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The Synthesis and Evaluation of Benzofuranones as β-Lactamase Substrates

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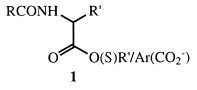
Abstract—6- and 7-Carboxy-3-phenylacetamido-3*H*-1-benzofuran-2-one have been synthesized as potential β-lactamase substrates and/or inhibitors. These compounds were prepared by lactonization of the corresponding, appropriately substituted phenylglycines. The latter compounds were prepared by either the Strecker or the Bücherer–Berg method. The benzofuran-2-ones were less stable in aqueous solution than the analogous acyclic phenaceturate esters but comparably stable to analogous benzopyran-2-ones. They differed from the latter compounds however in that the C-3 hydrogen of the furan-2-ones, adjacent to the lactone carbonyl group, was distinctly acidic; 7-carboxy-3-phenylacetamido-3*H*-1-benzofuran-2-one exists largely as an enolate at pH 7.5. The furan-2-ones were β-lactamase substrates with reactivity very similar to the analogous acyclic phenaceturates. They were not, however, DD-peptidase inhibitors and are thus unlikely to have antibiotic activity. The structural basis for these observations is discussed.

Introduction

The β-lactam antibiotics are, arguably, still the most successful antibiotics that we have available to combat bacterial infection. Their effectiveness has however been compromised in recent years by the appearance of large numbers of resistant bacterial strains, selected for by our use and, to some extent, overuse of these same antibiotics.[1] The molecular targets of the β-lactams, the bacterial cell wall DD-peptidases, remain, however, as enticing targets for antibiotics comprised of new molecular entities. Thus the search for new classes of molecule that interact with these enzymes, and with the derivative β-lactamases, the enzymes of bacterial β-lactam resistance, continues.

It has been known for some years now that acyclic (thio)depsipeptides of general structure **1** are substrates of both β-lactamases and DD-peptidases and react with them via the same double-displacement mechanism and acyl-enzyme intermediate as do β-lactams.[2–5] These depsipeptides therefore represent leads to novel mechanism-based and transition state analogue inhibitors which may prove to be antibiotics.



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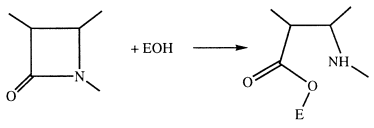
Molecules of general structure **1** differ from β-lactams in that on acylation of the enzyme during the first step of turnover, the leaving group $^{-}O(S)R'/Ar(CO_2^{-})$, departs from the active site. This contrasts with the situation with β-lactams where, because of the ring structure, the leaving group remains attached to the substrate in the active site on acylation of the enzyme (Scheme 1).

This difference is important since the pendant leaving group remains in the active site, and while there, in the case of the DD-peptidases at least, appears to block the deacylation step of turnover; β-lactams are thus DD-peptidase inhibitors.

In recent years, we have been exploring the motif of the pendant leaving group in cyclic esters rather than amides. For example, compounds **2** and **3**, cyclic analogues of the depsipeptide substrate **4**, are β-lactamase



Scheme 1.

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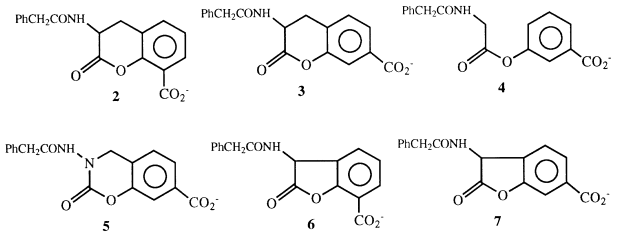
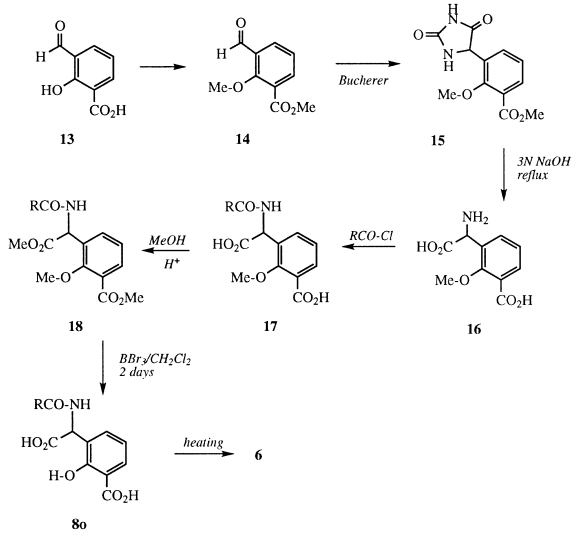
substrates; **2** is a slow inhibitor of DD-peptidases.[6,7] On the other hand, the cyclic carbamate **5**, is not a substrate/inhibitor of these enzymes.[8] In this paper we describe a further venture along these lines where the γ-lactones **6** and **7** have been prepared and their reactivity with β-lactam-recognizing enzymes examined.



