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A "CEPHALOSPORIN-LIKE" CYCLIC DEPSIPEPTIDE: SYNTHESIS AND REACTION WITH ß-LACTAM-RECOGNIZING ENZYMES

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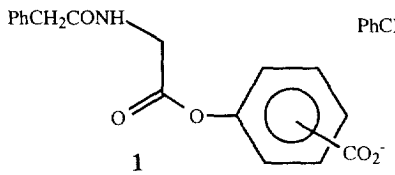
Abstract: The cyclic depsipeptide 8-carboxy-3-phenylacetamido-3,4-dihydro-2*H*-1-benzopyran-2-one, a cyclic analog of aryl phenaceturates with structural similarity to cephalosporins, has been synthesized as a potential substrate/inhibitor of ß-lactam-recognizing enzymes. It was found to be a tight-binding, poor substrate of class A ß-lactamases and an irreversible inhibitor of several DD-peptidases.

In view of the increasing resistance of bacteria to all currently employed antibiotics including ß-lactams,[1] the search continues for new molecules that react with the active sites of ß-lactam-recognizing enzymes. Such molecules may be lead compounds in the search for new antibiotics. Acyclic depsipeptides of structure such as **1** have been shown to be substrates of both ß-lactamases and also of certain DD-peptidases, for example that of *Streptomyces* R61.[2-4] Recently, we have shown that the cyclic depsipeptide **2**, a δ-lactone, is also a ß-lactamase substrate.[5]



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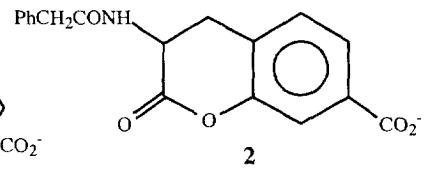

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The serine ß-lactamases and DD-peptidases turn over **1** and, presumably, **2**, by means of a double-displacement mechanism involving an acyl-enzyme intermediate[6] (Scheme 1). The cyclic depsipeptide **2** differs from

Scheme 1

$$E + S \underset{K_s}{\rightleftharpoons} ES \xrightarrow{k_2} \underset{\text{acyl-enzyme}}{E\text{-}S} \xrightarrow{k_3} E + P$$

1, from the point of view of an enzyme, first by the presence of a rigidly placed carboxylate that must impinge particularly on the acylation step of turnover, and second because the *m*-hydroxybenzoate leaving group would not dissociate from the enzyme at the acyl-enzyme stage but remain tethered in a way that might negatively affect

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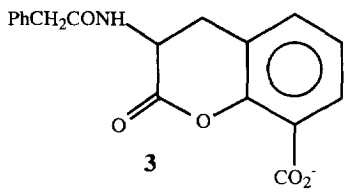
deacylation (k_3). This slower deacylation of **2** was in fact observed with the class C ß-lactamase of *Enterobacter cloacae* P99.[5] The cyclic compound **2**, however, did not interact with the DD-peptidase of *Streptomyces* R61 as either a substrate or an inhibitor.

To further investigate the theme represented by **2**, we have now prepared its isomer **3**. Compound **3** also has a rigidly placed carboxylate group, but closer to scissile ester linkage, giving the molecule the appearance, in two dimensions at least, of a cephalosporin. The distance between the ester oxygen and the carboxylate carbon in **3** is 4.86 Å,[7] which is closer to the limit of around 4 Å proposed by Cohen for an active antibiotic.[8] Depsipeptide **3** is also of interest since the boronate **4**, which yields an analog of the tetrahedral intermediate generated during



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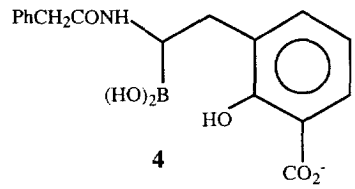

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deacylation of an acyl-enzyme from **3**, has been prepared by Martin and Jones, and shown to be a potent inhibitor of the class A TEM ß-lactamase.[9,10]

In this paper we describe the synthesis of **3** and the kinetics of its interaction with typical ß-lactam recognizing enzymes, emphasizing the features that distinguish it from **2**.

