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PAPER

Kinetics and stereochemistry of hydrolysis of an *N*-(phenylacetyl)-α-hydroxyglycine ester catalyzed by serine β-lactamases and DD-peptidases†

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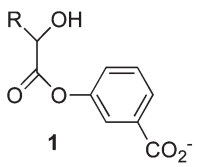
The α-hydroxydepsipeptide 3-carboxyphenyl *N*-(phenylacetyl)-α-hydroxyglycinate (**5**) is a quite effective substrate of serine β-lactamases and low molecular mass DD-peptidases. The class C P99 and ampC β-lactamases catalyze the hydrolysis of both enantiomers of **5**, although they show a strong preference for one of them. The class A TEM-2 and class D OXA-1 β-lactamases and the *Streptomyces* R61 and *Actinomadura* R39 DD-peptidases catalyze hydrolysis of only one enantiomer of **5** at any significant rate. Experiments show that all of the above enzymes strongly prefer the same enantiomer, a surprising result since β-lactamases usually prefer L(*S*) enantiomers and DD-peptidases D(*R*). Product analysis, employing peptidylglycine α-amidating lyase, showed that the preferred enantiomer is D(*R*). Thus, it is the β-lactamases that have switched preference rather than the DD-peptidases. Molecular modeling of the P99 β-lactamase active site suggests that the α-hydroxyl of **5** may interact with conserved Asn and Lys residues. Both α-hydroxy and α-amido substituents on a glycine ester substrate can therefore enhance its productive interaction with the β-lactamase active site, although their effects are not additive; this may also be true for inhibitors.

Introduction

The bacterial DD-peptidases remain well-validated antibiotic targets and the β-lactams remain their major weakness.[1] Resistance to β-lactam antibiotics is largely associated with bacterial production of β-lactamases, enzymes that catalyze β-lactam destruction by hydrolysis. β-Lactamase inhibitors are therefore of considerable interest in that they may be used to protect β-lactams in their assault on bacteria and thus lead to therapeutic success.[2–4] The search for new chemical entities that interact specifically with DD-peptidases or β-lactamases is thus an ongoing enterprise.

Recently, we have shown that α-hydroxyalkyl esters such as **1** can be good substrates of class A and C β-lactamases.[5] The incorporation of substituents



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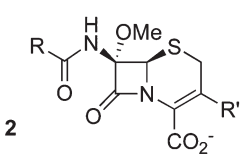

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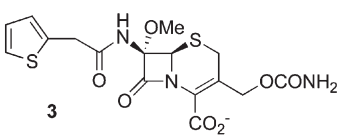
α to the scissile amide or ester bond of β-lactamase and DD-peptidase substrates and inhibitors has quite a long history, beginning with the example of the cephamycins, a class of β-lactam natural products containing the 7-α-methoxy group, **2**.[6] Synthetic analogues of these, such as cefoxitin, **3**, soon followed.[7] Often, on interaction with β-lactamases, these substituents lead to quite inert acyl–enzyme complexes and thus to inhibitory substrates.[8–14] The effects of α-substituents (X = Me, OMe, CH_2OH, $CH_2NH_3^+$, CO_2^-) on the reactivity of acyclic β-lactamase and DD-peptidase substrates such as **4** have also been examined.[15]



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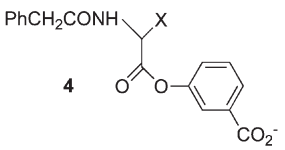

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The effect of the α-hydroxy substituent on β-lactamase and DD-peptidase substrates and inhibitors has not, to our knowledge, been studied, although 7-α-hydroxy-penicillins and cephalosporins have been synthesized.[16–18] In this paper we describe the preparation of the α-hydroxy-depsipeptide **5** and its reactivity with typical β-lactamases and DD-peptidases. In particular, an unusual specificity of β-lactamases for the α-D(*R*)-enantiomer was observed. [Throughout the paper we refer to the enantiomers of **5** as D and L in analogy to familiar amino acid terminology, where the OH group of **5** is seen as replacing the usual amino

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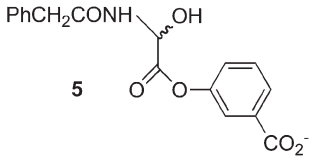
acid side chain. We do this to enable the discussion to be easily understood in the framework of the β-lactamase literature where acyclic substrates are amino acid derivatives. In the present text, however, we also give the formal *RS* descriptor when enantiomers of **5** are referred to.] The specificity of the enzyme peptidylglycine α-amidating lyase (PAL) for α-hydroxyglycine derivatives was employed to make this stereochemical assignment. Molecular modeling was employed to help understand this result.