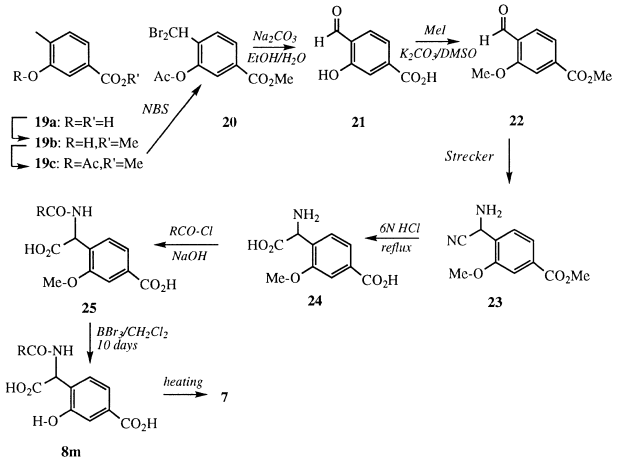
Results and Discussion

Synthesis

Reaction of *p*-hydroxybenzoic acid with α-hydroxyhippuric acid in sulfuric acid gives a 3-benzamido-3*H*-l-benzofuran-2-one possessing a meta-carboxy group.[9]



Scheme 2. Method of preparation of benzofuranone **6**.



Scheme 3. First method for the preparation of benzofuranone **7**.

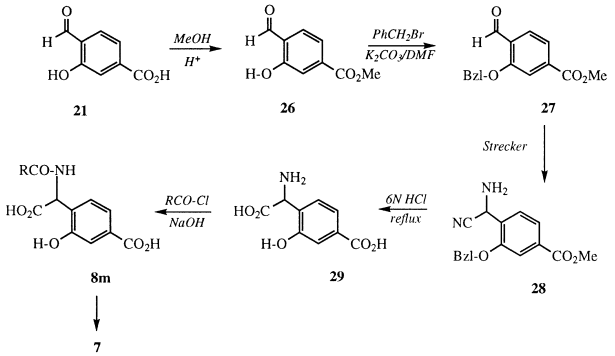
However, this amidoalkylation reaction requires the para-position of the starting phenol to be blocked.[10,11] Therefore, we turned our attention towards the preparation of a substituted α-phenylglycine from a substituted salicylaldehyde by the Strecker or the Bücherer–Berg method.[12,13] From the literature, *O*-alkylated derivatives of substituted salicylaldehydes seemed necessary starting materials for these amino acid syntheses.[14]

The benzofuranone **6** was synthesized using a methoxy protecting group for the phenol function and a Bücherer reaction for the preparation of the intermediate hydantoin **15** (Scheme 2).

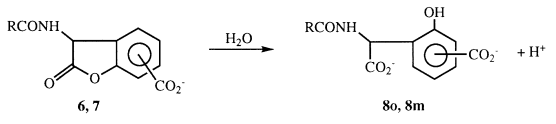
For the synthesis of the meta isomer **7** the amino-nitrile intermediate **23** was obtained by the Strecker method (Scheme 3). Cleavage of the methoxy protecting group by treatment with boron tribromide proved to be difficult, particularly in the case of the synthesis of the meta isomer **7**.

The preparation of this molecule was improved by following a second route using a benzyl protecting group which was easily cleaved in acidic conditions (Scheme 4). In both cases, lactonization of the hydroxy acid, **8o** or **8m**, was achieved by heating. The products **6** and **7** are of course racemic mixtures of the 3-α and 3-β-substituted benzofuran-2-ones.

Behavior of 6 and 7 in aqueous solution. In $NaHCO_3$ buffer, the ^1H NMR spectra of **6** and **7** changed over a time period of about 1 h, which correlated well with the time course of the spectrophotometrically observed reaction (see below). Only small changes in the chemical shifts of aromatic and side chain methylene protons were observed. The final spectrum from **7**, was identical to that of an authentic sample of the hydrolysis product **8m**; the observed reaction is therefore hydrolysis of the γ-lactone (Scheme 5). The methine proton of **6** and **7** was not observed in the NMR spectra of either reac-



Scheme 4. Second route for the preparation of benzofuranone **7**.



Scheme 5.

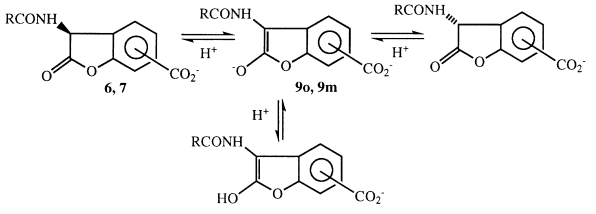
tants or products. This could be because these peaks lie under the water peak[18] or because of exchange with solvent.

The former explanation is likely for **8** since this proton, adjacent to a carboxylate, is unlikely to exchange rapidly with solvent in D_2O. On the other hand, the latter explanation is probably true for **6** and **7**. The addition of a drop of D_2O to d_6-Me_2CO solutions of these compounds led to immediate ($t_{1/2} < 1$ min) disappearance of the methine protons. This exchange is presumably mediated by facile equilibration of **6** and **7** with their respective enols/enolates (Scheme 6).

