-lactam-recognizing enzymes. For a particular compound, however, that access may only be available for a narrow range of enzymes. Of particular interest are the reaction of **8** with the P99 β-lactamase, where a slowly hydrolyzing acyl-enzyme is probably generated, and the very positive influence of the methoxy substitution in **9** on both DD-peptidases examined. Further investigation of these leads may produce novel inhibitors of specific enzymes. The retro-amide phosphonates may also be worthy of closer scrutiny.

4. Experimental

4.1. Syntheses

4.1.1. 3-(2-Benzylcarbamoyl-propionyloxy)-benzoic acid (7) (Scheme 1)

4.1.1.1. *N*-Benzyl 2-methylmalonamic acid 17a. Benzylamine (545 μL, 5.0 mmol) and 2,2,5-trimethyl-1,3-dioxan-4,6-dione (790 mg, 5.0 mmol) were partially dissolved in dry toluene (10 mL) in a round-bottomed flask fitted with a condenser and a $CaCl_2$ trap. The mixture was heated at 105 °C for 16 h. After cooling at room temperature, a white precipitate was collected by filtration and recrystallized from ethyl acetate and cyclohexane to afford the title product **17a** as colorless needles (610 mg, 2.95 mmol, 59% yield). Mp 138 °C; R_f 0.35 (ethyl acetate/acetic acid: 99/1); ^{1}H NMR: δ (CD_3COCD_3) 1.37 (d, 3H, J = 7.2 Hz), 3.27 (q, 1H, J = 7.2 Hz), 4.37 (dd, 2H, J = 5.8, 14.9 Hz,), 7.13–7.29 (m, 5H), 7.79 (br s, 1H). ^{13}C NMR: δ (CD_3COCD_3) 15.08, 43.66, 43.78, 46.67, 46.72, 127.87, 128.33, 129.28, 140.16, 171.29, 172.44. Anal. Calcd for $C_{11}H_{13}NO_3$: C, 63.75; H, 6.32; N, 6.76. Found: C, 63.93; H, 6.65; N, 6.57.

4.1.1.2. 3-(2-Benzylcarbamoyl-propionyloxy)-benzoic acid benzyl ester 18a. *N*-Benzyl 2-methylmalonamic acid **17a** (232 mg, 1.12 mmol) was dissolved in a mixture of dichloromethane (15 mL) and DMF (few drops), then was added 3-hydroxybenzoic acid benzyl ester (269 mg, 1.18 mmol) followed by dicyclohexyl-

Table 1
Steady-state parameters for turnover of retro-amide

Substrate	Enzyme[a] P99 k_{cat} (s^{-1})	P99 K_m (μM)	P99 k_{cat}/K_m (s^{-1} M^{-1})	TEM k_{cat} (s^{-1})	TEM K_m (μM)	TEM k_{cat}/K_m (s^{-1} M^{-1})	R61 k_{cat} (s^{-1})	R61 K_m (μM)	R61 k_{cat}/K_m (s^{-1} M^{-1})	R39 k_{cat} (s^{-1})	R39 K_m (μM)	R39 k_{cat}/K_m (s^{-1} M^{-1})
6[b]	41	170	2.45×10^5	28	1.42	1.95×10^4	0.7	10^4	70			⩽1.5[c]
7[d]	1.17 ± 0.01	60 ± 5	2.13×10^4 (*R*)	$>7.2 \times 10^{-4}$	>200	3.6 ± 0.2	7.63 ± 0.03	320 ± 20	2.37×10^4 (*S*)	>0.73	>1000	$(7.28 \pm 0.03) \times 10^2$ (*S*)
	6.6 ± 0.6	2800 ± 300	2.36×10^3 (*S*)				0.12 ± 0.02	90 ± 6	1.31×10^3 (*R*)			
8	0.05 ± 0.01	2.6 ± 0.5	1.9×10^4[e]	$>9.4 \times 10^{-4}$	>200	4.7 ± 0.7			⩽0.2[c]		nd[f]	
9	>7.4	>1000	$(7.4 \pm 0.1) \times 10^3$ (*S*)			⩽6[c]	9.4 ± 0.3	280 ± 20	3.3×10^4 (*S*)	2.68 ± 0.01	53 ± 1	5.0×10^4 (*S*)
	>2.9	>1000	$(2.9 \pm 0.1) \times 10^3$ (*R*)									
10	0.33 ± 0.03	700 ± 140	4.7×10^2			⩽0.2[c]			⩽0.15[c]	$>3 \times 10^{-3}$	>1000	3.0 ± 0.4
11	0.25 ± 0.02	32 ± 7	7.9×10^3	0.21 ± 0.01	1800 ± 80	1.2×10^2			⩽0.35[c]		nd	
12	0.12 ± 0.01	2500 ± 300	50	$>1.1 \times 10^{-4}$	>220	0.54 ± 0.04	$>3.6 \times 10^{-3}$	>200	17.8 ± 0.1	$>8 \times 10^{-3}$	>1000	8.0 ± 0.1
13	0.41 ± 0.24	1400 ± 1000	290[g]			⩽0.9[c]	$>4.2 \times 10^{-3}$	>200	21 ± 1			⩽20[c]

[a] Enzymes: P99, the class C β-lactamase of *Enterobacter cloacae* P99; TEM, the class A β-lactamase of the TEM-2 plasmid; R61, the DD-peptidase from *Streptomyces* R61; R39, the DD-peptidase from *Actinomadura* R39.
[b] Data from Ref. 15.
[c] Assuming 10% of the background hydrolysis rate as an upper limit to the enzyme-catalyzed reaction.
[d] Two entries represent separate enantiomers (see text); one entry represents the apparent value (see text).
[e] Substrate activation observed at concentrations greater than K_m.
[f] nd = not determined.
[g] Substrate inhibition observed at concentrations greater than K_m.

carbodiimide (243 mg, 1.18 mmol). The reaction mixture was stirred overnight and DCU was removed by filtration. The organic solution was washed with saturated aqueous $NaHCO_3$ (15 mL), brine (15 mL) and dried. The solvent was evaporated under vacuum to give a colorless solid which was recrystallized from ethyl acetate/cyclohexane to give the title product **18a** (230 mg, 0.55 mmol, 49% yield). Mp 117 °C. R_f 0.41 (ethyl acetate/cyclohexane 40/60); ^{1}H NMR: δ (CD_3COCD_3) 1.46 (d, 3H, *J* = 7.2 Hz), 3.83 (q, 1H, *J* = 7.2 Hz), 4.48 (dd, 2H, *J* = 6.2, 14.9 Hz), 5.39 (s, 2H), 7.18–7.61 (m, 12H), 7.76 (m, 1H), 7.94 (m, 1H). ^{13}C NMR: δ (CD_3COCD_3) 14.28, 43.86, 47.80, 67.57, 123.48, 127.48, 127.79, 127.90, 128.33, 129.16, 129.21, 129.31, 129.50, 130.74, 132.69, 137.30, 140.28, 152.08, 165.91, 169.80, 170.31. Anal. Calcd for $C_{25}H_{23}NO_5$: C, 71.93; H, 5.55; N, 3.36. Found: C, 71.72; H, 5.67; N, 3.33.

