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Synthesis and characterisation of a ligand that forms a stable tetrahedral intermediate in the active site of the *Aureobacterium* species (−) γ-lactamase†

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The crystal structure of a (−) γ-lactamase from an *Aureobacterium* species showed a molecule bound covalently to the active site serine residue. This enzyme complex represented the first structure of a stably bound tetrahedral intermediate for an α/β hydrolase fold enzyme. The structural elucidation of tetrahedral intermediates is important for the understanding of enzymatic mechanism, substrate recognition and enzyme inhibition. In this paper, we report the synthesis and subsequent characterisation of (3*aR*,7*aS*)-3*a*,4,7,7*a*-tetrahydrobenzo-[1,3]-dioxol-2-one (BD1), the molecule modelled into the *Aureobacterium* (−) γ-lactamase active site. This molecule has been confirmed to be an inhibitor and to be displaced from the enzyme by the racemic γ-lactam substrate.

Enzymes capable of the enantiospecific resolution of γ-lactam ((±)-2-azabicyclo[2.2.1]hept-5-en-3-one), are called γ-lactamases.[1] This name is given after the enzymes' industrial activity since their *in vivo* activity is unknown. These enzymes have been used in the selective generation of useful chiral precursors for the synthesis of carbocyclic nucleosides.[2–4] We have previously reported the over-expression, purification and structural determination of a (−) γ-lactamase enzyme from an *Aureobacterium* species.[5]

During the refinement of the (−) γ-lactamase structure, additional electron density was observed within the active site that was continuous with the catalytic serine (Ser98). The additional density and subsequent modelling experiments indicated that the ligand was bicyclic.[5] The first ring of this ligand was modelled with a high degree of confidence as the tetrahedral intermediate of cyclic ethylene carbonate. However cyclic ethylene carbonate proved to be a substrate and not an inhibitor of the enzyme.[5]

The modelling of the second ring of the ligand proved to be more difficult as this part of the molecule was less ordered or represented a mixture of different species.[5] The dictionary descriptions were built for a number of possible molecules using the program REFMAC[6] and these were placed into the electron density and then subjected to refinement (Fig. 1a). The single species which gave the best fit to the electron density maps and had the most consistent atomic *B*-factors was found to be the compound (BD1) (Fig. 1b).

The molecule BD1 forms a stable tetrahedral intermediate in the (−) γ-lactamase active site with the catalytic serine (Ser98) and also makes hydrogen bonds with His259, Tyr32 and Met99 (Fig. 2). A hydrophobic pocket formed from the side-chains of Tyr32, Met99, Leu125, Phe162, Phe166, Trp204, Ile232 and Leu233 binds the second ring of BD1 with the residue Trp204 interacting with BD1 through ring stacking interactions.[5]

† Electronic supplementary information (ESI) available: PDB coordinates of just the bound complex in the pocket, BD1 synthesis experimental data and graph of the observed *in vitro* results. See http://dx.doi.org/10.1039/b511078e

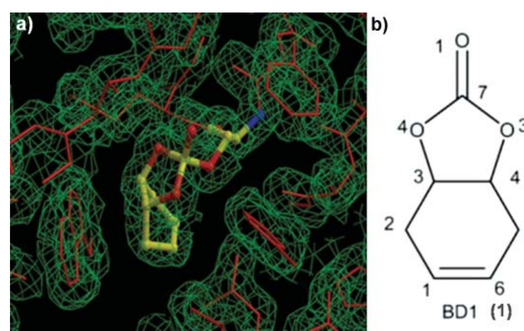

Fig. 1 (a) $2F_o$–F_c electron density map with the tetrahedral intermediate shown in ball and stick form. (b) Structure of BD1 with the numbering consistent with that of the PDB entry 1HL7.