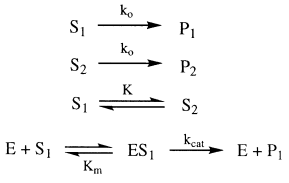
The absorption spectra of **6** and **7** in aqueous buffer, pH 7.5, are strikingly different. Compound **6** has maximal absorption at 286 nm ($\epsilon = 2.2\times10^3\,cm^{-1}\,M^{-1}$), closely similar to those of its acyclic (**4**) and δ-lactone (**2**) analogues. The spectrum of **7** however shows maximal absorbance at 330 nm ($\epsilon = 2.8\times10^3\,cm^{-1}\,M^{-1}$). The absorption spectrum of **7** at higher pH, 9.0 and 10.0, was very similar to that at pH 7.5, but at pH 5.0 the maximal absorbance moved back to 286 nm. The spectrum of **6** is unchanged at pH 5.0 but gains longer wavelength absorption at pH 9.0 and 10.0. These observations, supported by those of the ^1H NMR experiments described above, indicate that **7** exists largely as the enolate **9m** (Scheme 6) at pH 7.5, with a carbon acid (C_3-H) pKa between 5 and 7.5. On the other hand **6**, perhaps because of the presence of the negatively charged carboxylate closer to the enolate anion in **9o**, has a pK_a above 7.5 and thus exists largely as the carbon acid at that pH.[19]

The absorption spectra of **6** and **7** change as a function of time, presumably reflecting hydrolysis of the γ-lactone, yielding, at all pH values from 5 to 10, products with $\lambda_{max} = 302$ nm ($\epsilon = 5.1\times10^3\,cm^{-1}\,M^{-1}$) and 290 nm ($\epsilon = 3.3\times10^3\,cm^{-1}\,M^{-1}$), respectively. The final spectrum from **7** was identical to that of **8m**, the authentic hydrolysis product. Subsequently, the hydrolysis of **6** and **7** was monitored at 300 and 330 nm, respectively. Absorbance versus time measurements yielded pseudo-first order rate constants for the hydrolysis. At pH 7.5 in 100 mM MOPS buffer, these rate constants were $8.0\times10^{-4}\,s^{-1}$. and $6.6\times10^{-4}\,s^{-1}$ for **6** and **7**, respectively. Although **7** appears slightly less labile than **6**, the reverse is probably true since, as noted above, at pH 7.5, **7** exists mainly as the enolate **9m** which is presumably inert to hydrolysis. These rate constants can be compared with those of the analogous δ-lactones **2** and **3**, which are $5.4\times10^{-4}\,s^{-1}$ and $5.6\times10^{-4}\,s^{-1}$ respectively,[6,7] and of **1**, the acyclic analogue of **7**, which is $1.0\times10^{-5}\,s^{-1}$.[6] Thus, rather unexpectedly, the γ-lactones, **6** and **7**, appear to be more labile in solution than their δ-lactone analogues. This appears contrary to most precedent.[23,24] Izbicka and Bolen[25] have reported that the rate constant for alkaline hydrolysis of 2,3-dihydro-2*H*-1-benzofuran-2-one (coumaran-2-one) is about 10-fold smaller than that of the 3,4-dihydro-2*H*-1-benzopyran-2-one (3,4-dihydrocoumarin); on the other hand, the rates of spontaneous hydrolysis of their *p*-nitro derivatives are equal.[26] For benzofuranones, an E_1cB mechanism of hydrolysis with a ketene intermediate has been ruled out.[20–22] The greater lability of **6** and **7** in solution with respect to their δ-lactone analogues is therefore difficult to explain. It is possible that intramolecular catalysis by the side chain is involved, as may occur in bicyclic β-lactams, for example, under certain conditions.[27] From the point of view of achieving a more stable platform for inhibitor design however, this result was not helpful.

Enzymology. Spectrophotometric observations showed that the hydrolysis of **6** and **7** was catalyzed by the class C β-lactamase of *Enterobacter cloacae* P99. The complete spectrophotometric progress curve for both compounds appeared to indicate a single, smooth reaction phase. Since both **6** and **7** were racemic mixtures of the 3-α and 3-β-enantiomers, two explanations of this observation are possible. Either both enantiomers of each compound are comparably active substrates of the β-lactamase, or only one enantiomer of each is a substrate and racemization at C-3 is rapid enough to convert the non-substrate form into the substrate as the reaction proceeds, i.e., the enantiomers are effectively in rapid equilibrium in the time frame of the enzyme-catalyzed steady state reaction. The former possibility seems unlikely in view of known β-lactamase specificity and, in particular, in view of the fact that only one enantiomer of **3**, presumably the 3-β isomer, is a substrate.[6] The latter suggestion is supported by the rapid exchange of C_3-H of both **6** and **7** with solvent protons, and the existence of **7** as an enolate at pH 7.5. The two-phased reaction progress curve of **7** at pH 5 (see below) also supports the latter explanation. Thus, Scheme 7 was applied to the P99 β-lactamase-catalyzed hydrolysis of **6** and **7** at pH 7.5. In this scheme, S_1 and S_2 represent the two enantiomers of **6** or **7**, P_1 and P_2 their respective hydrolysis products, and S_1 the β-lactamase substrate. If S_2 is not a significant competitive inhibitor (as is likely), Scheme 7 predicts that the apparent K_m will be given by $K_m(1+K)$, and thus by 2 K_m if, as is presumably true, $K=1$; the apparent k_{cat} however will be the k_{cat} of Scheme 7. The steady state parameters for the hydrolysis of **6** and **7** are presented in Table 1, where the K_m (and k_{cat}/K_m) values have been adjusted as indicated above.



Scheme 6.



Scheme 7.

Compounds **6** and **7** were also substrates of two class A β-lactamases, the TEM-2 enzyme and the β-lactamase of *Staphylococcus aureus* PC1. The steady state parameters for these enzymes, obtained as described above, are also given in Table 1. Kinetic data for **2** and **3** and the acyclic parent compound **4** are also shown in Table 1 for comparison.