Results and discussion

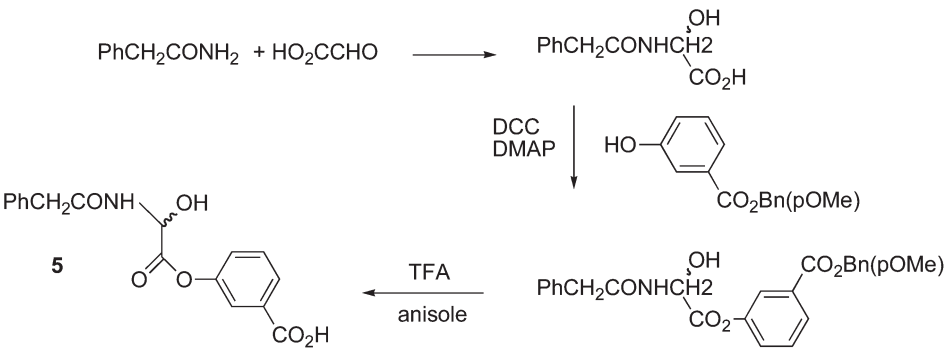
The synthesis of **5** was straightforward, following well-established precedents (Scheme 1). Preliminary experiments showed that at least one enantiomer of **5** was a substrate of several β-lactamases and DD-peptidases. These enzymes catalyzed its hydrolysis to a mixture of the α-hydroxy acid and *m*-hydroxybenzoic acid (see below). The issues of interest were then the stereochemical preferences of the various enzymes for the enantiomers of **5** and the magnitudes of the steady state parameters for turnover of these enantiomers. The former of these is first addressed below.

In a buffered solution at pH 7.5, **5** spontaneously hydrolyzes to a mixture of *N*-(phenylacetyl)-α-hydroxyglycine, **6**, and *m*-hydroxybenzoate (Scheme 2), as demonstrated by ^{1}H NMR (Fig. S4–S7, ESI†) and HPLC experiments (see below). The pseudo-first order rate constant for this reaction in 20 mM MOPS buffer, pH 7.5, was found to be $(1.1 \pm 0.1) \times 10^{-4}$ s^{-1}, a value quite comparable to that of **4** (X = OMe), *viz.* 2.5×10^{-4} s^{-1} [15] (although the latter represents a value in 100 mM MOPS buffer). The same products were obtained from the enzyme-catalyzed hydrolyses (Fig. S8, ESI†). Fig. 1A shows spectrophotometric data for turnover of **5** by the class C P99 β-lactamase. Two equal phases of reaction are seen, both faster than the spontaneous hydrolysis. It is clear, therefore, that this enzyme catalyzes the hydrolysis of both enantiomers of **5**, unless the slower phase represents the rate of racemization of the enantiomers (see below). The class C ampC β-lactamase behaved similarly. On the other hand, Fig. 1B shows that the class A TEM β-lactamase only measurably catalyzes the hydrolysis of one enantiomer of **5**. Since the second slower phase of reaction in this case represents spontaneous hydrolysis, it is clear that the second phase of the P99 reaction of Fig. 1A must represent enzyme-catalyzed hydrolysis of the second enantiomer rather than racemization.

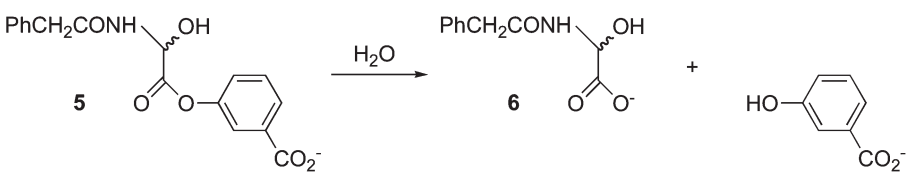
Addition of the P99 enzyme to the reaction mixture directly after the TEM enzyme had hydrolyzed its preferred enantiomer led to the data of Fig. 1C. The second phase shows a hydrolysis rate equal to that of the slower enantiomer in reaction with the P99 enzyme, *i.e.* P99 and TEM enzymes preferentially hydrolyze the same enantiomer of **5**. The converse experiment (not shown) where the TEM enzyme was added directly after the first phase of the P99 reaction was also performed. No acceleration in the rate of hydrolysis, above that of P99 catalysis, of the remaining enantiomer was then observed, confirming the conclusions reached above.

