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Benzopyranones with retro-amide side chains as (inhibitory) β-lactamase substrates

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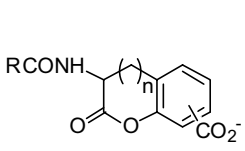
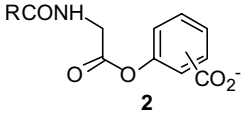
Abstract—3-(*N*-Benzylcarbamoyl)-7-carboxy-3, 4-dihydro-2*H*-1-benzo-pyran-2-one and its 8-carboxy analogue have been synthesized and evaluated as potential (inhibitory) substrates of β-lactam-recognizing enzymes. These compounds are bicyclic δ-lactones with retro-amide (with respect to classical β-lactams) side chains. They were found to be comparably effective as substrates of typical class A, C and D β-lactamases as analogous benzopyranones bearing 'normal' amide side chains. The new 8-carboxy derivative, however, formed a much more (1000-fold) tightly-bound acyl-enzyme with a class C β-lactamase than did its 'normal' analogue, and thus provides a structural lead to new inhibitors of this class of β-lactamase.

1. Introduction

In view of the present-day and growing resistance of bacteria to β-lactam antibiotics,[1] it is important to closely examine indications of novel specificity and reactivity in substrates of the relevant enzymes. The latter include the DD-peptidases (penicillin-binding proteins) that are essential for bacterial cell wall biosynthesis and are the β-lactam targets, and the β-lactamases that provide protection to bacteria by catalyzing β-lactam hydrolysis. The appearance of new chemistry in substrates, of course, can be translated into new inhibitors.



1; n = 1,2



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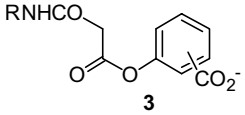

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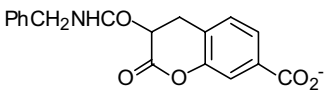
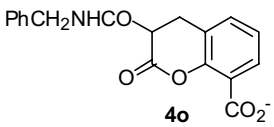
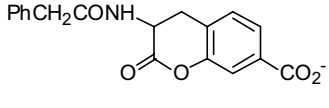
Some time ago, we showed that certain δ-lactones, of general structure **1**, were β-lactamase substrates. In some cases, these compounds produced relatively inert acyl-enzymes and thus may be a source of inhibitory substrates. More recently, we showed that in analogous acyclic depsipeptide substrates of β-lactamases such as **2**,[2,3] replacement of the normal amide side chain, RCONH–, with a retro-amide, RNHCO–, led to compounds, **3**, that, rather surprisingly, were also β-lactamase substrates and, in some cases, better than the original compounds.[4] Since the retro-amide side chain has not previously been seriously explored in β-lactamase substrates or inhibitors, we decided to examine it further. In this paper, we report the synthesis and reactivity with typical β-lactamases of the benzopyranones **4m** and **4o**, each carrying the retro-amide side chain. These compounds are also of interest since, as with



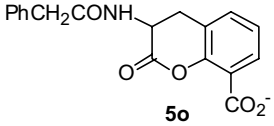
4m



4o



5m



5o

Keywords: Enzyme substrates; Inhibitors; β-Lactam antibiotics; Substrate analogues.
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bicyclic β-lactams, following their acylation of a serine β-lactamase or DD-peptidase, the leaving group will remain attached, occupying the active site of the enzyme. This is generally believed to be an important factor in the antibiotic activity of bicyclic β-lactams.[5] The kinetics results obtained for **4m** and **4o** were compared to those from the 'normal' side chain analogues **5m** and **5o**.[6,7]