The meaning of k_{cat} for substrates of the P99 β-lactamase is readily available from the effects of alternative nucleophiles such as methanol or a D-amino acid[5] at substrate concentrations approaching saturation. In the case of rate-determining deacylation, as is usually observed with this enzyme, the rates increase with nucleophile concentration, because of the availability of a second channel of breakdown of the acyl-enzyme, as shown in Scheme 8. In Scheme 8, E-S′ represents the acyl-enzyme, P the hydrolysis product, and Q the alternative (methanolysis or aminolysis) product.

In the present study, the rate of hydrolysis of **6** ($S_o/K_m = 6.7$) was observed to increase linearly with methanol concentration. Application of the appropriate equations[5,6] to these data yielded a value for k_4/k_3 of 55±4. This is comparable to, although somewhat larger than, the values obtained for the δ-lactone **3**,[6] and the acyclic compound **4**.[5] Initial rates of reaction of **6** were also increased by the presence of D-phenylalanine. These results show that $k_{cat} = k_3$ for hydrolysis of **6** and that the acyl-enzyme derived from **6** is comparably accessible to nucleophiles as that from **4**.

In sharp contrast to the results with **6** however, the rate of P99 β-lactamase-catalyzed reaction of **7** ($S_o/K_m = 0.91$) was not increased by methanol or D-phenylalanine. In addition, a ^{1}H NMR experiment did not reveal a methanolysis product. This result is unusual. The acyl-enzyme derived from **4** is susceptible to both methanol and D-amino acids.[5] On the other hand, the δ-lactone **2** and benzylpenicillin itself are susceptible to methanolysis but not to aminolysis by D-phenylalanine.[6] It appears then, that the acyl-enzyme derived from **7** is even more shielded from nucleophiles than those of **2** and benzylpenicillin. The contrast between **6** and **7** in this regard is striking and presumably reflects specific and different interaction of the carboxylate groups with the enzyme, at the acyl-enzyme stage at least.

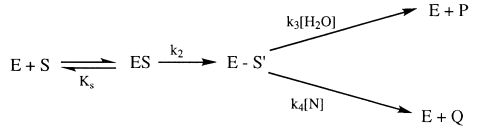
The data of Table 1 indicates that **6** is a much better substrate than **7** for both the P99 and TEM enzymes (although not, curiously, for the PC1 β-lactamase). This difference, which is not as evident in a comparison of **2** and **3**, the analogous ortho and meta substituted δ-lactones, is to a considerable extent apparent rather than real. As described above, **7**, but not **6**, exists essentially completely as the enolate **9** at pH 7.5, and it is unlikely that this species would be a substrate. Thus the kinetic parameters of **7** might well be more favorable than indicated by the raw data of Table 1. In order to investigate this point, steady state kinetic parameters for **6** and **7** were determined for the P99 β-lactamase at pH 5.0, where an absorption spectrum showed that **7** was largely present as the neutral lactone. At this pH, **6** reacted in what appeared spectrophotometrically as a single phase and thus Scheme 7 was employed for interpretation, while **7** appeared to react in a biphasic fashion. Approximately half of **7** reacted in an enzyme-catalyzed fashion while the remainder reacted more slowly although at a greater rate than the spontaneous reaction. Under the conditions employed for **7** at this pH, it appears that one enantiomer of **7**, presumably the 3-β isomer, reacted with the enzyme, while interconversion of the 3-α to the 3-β isomer was slow, leading to the slow second phase of reaction that was not enzyme-catalyzed. The steady-state parameters were therefore calculated from the initial spectrophotometric rates and concentrations of the reactive enantiomer, i.e., half the total of concentration of **7**.

The steady state kinetic constants obtained at pH 5.0 are shown in Table 2. It is clear that although **7** still appears a poorer substrate than **6**, it is relatively much better than at pH 7.5. The data obtained at pH 5.0 pre-

Table 1. Steady state kinetic parameters for β-lactamase-catalyzed lactone hydrolysis at pH 7.5

		Enzyme		
	Substrate	P99	TEM	PC1
6	k_{cat} (s^{-1})	33.5 ± 0.6[d]	29 ± 4	≥0.012[e]
	K_m (mM)[c]	(3.5 ± 0.5)×10^{-3}	1.0 ± 0.2	≥0.2[f]
	k_{cat}/K_m ($s^{-1}M^{-1}$)	9.6×10^5	2.9×10^4	60 ± 3
7	k_{cat} (s^{-1})	1.8 ± 0.2	≥0.021[e]	≥0.048[e]
	K_m (mM)[c]	0.55 ± 0.1	≥0.2[e]	≥0.2[e]
	k_{cat}/K_m ($s^{-1}M^{-1}$)	3.4×10^3	107 ± 1	240 ± 6
2[a]	k_{cat} (s^{-1})	5.4	2.1	4.7×10^{-3}
	K_m (mM)[c]	0.62	0.053	3.0×10^{-4}
	k_{cat}/K_m ($s^{-1}M^{-1}$)	8.7×10^3	3.9×10^4	1.55×10^4
3[a]	k_{cat} (s^{-1})	5.6	> 1.2	0.024
	K_m (mM)[c]	0.29	> 1	0.015
	k_{cat}/K_m ($s^{-1}M^{-1}$)	1.9×10^4	1.2×10^3	1.6×10^3
4[a]	k_{cat} (s^{-1})	125	25	0.030
	K_m (mM)	0.23	2.2	0.19
	k_{cat}/K_m ($s^{-1}M^{-1}$)	5.4×10^5	1.2×10^4	1.6×10^2
BP[b]	k_{cat} (s^{-1})	50	2000	30
	K_m (mM)	0.015	0.02	≤0.01
	k_{cat}/K_m ($s^{-1}M^{-1}$)	3.3×10^6	10^8	≥3×10^6