Similar experiments were carried out with the class D OXA-1 β-lactamase and the R61 and R39 DD-peptidases, with the same result as that obtained with the TEM β-lactamase, *viz.* all three enzymes catalyzed the hydrolysis of only one enantiomer of **5**, and that enantiomer was the same as that preferred by the P99 and TEM enzymes.

These results were rather interestingly different to those obtained previously for **4** (X = OMe, CH_2OH, Me). In those cases, as above, only one enantiomer was a substrate of the R61 and R39 DD-peptidases. On the basis of the structure of the likely



Scheme 1 Synthesis of **5**.



Scheme 2 Hydrolysis of the depsipeptide **5**.

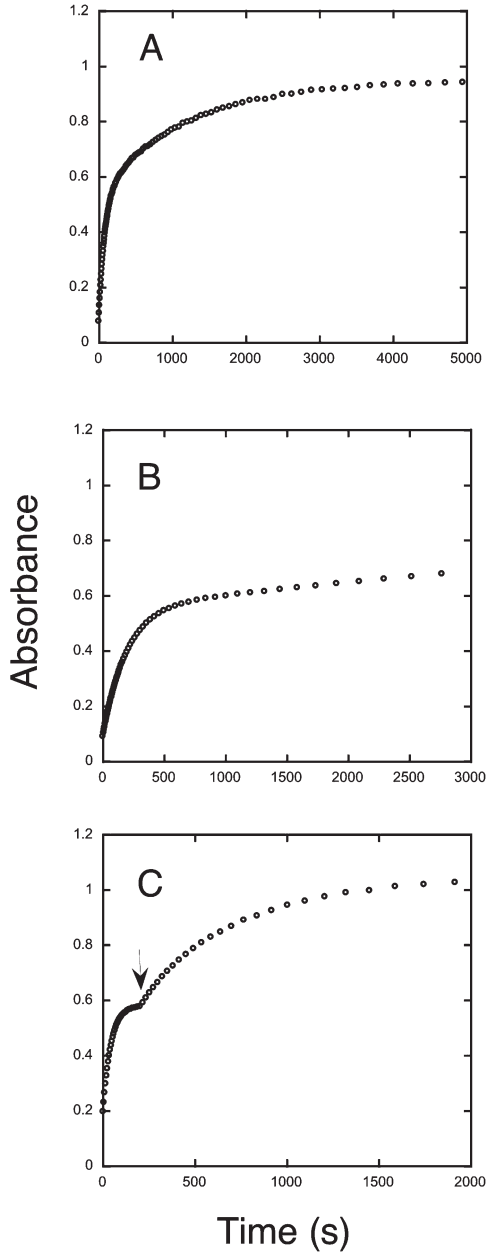


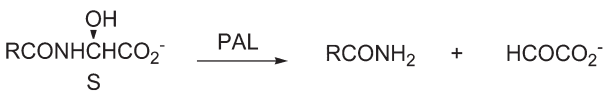
Fig. 1 Total progress curves (absorption at 290 nm) for the β-lactamase-catalyzed turnover of **5** (0.5 mM). A: P99 β-lactamase (35 nM). B: TEM β-lactamase (0.25 μM). C: On addition of the P99 β-lactamase (70 nM) immediately after (arrow) the TEM (1.0 μM)-catalyzed reaction.

in vivo substrate of these enzymes, a carboxyl-terminating D-alanyl-D-alanine peptide,[19] it was assumed that the reactive isomer was the D(*R*)-enantiomer. Given this assignment, the P99 and TEM β-lactamases therefore preferred the L(*S*)-enantiomer, although, as in the present case, the P99 enzyme also catalyzed more slowly the hydrolysis of the other [D(*R*)] enantiomer. The structural basis of these preferences has been discussed,[5] where the major point is the presence in DD-peptidases of a small hydrophobic binding pocket for the penultimate D-alanine methyl group, which is not present in β-lactamases. Indeed, the deletion of such a pocket has been proposed to be a major element of β-lactamase evolution.[20–22]

The dilemma presented by the current results is now clear. If the DD-peptidases prefer the D(*R*)-enantiomer of **5**, then the enantio-preference of the β-lactamases has switched. But do the DD-peptidases still prefer (absolutely) the D(*R*)-enantiomer? Or is it possible that, because of the very different chemical properties of a methyl and a hydroxyl group, the DD-peptidase preference is switched instead? We felt that a direct experimental resolution of these questions was required.