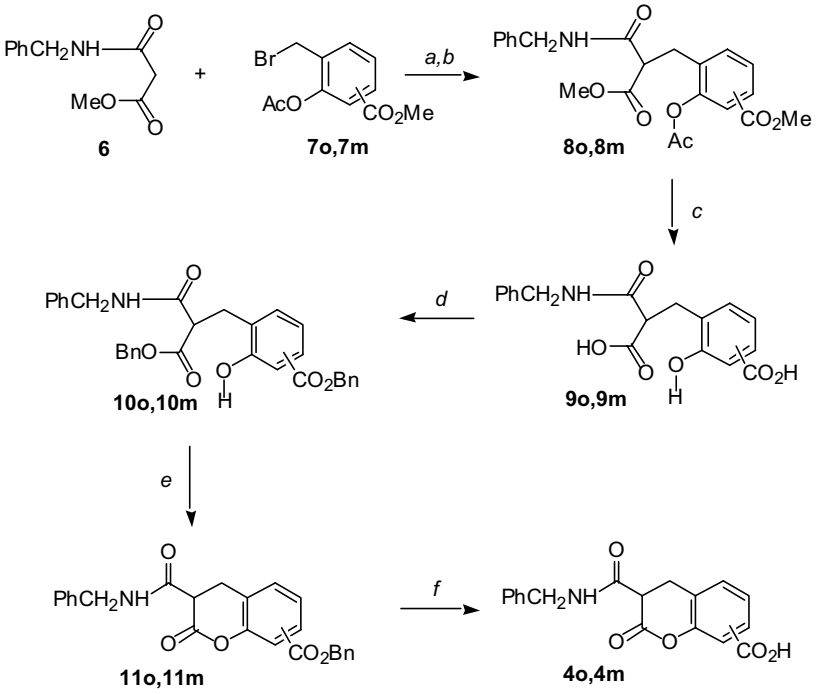
2. Results and discussion

For the syntheses of the target benzopyranones of type **4m** and **4o** (dihydrocoumarins possessing a 3-carbamoyl substituent), we considered the alkylation of the methyl benzylaminocarbonylacetate **6**[8] with a substituted benzyl bromide **7** (Scheme 1). The alkylation of malonamate anions has, however, been seldom used in synthesis.[9]

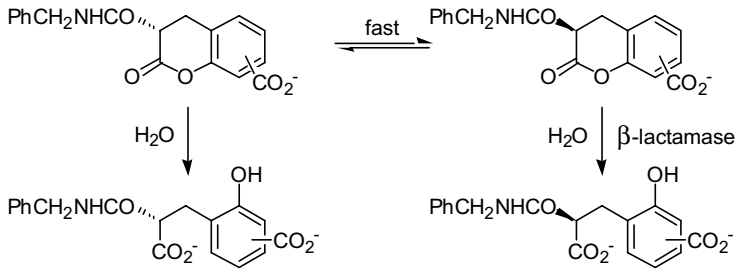
The anion of **6** was generated in dry DMF, using sodium hydride as a base, and reacted with different substituted benzyl bromides derived from salicylic acid[10] or 3-hydroxybenzoic acid;[6] the methoxycarbonyl substitutent was therefore in either the *ortho* or *meta* position relative to the acetoxy function. The reaction mixture contained the monoalkylated product **8** as well as some dialkylated material in the case of the *meta* series. After purification by column chromatography of the monoalkylated compounds and alkaline hydrolysis of the ester functions of **8**, direct thermal lactonization of the diacids **9** failed, leading to decarboxylated products. Therefore, selective benzylation of the carboxyl groups of **9** was achieved in DMF or DMSO using cesium carbonate and benzyl bromide as reactants. Then lactonization of the dibenzyl esters **10** gave monoesters **11**. Finally, hydrogenolysis of the remaining benzyl protecting group furnished the target benzopyranones **4m** and **4o**.

The hydrolysis of **4m** and **4o** could be monitored spectrophotometrically at 290 nm ($\Delta\varepsilon = 1760\,cm^{-1}\,M^{-1}$) and 300 nm ($\Delta\varepsilon = 950\,cm^{-1}\,M^{-1}$), respectively. Pseudo-first order rate constants for hydrolysis of **4m** and **4o** in 0.1 M MOPS buffer, pH 7.5, 25 °C (the buffer conditions employed in all kinetics studies reported in this paper unless otherwise noted) were 3.0×10^{-4} and $1.7 \times 10^{-4}\,s^{-1}$, respectively, and thus similar to those obtained previously[6,7] for **5m** and **5o,** viz. 5.6×10^{-4} and $5.4 \times 10^{-4}\,s^{-1}$, respectively. In all cases, the rates are higher than those for acyclic analogues;[6] δ-lactones are less stable to nucleophilic cleavage than analogous acyclic esters.[11]