[a]The data for **2**, **3**, & **4** are from refs 6, 7, and 32 respectively.
[b]Benzylpenicillin: The P99 data (0.1 M phosphate, pH 7.5, 25 °C), the TEM data (0.1 M phosphate, pH 7.0, 30 °C), and the PC1 data (0.1 M phosphate, pH 7.5, 25 °C) are from ref 6.
[c]K_m values are calculated from the apparent values obtained from experiment as described in the text accompanying Scheme 7.
[d]Experimentally determined parameters from the present work are given as means with standard deviations.
[e]Limits were obtained from rates at the highest concentration employed.



Scheme 8.

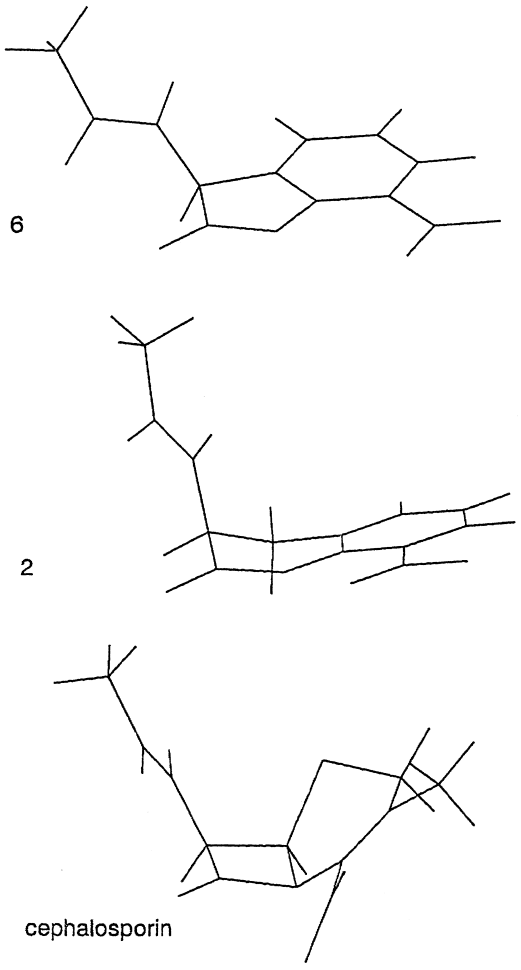
Table 2. Steady state kinetic parameters for the P99 β-lactamase-catalyzed hydrolysis of **6** and **7** at pH 5.0

Compound	k_{cat}(s^{-1})	K_m(mM)	k_{cat}/K_m($s^{-1}M^{-1}$)
6	5.4 ± 0.1	(8.5 ± 1.0)×10^{-3}	6.1×10^5
7	0.88 ± 0.02	(1.15 ± 0.05)×10^{-2}	7.7×10^4

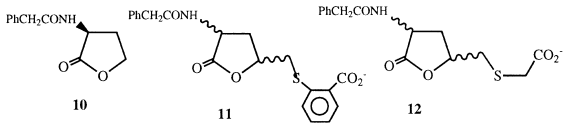
sumably gives a better estimate of the relative effectiveness of **6** and **7** as lactone substrates than that at pH 7.5.

The data indicate that **6** and **7** are comparably effective β-lactamase substrates as the other depsipeptides of Table 1, although there is a little variation depending on compound and enzyme. Compound **6** is remarkably similar in its activity to the acyclic compound **4**, for example. As with the acyclic compound, **6** and **7** are more specific substrates of the typical class C enzyme than of the class A representatives. None of the depsipeptides is of course as good a substrate as benzylpenicillin itself, but it is clear that all of them interact as substrates in an analogous fashion to β-lactams (according to Scheme 5). Any of the depsipeptide motifs would therefore be a viable platform for the design of mechanism-based inhibitors.

A preliminary assessment of monocyclic γ-lactones suggested that they would, in general, interact with β-lactamases less strongly than the bicyclic compounds **2**, **3**, **6** and **7**. For example, compounds **10**–**12**, at concentrations up to 0.5 mM, showed only weak second-order inhibition ($k_i \leq 2\,s^{-1}M^{-1}$) of the P99 β-lactamase, and had no effect on the TEM enzyme. This probably reflects the effect of poorer leaving groups than present in the bicyclic compounds and, perhaps, the absence of optimally placed hydrophobic functionality.[3] It is possible that these results might be improved by further design.



Scheme 9.