The enzyme peptidylglycine α-amidating lyase (EC 4.3. 2.5) (PAL, also known as peptidylglycolate lyase or PGL) catalyzes the reaction of Scheme 3, with an absolute preference for the (L) *S* configuration at the α-position of the substrate.[23] It has been used previously to determine the absolute configuration of α-hydroxyglycine derivatives, including *N*-(phenylacetyl)-α-hydroxyglycine, **6**.[24] We have therefore employed it to determine the absolute configuration of **6** produced on hydrolysis of **5** by the β-lactamases and DD-peptidases (Scheme 1). First, a sample of PAL was added to a solution of **6** in deuterated buffer and the ensuing reaction monitored by ^{1}H NMR spectroscopy. One half of **6** was converted to phenylacetamide and glyoxylate, the identity of the products was confirmed by the addition of authentic samples. PAL therefore behaved in our hands as expected. It was then added to the products of the TEM and P99 β-lactamase catalyzed hydrolysis of **5**.

In one experiment, solid TEM (*ca.* 0.1 mg) was added to a solution of **5** (1.0 mM) and the subsequent reaction monitored



Scheme 3 The PAL reaction.

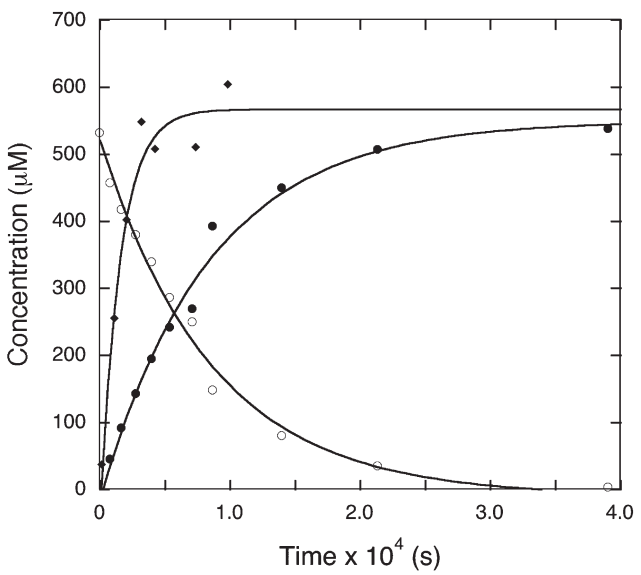
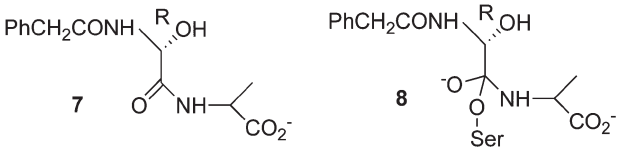


Fig. 2 HPLC-generated progress curves for the PAL-catalyzed turnover (monitoring the appearance of phenylacetamide) of reaction product **6** from hydrolysis of **5** by the P99 (closed diamonds) and TEM (closed circles) β-lactamases. The open circles describe the disappearance, by spontaneous reaction, of the single enantiomer of **5** left intact by the TEM β-lactamase. The lines represent exponential fits to the data.

spectrophotometrically as described above (Fig. 1B). Immediately after the first phase (when the enzyme-catalyzed reaction was complete), PAL (2 μM) was added and the subsequent reaction monitored by HPLC with the results shown in Fig. 2 (an example of the raw data is shown in Fig. S3 of the ESI†). The PAL-catalyzed appearance of phenylacetamide occurred at the same rate as disappearance of the unreacted isomer of **5** from spontaneous hydrolysis. This proves that the TEM β-lactamase has absolute specificity for the D(*R*) enantiomer of **5**, yielding the D(*R*) enantiomer of **6**, which does not react with PAL. To support this conclusion, PAL (2 μM) was added to the products of the P99-catalyzed reaction of **5**. In this case, both enantiomers are hydrolyzed (Fig. 1A). Addition of PAL then led to the initial appearance of phenylacetamide (0.5 mM) at a much faster rate than from the TEM experiment. This result shows that if the L(*S*) enantiomer of **5** were hydrolyzed by TEM, the PAL catalyzed formation of **6** would be observed at a rate faster than that of the spontaneous hydrolysis of **5**.