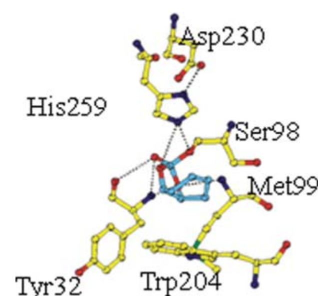

Fig. 2 Binding of the ligand BD1 in the active site of the (−) γ-lactamase (see supplementary material for the 3D model).†

Analysis of the structure revealed that the BD1 ring oxygen O4 is bound in the oxyanion hole and not the carbonyl O1 as would be required for the collapse of the tetrahedral intermediate.[5] Once the BD1 tetrahedral intermediate has formed, thermal motion around the Ser98 OG–BD1 C7 bond could position the BD1 second ring into the hydrophobic pocket leading to its subsequent retention in the active site.[5] In this position the active site histidine is sterically prohibited from protonating one of the ring oxygen atoms which is required for collapse of the tetrahedral intermediate. The results suggest that BD1 has a high affinity for the enzyme active site but becomes trapped until it is displaced by the γ-lactam substrate.

At no point was BD1 or a related compound added during the purification or crystallisation of the (−) γ-lactamase therefore it is likely to originate from the expression host *Escherichia coli*.

There are examples of other enzyme structures reported in the literature which have unexpected ligands bound to the active site.[7–10] Studies to inhibit (−) γ-lactamase with phenylmethyl sulfonylfluoride (PMSF) serine hydrolase inhibitor showed no inhibition of activity with 1 mM PMSF with crude enzyme extract and only partial inhibition with the pure enzyme.[5] This result could be explained by the presence of BD1 in the active site. BD1 is not commercially available and there are no reports of its synthesis. There are no reports of compounds related to BD1 as

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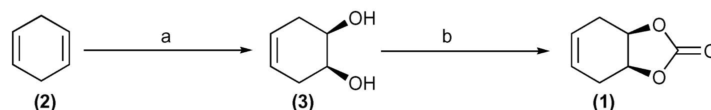

Fig. 3 Reaction scheme for the two step synthesis of BD1: (a) OsO_4, 1 eq NMO, acetone, *tert*-butanol, H_2O; (b) diethyl carbonate, reflux, 1 h.

intermediates in metabolic pathways of the expression host. The crystal structure of the (−) γ-lactamase has only been solved from the recombinant enzyme and little is known about the metabolism of the native *Aureobacterium* species. It is considered unlikely that BD1 has been introduced as a contaminant during the enzyme purification. However, it is present in sufficient amounts to enable it to fully occupy all the active sites of the recombinant enzyme. Since the modelling of the second ring of the bound molecule in the crystal structure was ambiguous we sought to synthesise the compound BD1 and to characterise its interaction with (−) γ-lactamase.

The synthesis of BD1 (**1**) was successfully carried out in an overall yield of 77% (Fig. 3). Firstly the synthesis of 4-cyclohexene-*cis*-diol (**3**) was achieved through a stoichiometric selective di-hydroxylation reaction of 1,4-cyclohexadiene (**2**) using osmium-tetroxide and 1 eq of *N*-methyl morpholine (NMO).[11,12] Then *via* the condensation of diethyl carbonate to the diol by refluxing (**3**) in neat anhydrous diethyl carbonate, the compound (3*aR*,7*aS*)-3*a*,4,7,7*a*-tetrahydrobenzo-[1,3]-dioxol-2-one (BD1) (**1**) was given in quantitative yields. This is, to our knowledge, the first fully characterized report of the synthesis of BD1. The full experimental and spectra are included as online supplementary material.†

To determine the inhibition profile of the γ-lactamase with PMSF, the purified enzyme was incubated with 0–1 mM of this inhibitor (diluted from a 100 mM stock in ethanol) prior to and during the enzymatic reaction. Lactamase activity was assayed using racemic γ-lactam as the substrate using the method previously described.[13] Reactions were carried out in a total of 1 ml containing 800 μl 0.1 M Tris-HCl pH 7.5, 100 μl enzyme solution (0.95 mg ml^{-1}) and 100 μl 100 g l^{-1} racemic (−) γ-lactam in the same Tris-HCl buffer. Reactions were left to incubate for 30 min at room temperature and were stopped by the removal of 100 μl of reaction mixture into 900 μl of HPLC mobile phase (50% methanol, 50% 10 mM potassium phosphate pH 7.0). 10 μl samples were then applied to a HPLC reverse flow C8 column for separation of the products, and monitored at 225 nm. Control incubations were carried out with the enzyme in buffer alone, substrate with inhibitor and enzyme with 1% (v/v) ethanol (final concentration), as would be present from the solubilised inhibitor. These experiments were conducted with both purified enzyme and purified enzyme recovered after catalytic turnover with the γ-lactam substrate. To determine the inhibition profile of BD1 with the γ-lactamase, the BD1 was solubilised in 50% (v/v) PEG 400 at a concentration of 66 mM and varying concentrations of BD1 (0–10 mM) were added to the enzymatic reaction. The γ-lactam concentration was reduced to 50 mM and assayed as above. Control reactions contained 50% (v/v) PEG 400 to ascertain its effect upon the lactamase reaction.