A final question to be addressed is that of whether **6** and **7** might resemble a β-lactam sufficiently to inhibit a DD-peptidase and thus provide a platform for antibiotic development. Unfortunately, neither **6** or **7** (at 1 mM concentration) were substrates or inhibitors of the DD-peptidase of *Streptomyces* R61 or of penicillin-binding protein 2 of *E.coli*. Thus despite their chemical reactivity, neither of these compounds can productively interact with a DD-peptidase active site. AM1 structures of **6**, **2** and a generic cephalosporin are shown in Scheme 9 (where the presumably-reactive β-side chain enantiomers of the former two compounds are shown). There are obviously similarities and differences in structure between the lactones and cephalosporin, where the major difference lies in the coplanarity of the rings in **6** and **2**; this has the effect of separating the β-lactam carbonyl group and the carboxylate group. The distance between these groups has been proposed as an important indicator of antibiotic activity.[28] Indeed, this distance in **6** (5.39 Å) is even greater than that in **2** (4.86 Å[7]), and each are much greater than that in a cephalosporin (ca. 3.2 Å[28]). The compound **2**, unlike **6**, does weakly inhibit DD-peptidases.[7] The carboxylate group of bicyclic β-lactams is observed in crystal structures to interact strongly with the active site functional groups of these enzymes.[29] None of the monocyclic compounds **10**–**12** was a substrate or inhibitor of the R61 DD-peptidase.

The new molecules, **6** and **7**, therefore, appear to be β-lactamase substrates but not DD-peptidase inhibitors. With regard to the former activity, they appear to have major specificity for a class C β-lactamase. These structures may lead, by appropriate side chain selection for example, to specific inhibitory substrates for these enzymes.

Experimental

Enzymes: The β-lactamases were purchased from the Centre for Applied Microbiology and Research, Porton Down, Wilts., UK. The DD-peptidase of *Streptomyces* R61 was the generous gift of Dr. J.-M. Frère of the University of Liège, Liège, Belgium.

Analytical and kinetic methods

Stock solutions of the lactones were prepared in DMSO rather than in aqueous buffer because of the instability of these compounds in the latter solvent (see Results). All kinetics measurements were made in 100 mM MOPS buffer, pH 7.5, 25 °C. The total DMSO concentration after addition of the substrate was 5% v/v.

Reactions of **6** and **7** were monitored spectrophotometrically at 300 and 330 nm respectively. Steady

state kinetics parameters were obtained from spectrophotometric initial rates by the method of Wilkinson.[31] The initial rates of the enzyme-catalyzed reaction were obtained by subtraction of the initial spontaneous hydrolysis rates from the observed initial rates. Inhibition of the R61 DD-peptidase by **6** and **7** was tested for by monitoring the activity of enzyme that had been incubated in the presence of these compounds against *N*-(phenylacetyl)glycyl-D-thiollactate.[31]

To obtain the structures of Scheme 9, molecules were constructed by means of the Builder Module of INSIGHT II, version 97.0 (MSI, San Diego, CA), run on an IBM 3CT computer. Initial structural relaxation was performed by the DISCOVER module. Final energy and structural minimization was achieved by semiempirical AM1 calculations (MOPAC 6.0).

Synthesis

Methyl 2-methoxy-3-formylbenzoate (14). 2-Hydroxy-3-formylbenzoic acid **13** (332 mg, 2 mmol) was dissolved in dimethyl sulfoxide (5 mL). Potassium carbonate, 1.66 g (12 mmol) was added, then while stirring, methyl iodide, 0.5 mL (8 mmol), was added dropwise. Stirring was continued for 2 h. The reaction mixture was poured into water and the product extracted with ethyl acetate. The organic layer was washed with water then with a 1M sodium thiosulfate solution and dried over sodium sulfate. The solvent was evaporated and the residue purified by chromatography (pentane:ether 1:1), to give 346 mg (89%) of the title product (liquid); R_f 0.55; ^{1}H NMR ($CDC1_3$) δ: 3.96 and 4.01 (2s, 2×3H, CH_3), 7.29 (t, $J=7.72$, 1H, H_5), 8.02 (dd, $J=7.72$ and 1.86, 1H, H_4), 8.08 (dd, $J=7.72$ and 1.86, 1H, H_6), 10.46 (s, 1H, CHO); ^{13}C NMR ($CDCl_3$) δ: 52.57 and 64.97 (CH_3), 124.16–137.73 (C Ar), 163.27 (C phenol), 165.51 (CO) and 189.26 (CHO). Anal. calcd for $C_{10}H_{10}O_4$: C, 61.85; H, 5.19. Found: C, 61.61; H, 5.18.

2-Methoxy-3-methoxycarbonylphenyl-imidazoline-2,4-dione (15). Ammonium carbonate 540 mg (5.6 mmol) and sodium cyanide 40 mg (0.82 mmol) were added to a solution of 110 mg (0.57 mmol) of the aldehyde **20** in a mixture of methanol (3 mL) and water (3 mL). The mixture was heated at 50–60 °C and stirred for 3 h. The solvent was evaporated and the product purified by chromatography (dichloromethane:methanol 97.5:2.5) affording 109 mg (73%) of the hydantoin; Rf 0.65; mp 173 °C; ^{1}H NMR ($CDC1_3$) δ: 3.88 and 3.92 (2s, 2×3H, CH_3), 5.41 (s, 1H, CH), 7.15 (t, $J=7.7$, 1H, H_5), 7.44 (dd, $J=7.7$ and 1.47, 1H, H_4), 7.84 (dd, $J=7.7$ and 1.47, 1H, H_6); ^{13}C NMR ($CDC1_3$) δ: 52.57 and 58.13 (CH_3), 63.36 (CH), 124.01–133.15 (C Ar), 158.35 (C phenol), 159.05 and 174.31 (CO). Anal. calcd for $C_{12}H_{12}N_2O_5$: C, 54.54; H, 4.58; N, 10.60. Found: C, 54.58; H, 4.81; N, 10.76.