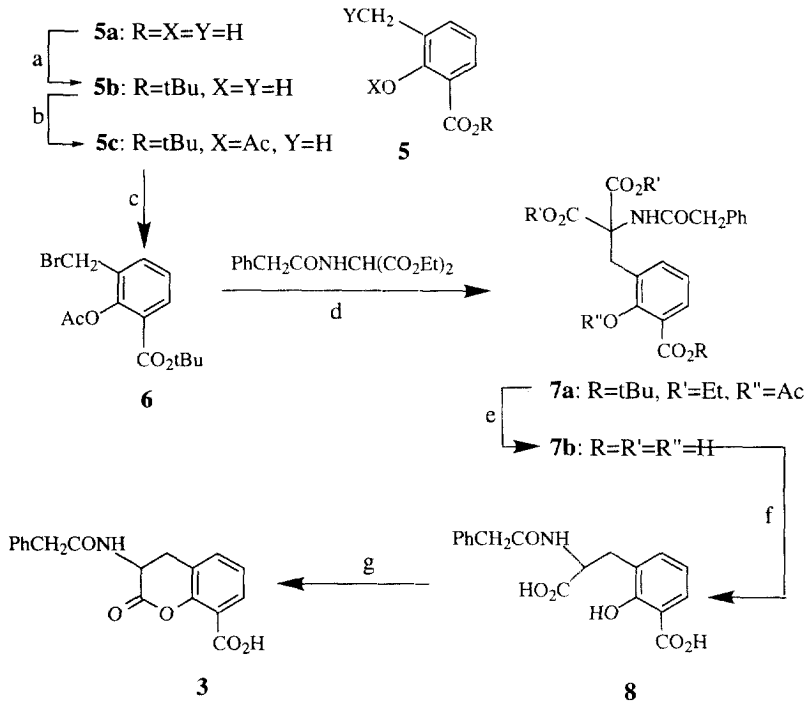
Results and Discussion

The cyclic depsipeptide 8-carboxy-3-phenylacetamido-3,4-dihydro-2*H*-1-benzopyran-2-one was prepared in a manner similar to that used to prepare **2**,[5] *viz.* by thermal lactonization of the corresponding o-tyrosine precursor **8**. This amino acid was prepared by the Sörensen method.[11,12] The substituted benzyl bromide **6** was prepared by bromination with NBS of a diester bearing a methyl substituent (**5c**), in an analogous manner to that of its *meta* isomer.[13] Alkylation of the anion of diethyl phenylacetamidomalonate[14] with bromide **6** led to the malonic ester **7a** (Scheme 2). Alkaline hydrolysis, then acid treatment, of this compound under mild conditions led to incomplete reaction (NMR) and was therefore repeated twice. Decarboxylation of the triacid **7b** furnished the racemic N-phenylacetyl-3-carboxy-2-hydroxyphenylalanine **8**. The action of heat on **8** in vacuo for a few minutes at 200 °C gave the substituted dihydrobenzopyranone **3**.[15]

As a δ-lactone,[16] **3** was noticeably labile to hydrolysis to form **8** in neutral buffer solution. For example, in 100 mM MOPS buffer at pH 7.5 and 25 °C, the pseudo-first order rate constant for hydrolysis of **3**, yielding **8**, was 5.4 x $10^{-4}s^{-1}$.[17] For comparison, the cognate values for **1**, **2**, and benzylpenicillin were 1.0 x $10^{-5}s^{-1}$, 5.6 x $10^{-4}s^{-1}$, and 1.5 x $10^{-5}s^{-1}$, respectively.[5] Depsipeptide **3** is thus comparably labile to **2** and considerably more so than **1** and benzylpenicillin.