Inhibition studies with the pure (−) γ-lactamase showed that only partial inhibition was observed with PMSF (see supplementary material†). Incubation with 1 mM PMSF resulted in a 3-fold decrease in γ-lactam conversion, however the inclusion of 1% (v/v) ethanol in the incubation and reaction resulted in the same loss of activity. This result suggests that the ethanol in which the PMSF is solubilised is responsible for the observed inhibitory effect upon the (−) γ-lactamase. Since the X-ray studies revealed a molecule bound to the active site serine, the inhibition by PMSF was investigated using enzyme recovered from a catalytic turnover with the γ-lactam substrate. A total inhibition by PMSF was seen with this enzyme where incubation with 0.6 mM PMSF resulted in a total loss of catalytic activity and lower concentrations of 0.2 mM and 0.4 mM PMSF resulting in less than 1% γ-lactam conversion (see supplementary material†). The presence of BD1 bound to the active site serine confers protection from the serine protease inhibitor PMSF.

Investigations into the inhibitory effect of BD1 were hampered by the poor solubility of this compound in water. The use of organic solvents was avoided since they inhibited the enzyme. The BD1 solubilised sparingly in 50% (v/v) PEG 400 and the presence of the same concentration of PEG 400 was found to have no effect on the lactamase reaction. Analysis of the 30 minute reactions revealed that the presence of BD1 resulted in a reduction in the conversion of the γ-lactam as shown in Fig. 4. The lactamase assay cannot be monitored continuously and as such it was not possible to monitor the initial rate of reaction. It was therefore not possible to obtain kinetic data for the γ-lactamase reaction, or to evaluate the kinetics of the inhibition. The presence of 2 mM BD1 in the γ-lactamase reaction resulted in 10% less conversion of the γ-lactam substrate. In the presence of 10 mM BD1, the conversion of γ-lactam was decreased almost 3-fold.

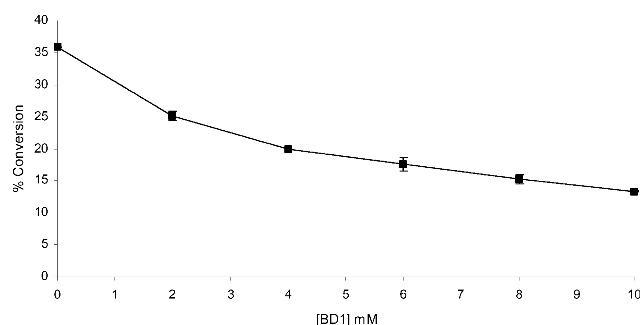

Fig. 4 Conversion of γ-lactam by purified (−) γ-lactamase in the presence of different concentrations of BD1.

In conclusion the results obtained have confirmed that BD1 interacts with the enzyme active site and can be removed by the γ-lactam substrate. BD1 acts as a competitive inhibitor of the (−) γ-lactamase enzyme by forming a stable reversible tetrahedral intermediate with Ser98. The BD1 molecule protects this catalytic serine from inhibition by PMSF. The chemical synthesis of BD1 described here confirms the identity of this molecule as that modelled into the electron density for the X-ray structure of the (−) γ-lactamase. We have also demonstrated that this compound inhibits the activity of the enzyme when γ-lactam is used as a substrate. These combined results have confirmed the previously proposed mechanism of inhibition of BD1 on the (−) γ-lactamase enzyme and its removal from this protein upon enzymatic turnover with the industrial substrate racemic γ-lactam.

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