4.1.1.3. 3-(2-Benzylcarbamoyl-propionyloxy)-benzoic acid 7.

3-(2-Benzylcarbamoyl-propionyloxy)-benzoic acid benzyl ester **18a** (163 mg, 0.39 mmol) was dissolved in ethyl acetate (15 mL), palladium on charcoa1 (49 mg, 30%/w) was added and the mixture was stirred under a hydrogen atmosphere for 24 h. The reaction mixture was filtered through Celite and the solvent was removed under vacuum. The residue was recrystallized from ethyl acetate/cyclohexane to afford the title product **7** as a cotton-like colorless solid (105 mg, 0.32 mmol, 82% yield). Mp 170 °C; R_f 0.67 (methanol/ethyl acetate: 30/70); ^{1}H NMR (300 MHz): δ (CD_3COCD_3) 1.48 (d, 3H, *J* = 7.0 Hz), 3.84 (q, 1H, *J* = 7.0 Hz), 4.49 (dd, 2H, *J* = 6.0, 14.1 Hz), 7.20–7.38 (m, 6H), 7.56 (t, 1H, *J* = 8.2 Hz), 7.77 (m, 1H), 7.94 (m, 1H), 7.99 (br s, 1H). ^{13}C NMR (300 MHz): δ (CD_3COCD_3) 14.19, 14.30, 43.75, 43.87, 47.76, 47.81, 123.72, 127.19, 127.90, 127.91, 128.34, 128.35, 129.32, 130.60, 133.05, 140.27, 152.04, 166.82, 169.81, 169.89, 170.33. Anal. Calcd for $C_{18}H_{17}NO_5$: C, 66.04; H, 5.24; N, 4.28. Found: C, 65.92; H, 5.33; N, 4.14.

4.1.2. 3-(2-Benzylcarbamoyl-3-phenyl-propionyloxy)-benzoic acid (8) (Scheme 1)

4.1.2.1. 2-Benzylmalonamic acid ethyl ester *N*-benzylamide 17b.

Benzylamine (865 μL, 7.94 mmol) and benzylmalonic acid monoethyl ester[33] (1.6 g, 7.21 mmol) were dissolved in dry dichloromethane (20 mL) under argon, dicyclohexylcarbodiimide (1.78 g, 8.64 mmol) was added, and the reaction mixture was stirred overnight. DCU was removed by filtration and the solvent was evaporated under vacuum to give a oil which was chromatographed on silica gel with cyclohexane/ethyl acetate (4/1) as eluent. The title product **17b** was isolated as a colorless solid (475 mg, 21% yield). Mp 74 °C; R_f 0.17 (ethyl acetate/cyclohexane 10/90); ^{1}H NMR: δ ($CDCl_3$) 1.15 (t, 3H, *J* = 7.0 Hz), 3.26 (dd, 2H, *J* = 6.6, 8.1 Hz), 3.52 (dd, 1H, *J* = 6.6, 8.1 Hz), 4.11 (q, 2H, *J* = 7.0 Hz), 4.43 (dd, 2H, *J* = 5.5,14.7 Hz), 6.71 (t, 1H, *J* = 5.5 Hz), 7.15–7.34 (m, 10H). ^{13}C NMR: δ ($CDCl_3$) 14.15, 36.65, 43.86, 55.11, 61.73, 127.01, 127.68, 127.82, 128.14, 128.72, 128.86, 129.13, 138.01, 167.79, 171.34. Anal. Calcd for $C_{19}H_{21}NO_3$: C, 73.29; H, 6.80; N, 4.50. Found: C, 72.96; H, 7.02; N, 4.67.

4.1.2.2. 3-(2-Benzylcarbamoyl-3-phenyl-propionyloxy)-benzoic acid benzyl ester 18b.

2-Benzylmalonamic acid ethyl ester *N*-benzylamide **18a** (350 mg, 1.13 mmol) was dissolved in warm ethanol (10 ml). After cooling, potassium hydroxide (63 mg, 1.13 mmol, 45% w/w in water) was added dropwise and stirring was maintained for 1 h. Ethanol and water were removed under vacuum and the residue was dissolved in water (15 mL). The aqueous layer was acidified to pH 5 using dropwise addition of 20% HCl at 0 °C and extracted with ethyl acetate (2 × 15 mL). The organic layer was washed with brine (15 mL), dried over $MgSO_4$ and evaporated to dryness to afford crude 2-benzylmalonamic acid *N*-benzylamide as a white solid. This acid (303 mg, 1.07 mmol) and 3-hydroxybenzoic acid benzyl ester (245 mg, 1.07 mmol) were

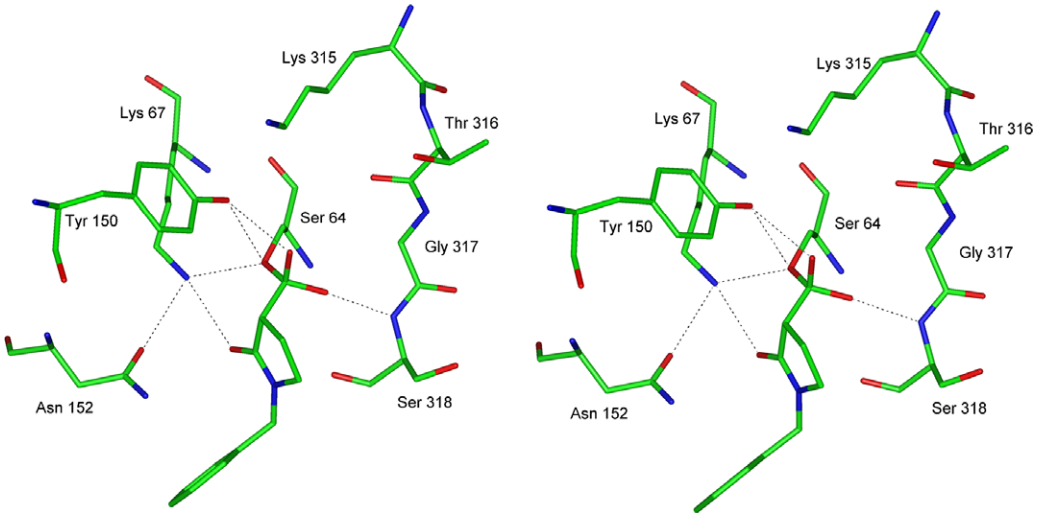


Figure 2. Stereoview of an energy-minimized tetrahedral intermediate structure, **16**, formed on reaction of the P99 β-lactamase with the cyclic retro-amide **12**. Only heavy atoms are shown.