A combination of the above results shows that all of the above enzymes prefer the D(*R*) enantiomer of **5** as a substrate. The L(*S*)-enantiomer is only hydrolyzed at a significant rate by the class C β-lactamases, but even these enzymes prefer the D(*R*) enantiomer. As noted above, this stereo-preference by the β-lactamases (but not the DD-peptidases) is contrary to most precedent with depsipeptide substrates of structure **4**. It is, however, in agreement with the stereo-preference of β-lactamases for the α-hydroxyalkyl esters **1**. It may be, therefore, that the α-hydroxydepsipeptides **5** bind and react at the β-lactamase active site in a conformation more closely resembling that of **1** than that of **4**. The occupancy of the active site by **1**[5] and by **4**[15] has been previously discussed. The interaction of **5** with the β-lactamase active site is explicitly addressed below.

Computational models were constructed of complexes between (*R*)-**7**, which contains the α-hydroxyacyl group of **5**, and the P99 β-lactamase. These were based on model complexes between the enzyme and the depsipeptides **4** (X = H) and D(*R*)-**4** (X = Me), previously constructed and described;[15,20,22] adducts of **7** rather than **5** were constructed since we have more experience with the conformation at the active site of the D-Ala leaving group than the *m*-hydroxybenzoate.[25] Energy-minimized structures of tetrahedral intermediates **8**, derived from attack of the enzyme on **7**, were obtained, following molecular dynamics exploration. Two conformations of the ligand were examined, corresponding to those previously identified, A and B, the former with the α-C directed into the enzyme and the latter with it out.[15,20]



An apparently stable complex, with active site functional groups strongly interacting with the ligand in familiar ways, was obtained when molecular dynamics runs were started from conformation A but not from B. Thus the complex derived from A, shown in Fig. 3, may be a good approximation to the tetrahedral complex **8**, and also to that derived from reaction of **5** with the enzyme. Much intramolecular hydrogen bonding is seen in this complex, as expected of a reactive intermediate in the β-lactamase active site,[15,20] including hydrogen bonds between the α-hydroxyl group (hydrogen bond acceptor) and Lys67-NH_3^+ and also the Asn152 side chain carbonyl (here the hydroxyl is a hydrogen bond donor). Interestingly, these same interactions were observed in one of the conformations that may occur on reaction of the P99 β-lactamase with **1**.[5] This structure is probably even more likely in this than in the latter case because of the presence in **5** of the amido side chain which is seen in Fig. 3

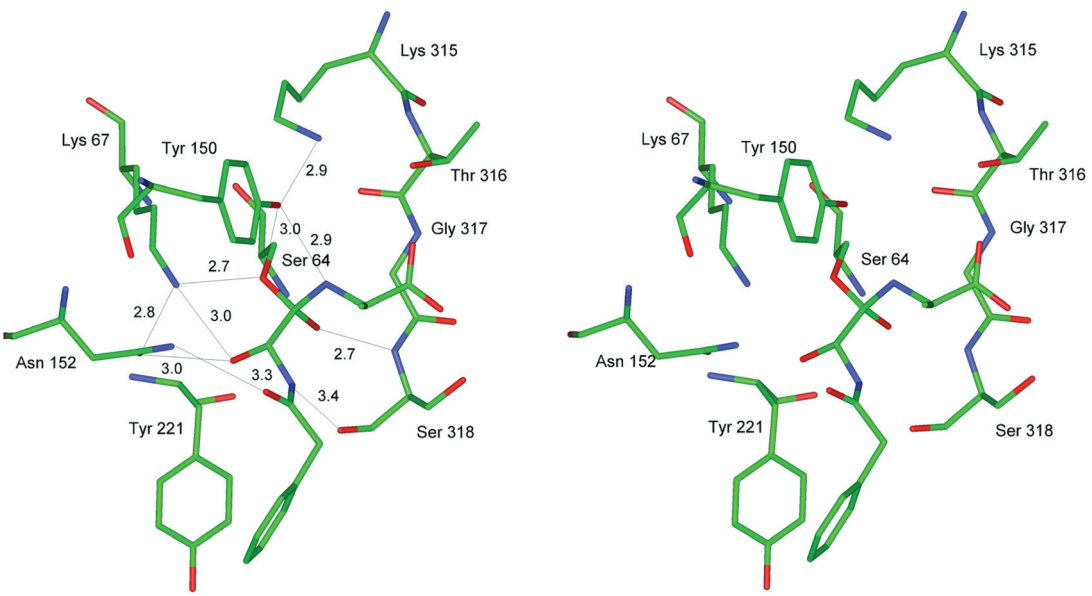


Fig. 3 Stereoview of an energy-minimized tetrahedral intermediate structure **8** formed on reaction of **7** with the P99 β-lactamase. Only heavy atoms are shown.