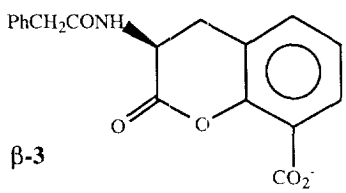
Preliminary experiments showed that the hydrolysis of one half of the sample of **3** was ß-lactamase catalyzed.[18] This was interpreted to mean that only one enantiomer of the racemic **3** was a ß-lactamase substrate, most likely the 6ß-isomer **ß-3**. The latter conclusion is simply based on the structural analogy with ß-lactams where the ß-amido epimers are substrates and the α-epimers generally are not. Scheme 3 depicts a cephalosporin,

Scheme 2



a) N,N-dimethylformamide di-*t*-butyl acetal/THF/reflux; *b*) Ac_2O/DMAP/NEt_3/CH_2Cl_2; *c*) NBS/CCl_4/ reflux; *d*) NaH/THF-DMF (1:3); *e*) (i) 20% aq NaOH-EtOH (1:1), 25 °C, 16h, (ii) H_3O^+/EtOAc, (iii) TFA/CH_2Cl_2 (1:1), 25 °C, 2h; *f*) 180 °C, 5 min; *g*) 220 °C (0.1 mm Hg), 20 min.

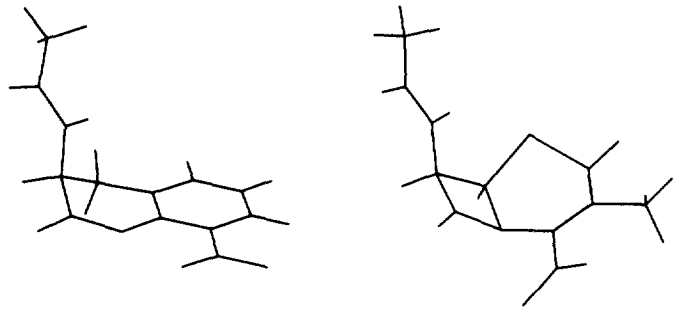
9, and the conformer of **ß-3** with the side chain in the axial position. This conformation more closely resembles the cephalosporin than does the equatorial isomer.[19]



ß-3

Steady state parameters for turnover of **ß-3** by typical serine ß-lactamases are given in Table 1. Comparison of these values with those of **ß-2**, which are also included in the Table, leads to two major conclusions. First, the class C P99 ß-lactamase appears indifferent to the position of the carboxylate group. This is also largely true for acyclic analogs of **2** and **3**.[2] Second, a negative charge is important for substrate reactivity, but this may reflect the general electropositive character of the P99 active site[20] rather than a specific interaction, at least in the immediate vicinity of the carboxylate groups of these depsipeptides.

Scheme 3



ß-3 9

In sharp contrast, the class A ß-lactamases, the TEM and PCl enzymes, have a distinct preference for the carboxylate in the ortho position as in **3**. This preference appears to be expressed most clearly in the K_m parameter, which probably largely reflects the stability of the acyl-enzyme intermediate. This is certainly true for the PCl enzyme where deacylation is essentially always found to be rate-limiting under conditions of substrate saturation.[4] The k_{cat} values are less affected, which would indicate that the deacylation transition states must be stabilized to a similar extent as are the acyl-enzymes by the shift of the carboxylate from the *meta* position in **2** to the *ortho* in **3**.

This is suggestive of a specific interaction between the *ortho* carboxylate and some active site functional group in the class A ß-lactamase active site. The crystal structure of the complex between the TEM ß-lactamase and the

Table 1. Steady State Kinetic Parameters for ß-Lactamase-Catalyzed Lactone Hydrolysis

Substrate		P99	TEM	PCl
ß-3	$k_{cat}(s^{-1})$	5.4 ± 1.4	2.07 ± 0.02	(4.70 ± 0.88) x 10^{-3}
	K_m (mM)	0.62 ± 0.02	0.053 ± 0.002	(3.04 ± 0.87) x 10^{-4}
	k_{cat}/K_m ($s^{-1}M^{-1}$)	8.7 x 10^3	3.9 x 10^4	1.55 x 10^4
ß-2[a]	$k_{cat}(s^{-1})$	5.6	>1.2	0.024
	K_m (mM)	0.29	>1	0.015
	k_{cat}/K_m ($s^{-1}M^{-1}$)	1.9 x 10^4	1.2 x 10^3	1.6 x 10^3
Cephalothin[b]	$k_{cat}(s^{-1})$	200	120	0.001
	K_m (mM)	0.009	0.2	≤.001
	k_{cat}/K_m ($s^{-1}M^{-1}$)	2.2 x 10^7	6 x 10^5	≥10^3

[a]These data are from 5.