The hydrolysis of **4m** and **4o**, again monitored spectrophotometrically, was accelerated on addition of β-lactamases. These enzymes therefore catalyze hydrolysis of **4m** and **4o** as they do of **5m** and **5o**.[6,7] All four compounds, as prepared, are racemic mixtures, with a chiral centre at C3. Progress curves for the hydrolysis of **5m** and **5o** in the presence of β-lactamases were biphasic,[6,7] the faster phase enzyme-catalyzed, the slower not. This was interpreted to mean that one enantiomer, presumably the 3β-isomer by analogy with β-lactams, was a β-lactamase substrate, while the other was not. In contrast, the spectrophotometric total progress curves for the hydrolysis of **4m** and **4o** in the presence of β-lactamases exhibited only one smooth phase of reaction. This can be interpreted in terms of Scheme 2, where fast equilibration of the 3α- and 3β-isomers would lead to observation of only a single reaction phase.



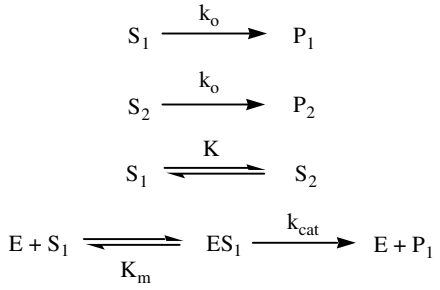
Scheme 1. Synthesis of the benzopyranones **4o** and **4m**. Reagents and conditions: (a) NaH/DMF/25 °C; (b) SiO_2/cyclohexane–EtOAc (7:3); (c) NaOH/MeOH–H_2O; (d) i. Cs_2CO_3 (1 equiv)/DMF, ii. $PhCH_2Br$ (2 equiv)/DMF/25 °C; (e) 220 °C/1.33 Pa; (f) H_2/Pd/C/EtOAc.



Scheme 2.

The hydrogen atoms at the 3-position of **4m** and **4o** must therefore be kinetically more acidic than those of **5m** and **5o**. This would not be unexpected since **4m** and **4o** are malonates with two carbonyls adjacent to C3. Indeed, ready exchange of these hydrogens without noticeable lactone hydrolysis was observed in NMR spectra of these compounds in acetone-d_6/D_2O. A similar result was found with benzofuranones analogous to **4** where exchange at C3 was also sufficiently facile as to lead to apparently simple kinetics.[12]

In view of the discussion above, the kinetics of β-lactamase-catalyzed hydrolysis of **4m** and **4o** were interpreted in terms of Scheme 3, where S_1 and S_2 represent the 3β- and 3α-isomers of **4m** and **4o**, and P_1 and P_2 their hydrolysis products, respectively. It can be shown that in terms of Scheme 3, $k_{cat}^{obs} = k_{cat}$ and $K_m^{obs} = 2K_m$, the latter assuming, reasonably, that $K = 1$. Steady state kinetics parameters, determined spectrophotometrically, were generally determined from initial rates of hydrolysis of **4m** and **4o** by the class A TEM-2 and *Staphylococcus aureus* PC1 β-lactamases, the class C β-lactamase of *Enterobacter cloacae* P99, and the class D OXA-1



Scheme 3.

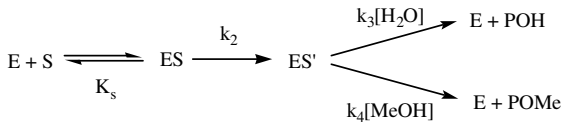
enzyme. The results of these experiments are presented in Table 1, along with comparable data for the analogous compounds with normal side chains, **5m** and **5o**, and for a good substrate, cephalothin.

Superficially, the first notable result is that, generally, the retro-side chain compounds **4m** and **4o** are β-lactamase substrates, and often as good as those with a normal amide side chain, **5m** and **5o**. This indicates that, as