Table 1 Steady state kinetics parameters for the enzyme-catalyzed hydrolysis of **5** and of **1** and **4** (X = OMe) for comparison

Enzyme	Substrate	Enantiomer	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})
P99[a]	**5**	D(*R*)	137 ± 13	0.13 ± 0.03	1.0×10^6
		L(*S*)	59 ± 4	0.84 ± 0.09	7.1×10^4
	1 (R = CH_2Ph)[5]	D(*R*)	6.9	0.17	3.9×10^4
	4 (X = OMe)[15]	D(*R*)	13.7	1.3	1.1×10^4
		L(*S*)	310	0.81	3.8×10^5
AmpC[b]	**5**	D(*R*)	≥510	≥1.5	$(3.4 \pm 0.2) \times 10^5$
		L(*S*)	≥14	≥1.5	$(9.4 \pm 0.1) \times 10^3$
TEM-2[c]	**5**	D(*R*)	46 ± 12	2.7 ± 0.9	1.7×10^4
		L(*S*)	No reaction observed		
	1 (R = CH_2Ph)[5]	D(*R*)	≥0.46	≥1	460
	4 (X = OMe)[15]	D(*R*)	No reaction observed		
		L(*S*)	≥0.02	≥0.5	47
OXA-1[d]	**5**	D(*R*)	≥4.5	≥2.5	$(1.8 \pm 0.17) \times 10^3$
		L(*S*)	No reaction observed		
	1 (R = CH_2Ph)[5]	D(*R*)	No reaction observed		
R61[e,f]	**5**	D(*R*)	6.9 ± 0.4	0.36 ± 0.05	1.9×10^4
		L(*S*)	No reaction observed		
	4 (X = OMe)[15]	D(*R*)	44	0.45	9.8×10^4
		L(*S*)	No reaction observed		
R39[f,g]	**5**	D(*R*)	10.4 ± 0.5	4.3 ± 0.3	2.4×10^3
		L(*S*)	No reaction observed		
	4 (X = OMe)[15]	D(*R*)	114 ± 7	0.20 ± 0.03	5.7×10^5
		L(*S*)	No reaction observed		

[a] Class C β-lactamase of *Enterobacter cloacae* P99. [b] Class C β-lactamase from *E. coli*. [c] Class A β-lactamase from the TEM-2 plasmid in *E. coli*. [d] Class D β-lactamase from *E. coli*. [e] LMM class B DD-peptidase from *Streptomyces* R61. [f] Compound **1** did not react with either the R61 or R39 DD-peptidase.[5] [g] LMM class C DD-peptidase from *Actinomadura* R39.

to hydrogen bond into the active site in just the same way as it is thought to do in natural substrates.[26] The interactive features of the structure of Fig. 3, therefore, may lead to the reactivity of both **1** and **5** with the P99 β-lactamase. The hydrogen bonding interaction of the α-hydroxyl group with Lys67 must overcome any unfavorable steric interaction that it might have with the β-C of Tyr221. Such a steric interaction has been proposed to reduce the reactivity of D-alanyl derivatives with the P99 β-lactamase.[20–22]

Steady state kinetics parameters for the turnover of **5** by the enzymes alluded to above are presented in Table 1, along with those of **1** (R = CH_2Ph) and **4** (X = OMe). It can be seen at once that **5** is quantitatively comparable to the latter compounds as a substrate of these enzymes. The weak apparent binding (high K_m values) of **5** to several of these enzymes is offset by high turnover numbers (k_{cat}), thus yielding quite large values for k_{cat}/K_m. Many β-lactams are turned over at comparable rates although the most specific β-lactams do have higher k_{cat}/K_m values (10^6–10^7 s^{-1} M^{-1}).[27,28]

The comparison of kinetic data from other *m*-hydroxybenzoates, **1** (R = CH_2Ph) and **4** (X = OMe), with those of **5** shows that the latter compound is a quite effective substrate of both serine β-lactamases (classes A, C and D) and DD-peptidases (low molecular mass, classes B and C). In all cases, the classical amido side chain of **5** leads to a better (k_{cat}/K_m) substrate than the alkyl (benzyl) side chain of **1**. Indeed, it has already been established that the presence of the α-hydroxyl group is essential to any substrate activity in **1**.[5] The effect of the amido side chain is particularly striking in the class A and D β-lactamases (TEM-2 and OXA-1) and even more so in the DD-peptidases where no activity is seen at all with **1**.