dissolved in dry tetrahydrofuran (15 mL), and DMAP (6 mg, 0.05 mmol) and dicyclohexylcarbodiimide (231 mg, 1.12 mmol) were added. The reaction mixture was stirred overnight. DCU was removed by filtration and the solvent was evaporated under vacuum to give a colorless solid which was recrystallized from ethyl acetate/cyclohexane to give the title product **18b** (268 mg, 51% yield). Mp 132 °C; R_f 0.28 (ethyl acetate/cyclohexane 30/70); ^{1}H NMR: δ ($CDCl_3$) 3.37 (dd, 2H, J = 6.9, 8.5 Hz), 3.78 (dd, 1H, J = 6.9, 8.5 Hz), 4.46 (dd, 2H, J = 5.9,14.7 Hz), 5.36 (s, 2H), 6.64 (t, 1H, J = 5.9 Hz), 7.03 (m, 1H), 7.16–7.45 (m, 16H), 7.54 (m, 1H), 7.94 (m, 1H). ^{13}C NMR: δ ($CDCl_3$) 36.84, 44.06, 55.16, 67.28, 122.79, 126.32, 127.37, 127.81, 127.87, 128.13, 128.56, 128.63, 128.86, 128.94, 128.96, 129.21, 129.70, 131.96, 135.92, 137.44, 137.77, 150.28, 165.49, 167.09, 169.89. Anal. Calcd for $C_{19}H_{21}NO_3 \cdot 1/2H_2O$: C, 74.09; H, 5.62; N, 2.79. Found: C, 74.11; H, 5.67; N, 2.87.

4.1.2.3. 3-(2-Benzylcarbamoyl-3-phenyl-propionyloxy)-benzoic acid 8. 3-(2-Benzylcarbamoyl-3-phenyl-propionyloxy)-benzoic acid benzyl ester **18b** (127 mg, 0.26 mmol) was dissolved in warm ethyl acetate (30 mL) and the solution was slowly cooled to room temperature. Palladium on charcoa1 (38 mg, 30%/w) was added and the mixture was stirred under a hydrogen atmosphere for 24 h. The reaction mixture was filtered through Celite and the solvent was removed under vacuum. The residue was recrystallized from ethyl acetate/cyclohexane to afford the title product **8** as a white solid (73 mg, 70% yield). Mp 174 °C; R_f 0.53 (methanol/ethyl acetate 10/90); ^{1}H NMR: δ ($CDCl_3$+MeOD) 3.24 (d, 2H, J = 11.8 Hz), 3.74 (t, 1H, J = 11.8 Hz), 4.30 (s, 2H), 6.98–7.05 (m, 3H), 7.11–7.24 (m, 8H), 7.32 (t, 1H, J = 11.8 Hz), 7.54 (m, 1H), 7.81 (m, 1H). ^{13}C NMR: δ ($CDCl_3$+MeOD) 35.52, 43.60, 54.76, 122.75, 125.90, 126.97, 127.37, 127.51, 127.59, 128.58, 128.67, 129.03, 129.44, 137.54, 137.67, 147.67, 150.27, 167.85, 169.05, 170.52. Anal. Calcd for $C_{24}H_{21}NO_5$: C, 71.45; H, 5.25; N, 3.55. Found: C, 71.41; H, 5.35; N, 3.55.

4.1.3. 3-Hydroxycarbonylphenyl *N*-benzyl-2-methoxymalonamate (9) (Scheme 2)

4.1.3.1. *N*-Benzyl-2-methoxymalonamic acid methyl ester 20. To a solution of methyl chlorocarbonylmethoxyacetate **19**[34] (825 mg, 5 mmol) in dichloromethane (10 ml) at 0 °C was added a solution of benzylamine (1.07 g, 10 mmol) in dichloromethane (10 ml). The reaction mixture was stirred for 1 h and then washed with a 10% aqueous HCl solution. The organic layer was dried over $MgSO_4$, the solvent evaporated, and the product purified by silica gel chromatography (pentane/AcOEt 1/1). The title product 20, 783 mg (67%), was thus obtained. Mp 94 °C. R_f 0.35. ^{1}H NMR ($CDCl_3$): δ = 3.46 (s, 3H), 3.85 (s, 3H), 4.37 (s, 1H), 4.48 (m, 2H), 6.90 (m, 1H), 7.26–7.34 (m, 5H). ^{13}C NMR ($CDCl_3$): δ = 43.45, 53.08, 58.66, 81.63, 127.87–137.66, 165.49, 168.11.

4.1.3.2. *N*-Benzyl-2-methoxymalonamic acid 21. Methyl *N*-benzyl-2-methoxymalonamate **20** (421 mg, 1.78 mmol) was dissolved in methanol (5 ml). NaOH solution (1 M, 5 ml) was added and the mixture stirred at room temperature overnight. The methanol was evaporated. The aqueous phase was washed with ethyl acetate, acidified and the product extracted with AcOEt. The organic layer was dried over $MgSO_4$ and the solvent evaporated to give 355 mg (86%) of the title product **21**. Mp 106 °C. ^{1}H NMR ($CDCl_3$): δ = 3.63 (s, 3H), 4.36 (s, 1H), 4.50 (m, 2H), 7.26–7.39 (m, 6H), 8.87 (s, 1H). ^{13}C NMR ($CDCl_3$): δ = 43.90, 60.09, 79.18, 128.08–136.76, 167.89, 168.45.

4.1.3.3. 3-Benzyloxycarbonylphenyl *N*-benzyl-2-methoxymalonamate 22. To a solution of 223 mg (1 mmol) of *N*-benzyl-2-methoxy-malonamic acid **21** in dichloromethane (2 ml) were added 228 mg (1 mmol) of benzyl 3-hydroxybenzoate[35] and 206 mg (1 mmol) of DCC. The reaction mixture was stirred at room temperature for 16 h and then filtered to remove DCU. The solvent was evaporated and the residue recrystallized from diethyl ether to give 236 mg (54%) of the title product **22**. Mp 86 °C. R_f 0.68 (pentane/AcOEt 1/1). ^{1}H NMR ($CDCl_3$): δ = 3.59 (s, 3H), 4.54 (m, 2H), 4.61 (s, 1H), 5.38 (s, 2H), 7.01 (s, 1H), 7.27–7.50 (m, 12H), 7.87 (t, 1H), 7.99 (dt, 1H). ^{13}C NMR ($CDCl_3$): δ = 43.55, 58.92, 67.24, 81.64, 122.77–137.56, 150.44, 165,10, 165,56, 166,15. Anal. Calcd for $C_{25}H_{23}NO_6$: C, 69.27; H, 5.35; N, 3.23. Found: C, 69.26; H, 5.37; N, 3.27.