[b]The P99 data (10 mM HEPES, pH 8.2) are from 25, the TEM data (0.1 M phosphate, pH 7.0, 30 °C) from 26, the PC1 data (0.1 M phosphate, pH 7.5, 20 °C) from 22.

boronic acid **4**,[10] which, as mentioned above, should generate a structure analogous to that of the transition state for deacylation of **3**, indicates that this specific interaction is likely that with Arg 244, a residue found in most class A ß-lactamases, including the TEM and PC1 enzymes. There appears to be no direct analog of this positively charged residue in class C ß-lactamases. One would expect the boronate **4** to be a less impressive inhibitor of class C ß-lactamases than of class A.

Both depsipeptides are considerably less specific than cephalothin as substrates for these enzymes. The rate of cephalothin deacylation from the P99 enzyme is certainly greater than those of the depsipeptides ($k_{cat} = k_3$[4,5]) and this is probably true for the TEM enzyme also. This result must reflect differences in the shape of the 6-membered ring and its interaction with the enzyme at the acyl-enzyme stage. In contrast, the deacylation of cephalothin from PC1 enzyme is slower than that of the depsipeptides. The difference here presumably relates to the fact that elimination of the 3'-acetoxy group of cephalothin occurs at the acyl-enzyme stage.[21] This may lead to a structure closer to that obtained from **3**.

Finally, the cyclic depsipeptide **3** was also found to irreversibly inactivate the DD-peptidase of *Streptomyces* R61[22] with a second order rate constant of 1.5 $s^{-1}M^{-1}$. This reaction distinguishes **3** from the previously studied isomer **2** which did not appear to react with the R61 enzyme in any way.[5] Further, 1 mM **3** completely inhibited acylation by nitrocefin of two class B DD-peptidases, PBP2 of *E.coli* and PBP2b of *Streptococcus pneumoniae*,[21,22] in two hours.

Cyclic depsipeptides therefore represent a new class of ß-lactamase substrates and, as now demonstrated, DD-peptidase inhibitors. For ß-lactamases at least, a new class of substrates represents a new platform from which inhibitors can, in principle, be designed since all clinically important ß-lactamase inhibitors to date are poor substrates/mechanism-based inhibitors or transition state analogs.[24] The new substrate described in this work, **3**, shows striking affinity for two class A ß-lactamases. This, taken together with the inhibitory activity of the boronate **4**, suggests a general direction of approach to inhibitors of class A ß-lactamases.

Acknowledgment This research was supported by the National Institutes of Health (RFP).

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7. This distance is taken from an AM1 energy-minimized structure of **3** (Scheme 2).[5]
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15. Characterization of **3**: mp 180 °C, ^{1}H NMR (CD_3COCD_3) δ: 3.23 (t, J = 13.3 Hz, 1H), 3.29 (dd, J = 7.4 and 15.5, Hz, 1H), 3.66 (s, 2H), 4.91 (td, J = 7.3 and 13.5 Hz, 1H), 7.20-7.80, 7.51 (d, J = 7.5 Hz, 1H) ppm. ^{13}C NMR (CD_3COCD_3) δ: 30.79, 43.30, 48.11, 112.71-136.74, 152.38, 166.98-173.48 ppm. MS (ES, 65V): 348 (MNa^+), 326 (MH^+). Anal. Calcd. for $C_{18}H_{15}NO_5$: C, 66.45; H, 4.65; N, 4.30. Found: C, 66.16, H, 4.86, N, 4.12.

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17. The hydrolysis of **3**, both spontaneous and enzyme-catalyzed, was followed spectrophotometrically at 300 nm, the absorbance maximum of **8**.

18. Addition of ß-lactamases to solutions of **3** led, as monitored by product absorption at 300 nm, to a two-phased reaction. The first phase was enzyme-catalyzed while the second, of equal amplitude to the first, was not. A similar phenomenon was previously observed with **2**.[5]

19. Scheme 3 shows AM-1 energy minimized structures. The side chains have been shortened for ease of computation. At the AM-1 level, the equatorial 6ß conformer was 0.88 kcal/mole more stable than the axial.

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