Comparison of the kinetics results with those from the compound **4** (X = OMe), bearing the electronically similar but sterically larger α-OMe group, is also interesting. In general, the D(*R*)-enantiomer of **4** is less reactive as a β-lactamase substrate than **5**. This difference presumably reflects the limited space available for bulky α-substituents in a substrate of a serine β-lactamase;[20–22] in fact, the L(*S*)-enantiomers of **4** are more reactive with the P-99 and TEM β-lactamases than the D(*R*) (Table 1, ref. 15). Strikingly, however, with both DD-peptidases, the D(*R*)-enantiomer of **4** (X = OMe) is more reactive than that of **5**. This may reflect the better fit of the bulkier OMe group into the methyl pocket of DD-peptidases, and perhaps, also, a negative effect of the α-hydroxyl group on catalysis, by its hydrogen bonding to the catalytically necessary active site lysine side chain (Lys67 of the P99 enzyme – see Fig. 3). Thus, incorporation of the D-α-hydroxyl substituent into a classical depsipeptide, **4** (X = H), does not enhance reactivity to the extent that it does with alkyl side chains, **1**, but the resulting molecule, **5**, certainly does maintain significant active site affinity.

The D-α-hydroxyl group, therefore, appears to activate certain β-lactamase (classes A and C) active sites by interaction with functional groups in a similar way to the classical amido side chain, thus inducing a reactive conformation. The effects of the two groups are not, however, additive, probably because they compete for the attention of the active site asparagine (Asn 152 in the P99 enzyme) (Fig. 3). The D-α-hydroxyl group appears to have less effect on the DD-peptidase active site despite its very similar structure. Incorporation of the D-α-hydroxyl group, instead of the L-amido group, into a molecule may therefore be a useful strategy to design new substrates and substrate and transition state analog inhibitors for serine β-lactamases.

Experimental

3-Carboxyphenyl *N*-(phenylacetyl)-α-D,L-hydroxyglycinate (5)

This compound was synthesized (Scheme 1) based on the procedures reported by Adediran *et al.*[15] A solution of phenylacetamide (13.0 g, 0.10 mol) and glyoxylic acid (11.5 g, 0.11 mol) in acetone (75 ml) was heated under reflux for 17 h. The solvent was evaporated and the residue dried overnight on an oil pump. The solid was then recrystallized from chloroform–dioxane (1 : 1), yielding *N*-(phenylacetyl)-α-hydroxyglycine as pale yellow crystals. This acid (4.2 g, 20 mmol), 3-(*p*-methoxy-benzyloxycarbonyl) phenol (5.09 g, 20 mmol) and DMAP (220 mg, 1.8 mmol) were dissolved in methylene chloride (50 ml). The stirred solution was cooled to 0 °C and DCC (5.01 g, 24.2 mmol) was added. The mixture was allowed to warm to room temperature as it was stirred overnight. It was then filtered and the filtrate washed sequentially with water, 10% citric acid, water, saturated sodium bicarbonate, and water again. The organic layer was dried over $MgSO_4$, filtered, and the solvent removed by rotary evaporation. The resulting crude residue was then purified by chromatography on silica gel with methylene chloride–ethyl acetate (3 : 1) as the eluent, yielding 560 mg (6%) of the *p*-methoxybenzyl ester of the required product. This protected product (560 mg, 1.2 mmol) was dissolved in methylene chloride (8 ml) and trifluoroacetic acid (10 ml) with anisole (135 µl, 1.2 mmol) and the solution stirred for 1 h at room temperature. The solvent was then removed and the crude product washed with benzene. The insoluble material was recrystallized from acetonitrile yielding 256 mg (62%) of colorless crystals (mp 176–178 °C). NMR: ^{1}H (DMSO): δ 13.21 (s, 1H), 9.29 (d, 1H, J = 8.3 Hz), 7.83 (d, 1H, J = 7.6 Hz), 7.67 (s, 1H), 7.56 (t, 1H, J = 7.9 Hz), 7.29 (m, 6H), 6.79 (d, 1H, J = 7.6 Hz), 5.63 (t, 1H, J = 8.0 Hz), 3.52 (s, 2H); IR (cm^{-1}): 1767, 1690, 1654. MS Calc. 329.30 ES$^-$ 328.07. HRMS (TOF, ESI$^+$) *m*/*z* [M + H$^+$] calc for $C_{17}H_{16}NO_6$ 330.0978; found 330.0977. Its purity is indicated by the NMR spectrum and HPLC chromatogram (Fig. S1 and S2, ESI†).