4.1.3.4. 3-Hydroxycarbonylphenyl *N*-benzyl-2-methoxymalonamate 9. To a solution of 95 mg of ester **22** in ethyl acetate (30 ml) was added 24 mg of 10% Pd/C. The hydrogenolysis was performed in a Parr apparatus for 16 h. The catalyst was removed by filtration and the solvent evaporated to give 60 mg (80%) of the title product **9**. Mp 152 °C. R_f 0.19 (pentane/AcOEt 1/1). ^{1}H NMR (CD_3COCD_3): δ = 3.58 (s, 3H), 4.51 (m, 2H), 4.75 (s, 1H), 7.23–7.42 (m, 12H), 7.60 (t, 1H), 7.80 (s, 1H), 7.96 (d, 1H), 8.15 (s, 1H). ^{13}C NMR

(CD_3COCD_3): δ = 43.39, 58.87, 82.52, 117.05–140.07, 151.64, 166.20, 166.74, 167.42. Anal. Calcd for $C_{18}H_{17}NO_6$: C, 62.97; H, 4.99; N, 4.08. Found: C, 62.86; H, 4.91; N, 4.35.

4.1.4. 3-[2-(*N*-Methyl-benzylaminocarbonyl)-ethanoyl]-oxybenzoic acid 10

4.1.4.1. Methyl *N*-methyl-*N*-benzylmalonamate. To a solution of methyl 3-chloro-3-oxopropionate 1.273 g (9.33 mmol) in dichloromethane (10 mL) at 0 °C, was added dropwise a solution of *N*-methylbenzylamine (2.26 g, 18.6 mmol) in dichloromethane (5 mL). The reaction mixture was stirred at 25 °C for 3 h. The solution was washed with 10% aqueous HCl, dried over $MgSO_4$, the solvent evaporated, and the residue purified by silica gel chromatography (eluent: cyclohexane/ethyl acetate 1/1). The title compound (1.72 g, 83%) was obtained as a liquid. R_f 0.51 (cyclohexane/ethyl acetate: 1/3). ^{1}H NMR ($CDCl_3$) (2 conformers) δ 2.91 and 2.97 (2s, 3H), 3.49 and 3.53 (2s, 2H), 3.71 and 3.76 (2s, 3H), 4.53 and 4.61 (2s, 2H), 7.16–7.37 (m, 5H). ^{13}C NMR ($CDCl_3$) (2 conformers) δ 34.2 and 35.5, 41.2 and 41.4, 51.1 and 54.1, 52.6, 126.5–136.9, 166.3 and 168.1.

4.1.4.2. *N*-Methyl-*N*-benzyl-malonamic acid 17c. Methyl *N*-methyl-*N*-benzylmalonamate (1.1 g, 5 mmol) was dissolved in methanol (15 mL). NaOH solution (1 M, 15 mL) was added and the mixture was stirred at room temperature overnight. After the methanol was evaporated, the solution was acidified with 20% aqueous HCl and extracted with ethyl acetate. The organic solution was dried ($MgSO_4$) and the solvent evaporated under vacuum to give quantitatively the title product **17c**. R_f 0.39 (acetic acid/ethyl acetate: 2.5/97.5). ^{1}H NMR (CD_3COCD_3) (2 conformers) δ 2.95 and 3.05 (2s, 3H), 3.57 and 3.60 (2s, 2H), 4.66 and 4.69 (2s, 2H), 7.29–7.40 (m, 5H). ^{13}C NMR ($CDCl_3$) (2 conformers) δ 34.1 and 35.4, 37.8 and 38.0, 51.4 and 53.8, 127.6–137.9, 168.5 and170.3.

4.1.4.3. Benzyl 3-[2-(*N*-methyl-benzylaminocarbonyl)-ethanoyl]-oxybenzoate 18c. To a solution of 310 mg (1.5 mmol) of *N*-methyl-*N*-benzyl-malonamic acid **17c** in dichloromethane (10 mL) were added 309 mg (1.5 mmol) of DCC and 340 mg (1.5 mmol) of benzyl 3-hydroxybenzoate. The mixture was stirred at room temperature overnight. DCU was removed by filtration. The solvent was evaporated and the residue purified by silica gel chromatography (eluent: cyclohexane/ethyl acetate: 7/3) to give 445 mg (71% yield) of title product **18c** as a liquid. R_f 0.43 (cyclohexane/ethyl acetate: 1/1). ^{1}H NMR ($CDCl_3$) (2 conformers) δ 2.99 and 3.04 (2s, 3H), 3.74 and 3.78 (2s, 2H), 4.59 and 4.67 (2s, 2H), 5.37 and 5.38 (2s, 2H), 7.21–7.51 (m, 12H), 7.82 and 7.85 (2t, 1H), 7.97 and 7.99 (2dt, 1H). ^{13}C NMR ($CDCl_3$) (2 conformers) δ 34.4 and 35.5, 41.2 and 42.6, 51.2 and 54.2, 67.1 and 67.2, 122.9–136.7, 150.6 and 152.0, 165.5, 165.8, 166.1.

4.1.4.4. 3-[2-(*N*-Methyl-benzylaminocarbonyl)-ethanoyl]-oxybenzoic acid 10. The benzyl ester **18c** (140 mg, 0.33 mmol) was dissolved in ethyl acetate (10 mL). 20 mg of 10% Pd/C was added and the solution was hydrogenated at room temperature for 2 h. The reaction mixture was filtered and the solvent evaporated. The solid residue was washed with pentane to give 96 mg (88% yield) of title product **10**, which was recrystallized from acetone. Mp 147 °C. R_f 0.48 (acetone). ^{1}H NMR (CD_3COCD_3) (2 conformers) δ 2.95 and 3.08 (2s, 3H), 3.89 and 3.93 (2s, 2H), 4.66 and 4.71 (2s, 2H), 7.26–7.47 (m, 6H), 7.57 and 7.58 (2t, 1H), 7.83 and 7.85 (2t, 1H), 7.94 and 7.95 (2dt, 1H). ^{13}C NMR (CD_3COCD_3) (2 conformers) δ 33.9 and 35.7, 41.7 and 41.9, 51.2 and 54.2, 123.9–138.5, 152.0 and 152.1, 166.8, 166.9, 167.3. Anal. Calcd for $C_{18}H_{17}NO_5$: C, 66.04; H, 5.24; N, 4.28. Found: C, 65.92; H, 5.32; N, 4.12.