2-Phenylacetamido-(2′-methoxy-3′-carboxy)phenylacetic acid (17). 175 mg (0.66 mmol) of the hydantoin **15** was dissolved in 3M NaOH (10 mL) and the solution was refluxed vigorously for 3 days. The reaction mixture containing the amino acid **16** was cooled to 0 °C, then a solution of 0.3 mL (2.27 mmol) of phenylacetyl chloride in ethyl acetate (3 mL) was added. The mixture was stirred at room temperature for 16 h, then cooled and acidified with concd hydrochloric acid. The solution was saturated with sodium chloride and extracted with ethyl acetate. The organic layer was dried over sodium sulfate and the solvent evaporated. The residue was purified by chromatography (ethyl acetate:acetic acid 95:5), affording 180 mg (79%) of the title product; Rf 0.38; mp 191 °C; ^{1}H NMR (CD_3OD) δ: 3.59 (s, 2H, CH_2Ph), 3.80 (s, 3H, OCH_3), 5.85 (s, 1H, CHAr), 7.17 (t, $J=7.20$, 1H, H_3), 7.20–7.27 (m, 5H, Ar), 7.54 (d, $J=7.20$, 1H, H_4), 7.80 (d, $J=7.20$, 1H, H_6); ^{13}C NMR (CD_3OD) δ: 43.44 (CH_2Ph), 52.93 (CH_3), 63.22 (CH Ar), 124.87–136.80 (C Ar), 159.71 (C phenol), 169.42, 173.59 and 175.35 (COO and CON). MS (FAB): 344 MH^+, 366 MNa^+. Anal. calcd for $C_{18}H_{17}NO_6$: C, 62.97; H, 4.99; N, 4.08. Found: C, 62.79; H, 5.04, N, 3.79.

2-Phenylacetamido-2-(2′-hydroxy-3′-carboxy)phenylacetic acid (8o). The previous diacid **17** was esterified in methanol (10 mL) by addition of a few drops of thionyl chloride and refluxing the solution for 16 h. The solvent was evaporated to dryness and the residue purified by chromatography (pentane:ether 1:3), affording methyl 2-phenylacetamido-2-(2′-methoxy-3′-methoxycarbonyl)-phenylacetate **18**; R_f 0.25; ^{1}H NMR ($CDC1_3$) δ: 3.61 (s, 2H, CH_2Ph), 3.68, 3.72, 3.92 (3s, 3×3H, OCH_3), 5.79 (d, $J=7.54$, 1H, CHAr), 6.69 (d, 1H, NH), 7.12 (t, $J=7.72$, 1H, H_3), 7.25–7.38 (m, 5H, Ar), 7.46 (dd, $J=7.53$ and 1.84, 1H, H_4), 7.79 (dd, $J=7.72$ and 1.84, 1H, H_6); ^{13}C NMR ($CDCl_3$) δ: 43.63 (CH_2Ph), 52.54, 52.94 and 53.00 (CH_3), 62.54 (CH Ar), 123.87 to 134.64 (C Ar), 158.46 (C phenol), 166.34, 170.49 and 171.29 (COO and CON).

A 1 M solution of BBr_3 in dichloromethane (3 mL) was added under argon to 83 mg (0.24 mmol) of the acid **18**. The mixture was stirred at room temperature for 2 days.Then 0.5 mL of water was added and the product extracted with ethyl acetate. The organic layer was dried over sodium sulfate and the solvent evaporated to leave a solid which was purified by chromatography (ethyl acetate:acetic acid 9:1), affording 55 mg (69%) of the title product **8o**; R_f 0.48; mp 168–170 °C; ^{1}H NMR (CD_3COCD_3) δ: 3.63 (s, 2H, $ArCH_2$), 5.88 (d, $J=7.89$, 1H, CH), 6.94 (t, $J=7.82$, 1H, H_3), 7.11–7.33 (m, 5H, Ar), 7.60 (dd, $J=7.54$ and 1.65, 1H, H_4), 7.77 (d, 1H, NH), 7.87 (dd, $J=7.90$ and 1.65, 1H, H_6). ^{13}C NMR (CD_3COCD_3) δ: 43.25 (CH_2Ar), 52.46 (CH-NH), 119.80 to 136.94 (C Ar), 160.81 (C phenol), 171.03, 171.89 and 172.98 (CO_2 and CON). MS (FAB): $C_{17}H_{15}NO_6$: m/z 330, MH^+ and 352, MNa^+.