Enzymes

The class C P99 β-lactamase from *Enterobacter cloacae* and the class A TEM-2 β-lactamase from *Escherichia coli* W3310 were purchased from the Centre for Applied Microbiology and Research (Porton Down, Wiltshire, UK). The ampC enzyme was provided by Dr Brian Shoichet of the University of California at San Francisco. The class D OXA-1 β-lactamase was generously provided by Dr Michiyoshi Nukaga, Jyosai International University, Japan. Purified samples of the *Streptomyces* R61 DD-peptidase and *Actinomadura* R39 DD-peptidase were generous gifts from Dr J.-M. Frère and Dr P. Charlier of the University of Liege, Liege, Belgium.

Analytical and kinetic methods

A Varian Gemini-300 MHz NMR spectrometer was used to collect ^{1}H NMR spectra.

The high resolution electrospray mass spectrum was obtained from the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois. Absorption spectra and spectrophotometric reaction rates were obtained from a Hewlett-Packard 8453 UV-VIS spectrophotometer. All enzyme concentrations were determined spectrophotometrically.

Hydrolysis kinetics. These studies were carried out at 25 °C, buffered in 20 mM 3-morpholinopropanesulfonic acid (MOPS) at a pH of 7.5, except in the case of the OXA-1 enzyme where the buffer also included 50 mM $NaHCO_3$ and 0.1% gelatin. The substrate was prepared in concentrated acetonitrile stock solutions and diluted to ≤5% acetonitrile in assays.

Hydrolysis of **5** was monitored spectrophotometrically (*m*-hydroxybenzoate release) at 290 nm ($\Delta\varepsilon$ = 1972 M^{-1} cm^{-1}), 300 nm ($\Delta\varepsilon$ = 1100 M^{-1} cm^{-1}), or 305 nm ($\Delta\varepsilon$ = 554 M^{-1} cm^{-1}) depending on the concentration employed. The spontaneous hydrolysis total progress curves were fitted to a first order rate equation by means of a nonlinear least-squares program and the rate constants from several runs thus obtained averaged.

Initial rates of hydrolysis of **5** by the P99 enzyme (35 nM), measured spectrophotometrically at a number of concentrations (0–1.0 mM), were fitted to the Henri–Michaelis–Menten equation by a non-linear least squares procedure to obtain the steady state kinetics parameters. This procedure was also used for the slower second phase from rates estimated after completion of the first phase. In both instances, the initial rates were corrected for spontaneous hydrolysis. Kinetic parameters for the hydrolysis of a single enantiomer by the R61 DD-peptidase (0.25 µM) and the TEM β-lactamase (0.25 µM) were also obtained from initial rate measurements as described above. Total progress curves for hydrolysis of **5**, catalyzed by the AmpC β-lactamase (69 nM) and the OXA-1 β-lactamase (0.25 µM), were fitted by means of the Dynafit program[29] to a two-substrate model (beginning with equal amounts of the D and L enantiomers). Total progress curves generated by the R39 DD-peptidase (0.25 µM) were treated in the same way. Substrate concentrations in these experiments were in the 0.1–2.5 mM range.

PAL assay. To a solution of the depsipeptide **5** (250 µl, 1.0 mM) in a buffer containing 150 mM MES, 1 mM $Cd(NO_3)_2$ and 0.02% Lubrol (Thesit), adjusted to pH 5.0, *ca.* 0.1 of solid β-lactamase, either the P99 or TEM-2 β-lactamase, was added and the rapid hydrolysis of **5** monitored at 290 nm. Upon completion of the hydrolysis reaction the enzyme PAL was added to a final concentration of 2 µM. Aliquots (20 µl) of the resulting solution, taken at suitable times, were then analyzed by HPLC to monitor progress of the subsequent reaction.

HPLC assay. An isocratic reverse phase HPLC separation of the β-lactamase/PAL reaction products was carried out using a Machery-Nagel C18 analytical column with a mobile phase consisting of 80% potassium phosphate solution (50 mM), adjusted to pH 6.0, and 10% methanol. The effluent of the column was monitored at 258 nm. Calibration curves for quantitation were obtained from authentic samples of the starting materials and products. Retention times were 9.0 min for **5**, 3.8 min for **6**, and 6.9 min for phenylacetamide.

Molecular modeling

The structure of Fig. 3 was derived from computational models of enzyme–substrate complexes that were set up essentially as previously described[15,20,22] 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 **8** was dianionic. Initially, the ligand was placed in conformations A and B[15,19,21] and the stability of these conformations explored by molecular dynamics (200 ps runs, where the entire protein together with solvating water molecules were unrestricted). Typical snapshots of dominant conformations were selected for energy minimization.

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