4.1.5. 3-[2-(Benzylaminocarbonyl)-ethanoyl]-oxybenzoic acid 11

4.1.5.1. Methyl *N*,*N*-dibenzyl malonamate. To a solution of methyl 3-chloro-3-oxopropionate (255 mg, 1.86 mmol) in dichloromethane (5 mL) at 0 °C was added dropwise a solution of dibenzylamine (736 mg, 3.73 mmol) in dichloromethane (2 mL). The reaction mixture was stirred at room temperature for 3 h. The solution was washed with 10% aqueous HCl, dried over $MgSO_4$, the solvent evaporated under vacuum, and the residue purified by silica gel chromatography (eluent: cyclohexane/ethyl acetate 7/3). The title compound (538 mg, 97%) was obtained as a liquid. R_f 0.34 (cyclohexane/ethyl acetate: 7/3). ^{1}H NMR ($CDCl_3$) 3.56 (s, 2H), 3.76 (s, 3H), 4.45 and 4.65 (2s, 2H), 7.16–7.42 (m, 10H). ^{13}C NMR ($CDCl_3$) δ 48.6, 50.8, 52.6, 126.6–136.8, 166.9, 168.2.

4.1.5.2. *N*,*N*-Dibenzylmalonamic acid 17d. Methyl *N*,*N*-dibenzylmalonamate (411 mg, 1.53 mmol) was dissolved in methanol (10 mL). NaOH solution (1 M, 10 mL) was added and the mixture was stirred at room temperature overnight. The methanol was removed by evaporation and the residual aqueous solution acidified with 20% aqueous HCl. The reaction mixture was extracted with ethyl acetate. The organic phase was dried ($MgSO_4$) and evaporated under vacuum to give 305 mg (78% yield) of the title product **17d**. R_f 0.53 (acetic acid/ethyl acetate: 2.5/97.5). ^{1}H NMR (CD_3COCD_3) δ 3.63 (s, 2H), 4.62 and 4.65 (2s, 2H), 7.27 to 7.41 (m, 10H). ^{13}C NMR ($CDCl_3$) δ 39.0, 49.2, 51.2, 127.8–137.9, 168.7, 170.1.

4.1.5.3. Benzyl 3-[2-(*N*,*N*-dibenzylaminocarbonyl)-ethanoyl]-oxybenzoate 18d. To a solution of 267 mg (0.94 mmol) of *N*,*N*-dibenzylmalonamic acid, **17d**, in dichloromethane (6 mL) were added 194 mg (0.94 mmol) of DCC and 214 mg (0.94 mmol) of benzyl 3-hydroxybenzoate. The mixture was stirred at room temperature for 2 h. DCU was filtered off. The solvent was evaporated and the residue purified by chromatography (eluents: cyclohexane/ethyl acetate: 9/1, then 8/2) to give 242 mg (52% yield) of the title product **18d** as a liquid. R_f 0.29 (cyclohexane/ethyl acetate: 8/2). ^{1}H NMR ($CDCl_3$) 3.80 (s, 2H), 4.52 and 4.71 (2s, 2H), 5.39 (s, 2H), 7.21–7.48 (m, 17H), 7.84 (t, 1H), 8.00 (dt, 1H). ^{13}C NMR ($CDCl_3$) δ 41.4, 48.7, 50.8, 67.2, 122.8–136.6, 150.6, 165.6, 166.2, 166.4.

4.1.5.4. 3-[2-(Benzylaminocarbonyl)-ethanoyl]-oxybenzoic acid 11. To a solution of the benzyl ester **18d** (131 mg, 0.27 mmol) in ethyl acetate (10 mL) was added 22 mg of Pd/C 10% and the mixture was hydrogenated at rt for 2 h. The reaction mixture was filtered and the solvent evaporated. The solid residue was washed with pentane to give 101 mg (94%) of title product **11**, which was recrystallized from acetone. Mp 137 °C R_f 0.59 (pentane/acetone: 1/1). ^{1}H NMR (acetone) δ 3.95 (s, 2H), 4.66 (s, 4H), 7.29–7.44 (m, 11H), 7.58 (t, 1H), 7.85 (t, 1H), 7.96 (dt, 1H). ^{13}C NMR (CD_3COCD_3) δ 41.8, 49.1, 51.5, 123.9–138.4, 152.1, 166.9, 167.3, 167.5. Anal. Calcd for $C_{24}H_{21}NO_5$: C, 71.45; H, 5.25; N, 3.47; Found: C, 71.38; H, 5.33; N, 3.32.

4.1.6. 3-Carboxylphenyl 1-benzyl-2-oxo-pyrrolidine-3-carboxylate 12 (Scheme 3)

4.1.6.1. Methyl 1-benzyl-2-oxo-pyrrolidine-3-carboxylate 23. 1-Benzyl-2-pyrrolidinone (262 mg, 1.5 mmol) was dissolved in anhydrous THF (3 mL). The solution was cooled to −78 °C under argon, and a 2 N solution of LDA (1 mL, 1.3 equiv) was added dropwise. After a further 30 min stirring at −78 °C, methyl chloroformate (107 mg, 1.12 mmol) was added. After 30 min at −78 °C, the temperature was allowed to rise to 25 °C and the mixture was stirred for 1 h. The reaction mixture was then poured into aqueous 1 M HCl and extracted with ethyl acetate. The organic phase was dried over $MgSO_4$, the solvent was removed by

evaporation, and the residue chromatographed on silica gel (eluent: cyclohexane/AcOEt 25/75) to give 91 mg (35% yield) of the title compound **23** as an oil. R_f 0.35. ^{1}H NMR ($CDCl_3$) δ 2.24 and 2.39 (2 m, 2H), 3.23 and 3.39 (2 m, 2H,), 3.52 (dd, J = 9.5, 4.8 Hz, 1H), 3.80 (s, 3H), 4.48 (s, 2H), 7.23–7.37 (m, 5H). ^{13}C NMR ($CDCl_3$) δ 22.17, 45.08, 46.97, 48.31, 52.63, 127.68–135.88, 169.70, 170.71.

4.1.6.2. 1-Benzyl-2-oxo-pyrrolidine-3-carboxylic acid 24. To a solution of methyl 1-benzyl-2-oxo-pyrrolidine-3-carboxylate **23** (178 mg, 0.76 mmol) in methanol (5 mL) was added NaOH solution (1 M, 5 mL) and the reaction mixture was stirred at 25 °C overnight. After evaporation of the methanol and extraction of the residual aqueous solution with ethyl acetate, the aqueous phase was acidified, extracted with ethyl acetate, and the extract dried over $MgSO_4$. Evaporation of the solvent gave quantitatively the title acid **24**. R_f 0.42 (AcOEt/AcOH 95/5). ^{1}H NMR (CD_3COCD_3) δ 2.31 (m, 2H), 3.36 (m, 2H), 3.55 (t, J = 8.7 Hz, 1H), 4.49 (dd, 2H), 7.27–7.34 (m, 5H). ^{13}C NMR (CD_3COCD_3) δ 22.75, 45.69, 47.16, 48.28, 128.27–137.43, 171.28, 171.68.