Table 1. Steady state kinetics parameters for β-lactamase-catalyzed hydrolysis

Enzyme		Substrate				
		β-**4m**	β-**4o**	β-**5m**[a]	β-**5o**[b]	Cephalothin
P99	k_{cat} (s^{-1})	1.18 ± 0.09	0.442 ± 0.003	5.6	5.4	200[g]
	K_m (mM)	0.43 ± 0.09	(2.0 ± 0.1) × 10^{-4}[c]	0.29	0.62	0.009
	k_{cat}/K_m ($s^{-1}M^{-1}$)	2.7 × 10^3	2.2 × 10^6	1.9 × 10^4	8.7 × 10^3	2.2 × 10^7
TEM	k_{cat} (s^{-1})	>0.02	25.5 ± 0.07	>1	2.07	120[g]
	K_m (mM)	>0.2	0.29 ± 0.02	> 1.2	0.053	0.2
	k_{cat}/K_m ($s^{-1}M^{-1}$)	92 ± 4	8.8 × 10^4	1.2 × 10^3	3.9 × 10^4	6 × 10^5
PC1	k_{cat} (s^{-1})	>0.005	(1.06 ± 0.01) × 10^{-2}[d]	2.4 × 10^{-2}	4.7 × 10^{-3}	0.001[g]
	K_m (mM)	>0.2	(1.8 ± 0.2) × 10^{-3}[d]	1.5 × 10^{-2}	3.0 × 10^{-4}	⩽0.001
	k_{cat}/K_m ($s^{-1}M^{-1}$)	25.0 ± 0.1	5.8 × 10^3	1.6 × 10^3	1.55 × 10^4	⩾10^3
OXA-1[e]	k_{cat} (s^{-1})		>0.15		0.25 ± 0.09	1.2
	K_m (mM)		>0.2		(7.4 ± 3.2) × 10^{-3}	0.0139
	k_{cat}/K_m ($s^{-1}M^{-1}$)	⩽40[f]	735 ± 5	⩽170[f]	3.39 × 10^4	8.6 × 10^4

[a] Data from Ref. 6; rate constants from the first kinetics phase (see text).
[b] Data from Ref. 7; rate constants from the first kinetics phase (see text).
[c] Determined from competition experiments with cephalothin as the reporter substrate.
[d] Determined from total progress curves.
[e] Buffer employed: 20 mM MOPS, 50 mM $NaHCO_3$, pH 7.50.
[f] No reaction observed above background.
[g] Data from Ref. 7.



Scheme 4.

with the acyclic analogues previously examined,[4] the retro-amide functionality must fit into the active site without significant difficulty, and perhaps, in some cases, with positive interactions. The lack of side chain specificity evident from this result may reflect the role of β-lactamases as resistance enzymes; evolutionary selection of such enzymes may reward breadth of specificity. The most dramatic result seen in Table 1, is that of **4o** with the P99 β-lactamase where a K_m value of 0.2 μM indicates tight binding of this molecule to the active site. The comparable parameter for **5o** is more than 1000 times larger (weaker binding). The class D OXA-1 β-lactamase appears to discriminate between these substrates more than the other enzymes: although β-**5o** reacts rapidly with the enzyme, β-**4o** and, more particularly, the *meta*-compounds are much less reactive.

Experiments with an additional nucleophile showed that k_{cat} for both **4m** and **4o** with the P99 enzyme must represent k_3, the deacylation rate constant [Scheme 4, where ES and ES′ represent noncovalent and covalent (acyl-enzyme) enzyme–substrate complexes, respectively]. Methanol accelerated the observed initial rates of reaction of **4m** and **4o** to an extent corresponding to k_4/k_3 values of 42 and 27, respectively. These values are comparable to those generally observed for depsipeptide substrates of the P99 β-lactamase[2–4,6,7,12] and indicate that methanol has unfettered access to the acyl-enzyme. On the other hand, D-phenylalanine (0–40 mM) had no effect on the rate of disappearance of **4**. This contrasts with results with acyclic depsipeptides with normal amido side chains,[13] but is in accord with results for bicyclic substrates, including **5** and β-lactams,[6] and with results for **3**.[4] More hindered nucleophiles have limited access to the congested active site of the acyl-enzymes derived from bicyclic substrates.

Thus, the rate constant for acylation of the P99 β-lactamase by **4o** is an impressive $2.2 \times 10^6\,s^{-1}\,M^{-1}$ and the deacylation rate is a modest $0.44\,s^{-1}$. These parameters suggest that **4o** may represent a lead compound to a new series of inhibitory substrates of class C β-lactamases. Compound **4o** also more rapidly inactivated the *Streptomyces* R61 DD-peptidase than did **5o**; the second order rate constants for such inactivation were 22 and $1.5\,s^{-1}\,M^{-1}$,[7] respectively.

Acknowledgements

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