4.1.6.3. 3-Benzyloxycarbonylphenyl 1-benzyl-2-oxo-pyrrolidine-3-carboxylate 25. To a solution of the acid **24** (305 mg, 1.39 mmol) in dichloromethane (5 mL) was added 318 mg (1.39 mmol) of benzyl 3-hydroxybenzoate, and then 287 mg (1.39 mmol) of DCC. The reaction mixture was stirred at 25 °C overnight. DCU was removed by filtration and the solvent evaporated under vacuum. Two column chromatographies on silica gel (eluent: hexane/AcOEt 70/30, then dichlorométhane/acetone 97.5/2.5) afforded 525 mg (88%) of the title ester **25** as an oil. R_f 0.48 (ether). ^{1}H NMR ($CDCl_3$) δ 2.39, 2.52 (2 m, 2H), 3.31, 3.44 (2 m, 2H), 3.76 (dd, J = 6.9, 9.4 Hz, 1H), 4.53 (s, 2H), 5.38 (s, 2H), 7.28- 7.45 (m, 12H), 7.85 (t, 1H), 7.98 (dt, 1H). ^{13}C NMR ($CDCl_3$) δ 22.43, 45.31, 47.35, 48.72, 117.25–135.97, 152.32, 165.64, 168.96, 169.36. Anal. Calcd for $C_{26}H_{23}NO_5$: C, 72.71; H, 5.40; N, 3.26. Found: C, 72.50; H, 5.49; N, 2.95.

4.1.6.4. 3-Carboxylphenyl 1-benzyl-2-oxo-pyrrolidine-3-carboxylate 12. To a solution of ester **25** (193 mg, 0.45 mmol) in ethyl acetate (10 mL) was added 20 mg of 10% Pd/C and the mixture stirred for 2 h under a hydrogen atmosphere. Filtration and evaporation of the solvent afforded the title acid **12** as a syrup. R_f 0.68 (AcOEt/AcOH 99/1). ^{1}H NMR (CD_3COCD_3) δ 2.49 (m, 2H), 3.44 (m, 2H), 3.83 (t, J = 8.5 Hz, 1H), 4.47, 4.58 (2d, J = 14.9 Hz, 2H), 7.27–7.35 (m, 5H), 7.44 (dt, 1H), 7.59 (t,1H), 7.83 (t, 1H), 7.97 (dt, 1H). ^{13}C NMR (CD_3COCD_3) δ 23.21, 45.81, 47.28, 49.42, 123.79–137.77, 151.99, 166.81, 167.96, 170.10. Anal. Calcd for $C_{19}H_{17}NO_5 \cdot 1/2H_2O$: C, 65.50; H, 5.20; N, 4.02. Found: C, 65.31; H, 5.14; N, 4.11.

4.1.7. 3-Carboxylphenyl *N*-benzyl-2-oxo-1,2,3,4-tetrahydroquinoline-3-carboxylate 13 (Scheme 4)

4.1.7.1. Methyl 2-nitrobenzylmalonate 26. To a solution of dimethyl malonate (1.056 g, 8.0 mmol) in DMF (18 mL) was slowly added 320 mg (8.0 mmol) of sodium hydride (60% in oil) and the mixture stirred for 15 min at 25 °C. 2-Nitrobenzyl chloride (343 mg, 2.0 mmol) was added and the reaction mixture stirred for a further 2 h., then poured into 10% HCl. After extraction of the acidified solution with ethyl acetate, the organic phase was washed three times with distilled water, brine, and then dried over $MgSO_4$. Dimethyl malonate was removed in a Büchi apparatus at 150 °C under vacuum (20 mm Hg). The residue (530 mg) was a mixture of monoalkylated and dialkylated products in a ratio 83/11 (NMR). Two spots were observed by tlc (eluent: cyclohexane/AcOEt 70/30). R_f monoalkylated derivative: 0.46; R_f dialkylated derivative: 0.42. These products were separated and purified by silica gel column chromatography.

Methyl 2-nitrobenzylmalonate **26**: ^{1}H NMR ($CDCl_3$) δ 3.52 (d, J = 7.7 Hz, 2H), 3.71 (s, 6H), 3.93 (t, 1H), 7.35 (d, 1H), 7.40–7.55 (m, 2H), 8.01 (d, 1H). ^{13}C NMR ($CDCl_3$) δ 32.43, 52.33, 52.90, 169.11.

4.1.7.2. Methyl 2-oxo-1,2,3,4-tetrahydroquinoline-3-carboxylate 27. To a solution of compound **26** (240 mg, 0.9 mmol) in ethyl acetate (15 mL) was added 20 mg of Pd/C and the mixture stirred for 3 h at 25 °C under an H_2 atmosphere. Filtration, evaporation of the solvent and column chromatography of the residue (eluent: cyclohexane/AcOEt 50/50) afforded 155 mg (84%) of the title product **27**. Mp 166.5 °C. R_f 0.38. ^{1}H NMR ($CDCl_3$) δ 3.13 (dd, J = 6.2, 15.8 Hz, 1H), 3.41 (dd, J = 9.0, 15.8 Hz, 1H), 3.66 (dd, 1H), 3.78 (s, 3H), 6.85 (d, 1H), 7.00 (t, 1H), 7.15–7.23 (m, 2H), 9.09 (s, 1H). ^{13}C NMR ($CDCl_3$) δ 29.03, 47.56, 52.94, 115.88–136.71, 167.65, 169.80. Anal. Calcd for $C_{11}H_{11}NO_3$: C, 64.38; H, 5.40; N, 6.83. Found: C, 64.27; H, 5.53; N, 6.64.

4.1.7.3. Methyl *N*-3-di-*tert*-butoxycarbonyl-2-oxo-1,2,3,4-tetrahydroquinoline-3-carboxylate 28. To a solution of ester **27** (95 mg, 0.5 mmol) in acetonitrile (5 mL) was added 218 mg (1.0 mmol) of Boc_2O and 61 mg of DMAP and the mixture was stirred at 25 °C overnight. Evaporation of the solvent and silica gel chromatography (eluent: cyclohexane/AcOEt 70/30) gave 155 mg (76%) of the title product **28**. Mp 108.4 °C. R_f 0.47. (24 mg of methyl 3-*tert*-butoxycarbonyl-2-oxo-1,2,3,4-tetrahydroquinoline-3-carboxylate **28** were also isolated). ^{1}H NMR ($CDCl_3$) δ 1.28 (s, 9H), 1.62 (s, 9H), 3.40 (d, J = 15.6 Hz, 1H), 3.60 (d, 1H), 3.81 (s, 3H), 6.91 (d, 1H), 7.09 (t, 1H), 7.09–7.24 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 27.56, 27.81, 33.36, 53.57, 63.57, 83.95, 85.75, 116.85–136.38, 151.26, 163.98, 165.16, 167.23.

4.1.7.4. Methyl 3-*tert*-butoxycarbonyl-2-oxo-1,2,3,4-tetrahydroquinoline-3-carboxylate 29. To a stirred solution of compound **28** (232 mg, 0.59 mmol) in dichloromethane (5 mL) at 0 °C was added TFA (0.5 mL). After 1 h, toluene (5 mL) was added and the solvents were evaporated under vacuum at room temperature. Silica gel column chromatography (eluent: cyclohexane/AcOEt 70/30) afforded 161 mg (92%) of the title product **29**, as an oil. R_f 0.27. ^{1}H NMR ($CDCl_3$) δ 1.36 (s, 9H), 3.47 (d, J = 15.6 Hz, 1H), 3.58 (d, 1H), 3.79 (s, 3H), 6.82 (d, 1H), 7.01 (t, 1H), 7.16–7.27 (m, 2H), 9.35 (s, 1H). ^{13}C NMR ($CDCl_3$) δ 27.70, 33.40, 53.49, 62.94, 83.76, 115.99–136.36, 165.76, 166.60, 167.80.

4.1.7.5. Methyl *N*-benzyl-3-*tert*-butoxycarbonyl-2-oxo-1,2,3,4-tetrahydroquinoline-3-carboxylate 30. To a solution of compound **29** (260 mg, 0.85 mmol) in acetonitrile (10 mL) was added 30 mg of benzyltriethylammonium chloride, 250 mg of K_2CO_3 then benzyl chloride (0.22 mL) and the mixture was heated at 60 °C for 4 h under stirring. After cooling of the mixture and its extraction with ethyl acetate, the organic phase was washed with water and dried over $MgSO_4$. Silica gel column chromatography (eluent: cyclohexane/AcOEt 80/20) afforded 278 mg (83%) of the title product **30**, as an oil. R_f 0.36. ^{1}H NMR ($CDCl_3$) δ 1.33 (s, 9H), 3.49 (d, J = 15.6 Hz, 1H), 3.60 (d, 1H), 3.81 (s, 3H), 5.18 (s, 2H), 6.88 (d, 1H), 7.01 (d, 1H), 7.13 (t, 1H), 7.18–7.42 (m, 6H). ^{13}C NMR ($CDCl_3$) δ 27.79, 33.36, 47.86, 53.61, 63.50, 83.83, 115.98- 139.39, 165.19, 165.89, 167.97.

4.1.7.6. Methyl *N*-benzyl-2-oxo-1,2,3,4-tetrahydroquinoline-3-carboxylate 31. To a solution of compound **30** (162 mg, 0.55 mmol) in dichloromethane (1 mL) was added TFA (1 mL) and the mixture was stirred for 1 h at 25 °C. Silica gel column chromatography (eluent: cyclohexane/AcOEt 70/30) gave 113 mg (93%) of the title product **31**. Mp 107 °C. R_f 0.40. ^{1}H NMR ($CDCl_3$) δ 3.18 (dd, J = 15.5, 5.9 Hz, 1H), 3.41 (dd, J = 15.5, 8.3 Hz, 1H), 3.80 (dd,

1H), 3.76 (s, 3H), 5.07, 5.33 (2d, J = 16.2 Hz, 2H), 6.90 (d, 1H), 7.01 (t, 1H), 7.13 (t, 1H), 7.17–7.37 (m, 6H). ^{13}C NMR ($CDCl_3$) δ 29.01, 47.81, 48.31, 52.83, 115.98–139.43, 166.75, 169.94.

4.1.7.7. *N*-Benzyl-2-oxo-1,2,3,4-tetrahydroquinoline-3-carboxylic acid 32. To a solution of ester **31** (108 mg, 0.37 mmol) in methanol (5 mL) was added 10% aqueous NaOH (5 mL). After the solution was stirred for 1 h at 25 °C, methanol was removed by evaporation and the aqueous phase extracted with ethyl acetate. The extract was dried over $MgSO_4$ and the solvent removed under vacuum, quantitatively affording the title acid **32** as an oil. R_f 0.56 (eluent: AcOEt/AcOH 97.5/2.5). ^{1}H NMR ($CDCl_3$) δ 3.27 (dd, J = 15.0, 6.1 Hz, 1H), 3.38 (dd, J = 15.0, 11.6 Hz, 1H), 3.70 (dd, 1H), 5.17, 5.27 (2d, J = 16.2 Hz, 2H), 6.95 (d, 1H), 7.07 (t, 1H), 7.18 (t, 1H), 7.20–7.35 (m, 6H). ^{13}C NMR ($CDCl_3$) δ 28.50, 45.21, 47.40, 115.07–138.71, 169.08. 172.12. Anal. Calcd for $C_{17}H_{15}NO_3$: C, 72.58; H, 5.38; N, 4.98. Found: C, 72.47; H, 5.51; N, 4.74.

4.1.7.8. 3-Benzyloxycarbonylphenyl *N*-benzyl-2-oxo-1,2,3,4-tetrahydroquinoline-3-carb-oxylate 33. To a solution of acid **32** (107 mg, 0.38 mmol) in dichloromethane (3 mL) was added 88 mg (0.38 mmol) of benzyl 3-hydroxylbenzoate and 79 mg (0.38 mmol) of DCC. After the mixture was stirred for 4 h at 25 °C, the solvent was evaporated and the residue chromatographed on silica gel (eluent : dichloromethane/acetone 99/1) to give 133 mg (71%) of the title product **33**, as an oil. R_f 0.41. ^{1}H NMR ($CDCl_3$) δ 3.32 (dd, J = 15.8, 5.9 Hz, 1H), 3.54 (dd, J = 15.8, 8.6 Hz, 1H), 4.04 (dd, 1H), 5.13, 5.34 (2d, J = 16.2 Hz, 2H), 5.36 (s, 2H), 6.94 (d, 1H), 7.02 (t, 1H), 7.15 (t, 1H), 7.18–7.44 (m, 12H), 7.73 (t, 1H), 7.97 (dt, 1H). ^{13}C NMR ($CDCl_3$) δ 29.16, 47.27, 48.49, 67.19, 114.97–139.51, 150.58, 165.55, 166.35, 168.12.

4.1.7.9. 3-Carboxylphenyl *N*-benzyl-2-oxo-1,2,3,4-tetrahydroquinoline-3-carboxylate 13. To a solution of ester **33** (115 mg, 0.23 mmol) in ethyl acetate (10 mL) was added 10% Pd/C (20 mg) and the mixture was stirred for 2 h under a hydrogen atmosphere. After removal of the Pd/C by filtration and evaporation of the solvent, the solid residue was recrystallised from dichloromethane to give 89 mg (95%) of the title product **13**. Mp 147 °C. R_f 0.63 (AcOEt/AcOH 99/1). ^{1}H NMR (CD_3COCD_3) δ 3.41 (dd, J = 15.7, 2.0 Hz, 1H), 3.51 (dd, J = 15.7, 9.7 Hz, 1H), 4.17 (dd, 1H), 5.23. 5.34 (2d, J = 16.4 Hz, 2H), 7.01–7.40 (m, 10H), 7.58 (t, 1H), 7.79 (t, 1H), 7.94 (dt, 1H). ^{13}C NMR (CD_3COCD_3) δ 29.34, 46.84, 48.93, 116.78–140.29, 151.88, 166.77, 167.07, 169.08. Anal. Calcd for $C_{24}H_{19}NO_5{\cdot}H_2O$: C, 68.72; H, 5.04; N, 3.34. Found: C, 68.67; H, 4.76; N, 3.32.

4.1.8. 3-[(Benzylcarbamoyl-methyl)-hydroxy-phosphinoyloxy]-benzoic acid 14 (Scheme 5)

4.1.8.1. Methyl 3-[(benzylcarbamoyl-methyl)-methoxy-phosphinoyloxy]-benzoate 35. The phosphite **34** (840 mg, 3.44 mmol) and *N*-benzyl-2-bromoacetamide (392 mg, 1.72 mmol) were heated together with stirring for 16 h at 110 °C. After cooling the reaction mixture, the product was isolated by silica gel chromatography (eluent: dichloromethane/acetone (4:1). Recrystallisation of the product from ethyl acetate/cyclohexane afforded 290 mg (45% yield) of **35** as a colorless solid. Mp 103 °C; R_f 0.18; ^{1}H NMR δ ($CDCl_3$) 3.06 (d, 2H, J = 21.1 Hz), 3.84 (d, 3H, J = 11.4 Hz), 3.91 (s, 3H), 4.44 (d, 2H, J = 5.9 Hz), 7.01 (t, 1H, J = 5.9 Hz), 7.20–7.45 (m, 7H), 7.81–7.91 (m, 2H); ^{13}C NMR δ ($CDCl_3$) 34.75 (d, J = 132 Hz), 43.91, 52.33, 53.86 (d, J = 6.7 Hz), 121.56 (d, J = 4.5 Hz), 125.09 (d, J = 3.9 Hz), 126.66 (d, J = 1.1 Hz), 127.49, 127.61, 128.65, 129.91 (d, J = 1.1 Hz), 132.10 (d, J = 1.1 Hz), 137.66, 149.86 (d, J = 8.4 Hz), 162.85 (d, J = 3.9 Hz), 165.86. Anal. Calcd for $C_{18}H_{20}NO_6P$: C, 57.30; H, 5.34; N, 3.71. Found: C, 57.22; H, 5.55; N, 3.94.

4.1.8.2. 3-[(Benzylcarbamoyl-methyl)-hydroxy-phosphinoyloxy]-benzoic acid 14. A mixture of 3-[(benzylcarbamoyl-methyl)-methoxy-phosphinoyloxy]-benzoic acid methyl ester **35** (98 mg, 0.26 mmol) and iodotrimethylsilane (150 μL, 1.05 mmol) was stirred and heated at 50 °C for 16 h. The reaction mixture was then allowed to cool and excess iodotrimethylsilane was removed under vacuum. The resulting pale yellow solid was recrystallised from methanol/dichloromethane affording the product **14** as a colorless powder (49 mg, 54%). Mp 201 °C; R_f 0.27 (ethyl acetate/methanol 1:1); ^{1}H NMR δ (MeOD) 3.09 (d, 2H, J = 21.7 Hz), 4.41 (s, 2H), 7.22–7.46 (m, 7H) 7.83- 7.88 (m, 2H); ^{13}C NMR δ (MeOD+ CD_3SOCD_3) 36.92 (d, J = 133 Hz), 44.39, 123.20 (d, J = 3.9 Hz), 126.73 (d, J = 4.5 Hz), 127.21 (d, J = 1.1 Hz), 128.38, 128.72, 129.74, 131.09, 133.93 (d, J = 1.1 Hz), 140.01, 152.23 (d, J = 7.9 Hz), 166.91 (d, J = 6.2 Hz), 168.52. Anal. Calcd for $C_{16}H_{16}NO_6P{\cdot}1/2H_2O$: C, 53.63; H, 4.78; N, 3.91. Found: C, 53.61; H, 4.76; N, 3.69.

4.2. Enzyme kinetics

The *Enterobacter cloacae* P99 and TEM-2 β-lactamases were purchased from the Center for Applied Microbiology and Research, Porton Down, Wilts., UK, and used as supplied. The *Streptomyces* R61[36] and *Actinomadura* R39[37] DD-peptidases were generously supplied by Professor J. -M. Frère of the University of Liège, Belgium and Dr. Paulette Charlier, also of the University of Liège, respectively. Cephalothin was a gift from Eli Lilly and Co.

All kinetics measurements were carried out in 100 mM MOPS buffer, pH 7.5 at 25 °C. Stock solutions of the substrates were prepared in DMSO. These stock solutions were then diluted into buffer such that the final DMSO concentration was less than 5%. At this level, DMSO did not affect the measured initial rates of the enzyme-catalyzed reactions. Steady-state kinetics parameters were obtained from initial rate measurements (spectrophotometric; appearance of *m*-hydroxybenzoate at 290 or 300 nm) at a series of substrate concentrations. Non-linear fits of the data to the Henri-Michaelis–Menten equation were then computed to obtain values of the kinetics parameters. In several cases, the linearity of these curves allowed only values of k_{cat}/K_m to be obtained, with lower limits to k_{cat} and K_m estimated. The K_m value for reaction of **8** with the P99 β-lactamase was obtained as the K_i value for inhibition of cephalothin (200 μM) turnover. Rates of inhibition of the P99 and TEM-2 β-lactamases by the phosphonates **14** and **15** were obtained from measurements of enzyme activity against 0.2 mM cephalothin as a function of time of incubation of the enzyme with the inhibitor. Reaction mixtures contained enzyme (0.2 μM) and phosphonate, 100 μM for the P99 enzyme and 3 mM for TEM-2. The measured initial rates decreased in a first order fashion. Exponential curve fitting yielded pseudo-first order rate constants of inhibition and thus second order rate constants.

4.3. Molecular modeling

The structure of Figure 2 was derived from a computational model of the enzyme–substrate complex that was set up essentially as previously described[15] and run on an SGI Octane 2 computer with INSIGHT II 2005 (Accelrys, San Diego, CA). In these models of the P99 β-lactamase, Lys 67 and Lys 315 were cationic, Tyr 150 was neutral, and the tetrahedral intermediate **16** was anionic. After the the C2–N ethylene bridge was constructed, the stability of the structure was explored by molecular dynamics (200 ps runs, where the entire protein together with solvating water molecules were unrestricted). A typical snapshot of the dominant conformation was selected for energy minimization.

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