7-Carboxy-3-phenylacetamido-3*H*-1-benzofuran-2-one (6). The acid **8o** was heated in vacuo at 200 °C for 30 min in a GKR 50 Büchi apparatus to give the title lactone **6**. The product was dissolved in acetone and precipitated by addition of pentane. When heated at 230 °C this solid lactone decomposed; ^{1}H NMR (CD_3COCD_3) δ: 3.62 (s, 2H, $ArCH_2$), 5.43 (d, $J=7.17$, 1H, CH), 7.23 (t, $J=7.72$, 1H, H_3), 7.20–7.31 (m, 5H, Ar), 7.47 (d, $J=7.4$, 1H, H_4), 7.88 (dd, $J=7.9$ and 1.5, 1H, H_6), 8.44 (d, 1H, NH); ^{13}C NMR (CD_3COCD_3) δ:

42.77 (CH_2Ar), 51.26 (CH-NH), 114.81–l36.28 (C Ar), 154.78 (C phenol), 165.18, 171.45 and 173.49 (CO_2 and CON). Anal. calcd for $C_{17}H_{13}NO_5$. 1/2 H_2O: C, 63.74; H, 4.41; N, 4.37. Found: C, 63.52; H, 4.21; N,4.19. MS (ES): m/z 312 (MH^+), 334 ($M+Na^+$), and 350 ($M+K^+$). HRMS (ES^-) calcd for $[C_{17}H_{12}NO_5]^-$: 310.0715. Found: 310.0721.

Methyl 3-acetoxy-4-dibromomethyl benzoate (20). A mixture of methyl 3-acetoxy-4-methyl benzoate **19c** (1.11 g, 5.34 mmol), NBS (1.9 g, 10.7 mmol) and benzoyl peroxide (50 mg) in $CC1_4$ (50 mL) was refluxed for 3 h under argon. The reaction mixture was cooled to room temperature and filtered. The filtrate was evaporated and the residue was purified by chromatography (dichloromethane:pentane 9:1). Methyl 3-acetoxy-4-dibromomethyl benzoate **20** was obtained, 1.64 g, 74% ; R_f 0.62; mp 74 °C; ^{1}H NMR ($CDCl_3$) δ: 2.42 (s, 3H, OAc), 3.92 (s, 3H, OCH_3), 6.81 (s, 1H, CH), 7.78 (d, 1H, H_2), 7.92 (d, 1H, H_5), 7.98 (dd, 1H, $J=8$ Hz, $J=1.5$ Hz, H_6); ^{13}C NMR ($CDCl_3$) δ: 20.97 ($COCH_3$), 32.97 ($CHBr_2$), 52.50 (OCH_3), 124.18–137.22 (Ar), 145.26 (C phenol), 165.28 and 168.12 (CO). Mass (FAB): m/z (%) 389 (45), 367 (30), 285 (40), 245 (100). Anal. calcd for $C_{11}H_{10}O_4Br_2$: C, 36.09; H, 2.75; O, 17.49. Found: C, 35.91; H, 2.74; O, 17.45.

Methyl 3-methoxy-4-formyl benzoate (22). Methyl 3-acetoxy-4-dibromomethyl benzoate **20** (494 mg, 2.33 mmol) was dissolved in ethanol (10 mL) and a solution of 3 g of Na_2CO_3 in water (2 mL) was added. The mixture was refluxed for 16 h, then ethanol was evaporated. The aqueous solution was acidified by addition of HCl and the product was extracted into ethyl acetate. 3-Hydroxy-4-formylbenzoic acid **21** was obtained; R_f 0.35 (dichloromethane:methanol:acetic acid 90:10:1). The crude product was dissolved in DMSO (4 mL), and K_2CO_3 (2 g) and methyl iodide (0.5 mL) were added to the stirred mixture. After stirring for 3 h at room temperature, water was added and the product extracted into ethyl acetate. The organic layer was washed twice with water, dried (Na_2SO_4) and evaporated. The residue was purified by chromatography (pentane:ether 1:1), affording 262 mg (58%) of the title product; R_f 0.69; mp 94–96 °C; ^{1}H NMR ($CDCl_3$) δ: 3.94 and 3.99 (2s, 6H, 2×CH_3), 7.67 (m, 2H, Ar), 7.86 (dd, 1H, $J=1$ Hz, $J=7$ Hz, H_6), 10.50 (s, 1 H, CHO); ^{13}C NMR ($CDC1_3$) δ: 52.63 ($COOCH_3$), 55.97 (OCH_3), 112.82–136.40 (Ar), 161.44 (C phenol), 166.08 (CO), 189.39 (CHO). Mass (FAB): m/z (%) 195 (100), 154 (75), 137 (55).Anal. calcd for $C_{10}H_{10}O_4$: C, 61.87; H, 5.19. Found: C, 61.67 ; H, 5.16.

2-Amino-2-(2′-methoxy-4′-methoxycarbonyl)phenylacetonitrile (23). The previous aldehyde **22** (99 mg) was dissolved in methanol (1 mL) and the solution was added at 0 °C to a mixture of 30 mg of NaCN and 50 mg of NH_4Cl in 33% ammonia (1 mL). After the mixture had been stirred for 4 h at room temperature, the methanol was evaporated, water was added, and the product was extracted into dichloromethane. The solution was dried over Na_2SO_4 and evaporated. Chromatography (ether) gave 105 mg (94%) of the title α-aminonitrile **23**; R_f 0.42; ^{1}H NMR ($CDC1_3$) δ: 3.93 and 3.98 (2s, 6H, 2×CH_3), 5.08 (s, CH), 7.50 (d, 1H, H_5), 7.60 (d, 1H, H_2), 7.69 (dd, 1H, $J=8$ Hz, $J=1.5$ Hz, H_6); ^{13}C NMR ($CDCl_3$) δ: 43.23 (CH), 52.37 ($COOCH_3$), 55.93 (OCH_3), 111.88–132.17 (Ar), 156.46 (C phenol), 166.31 (CO). Mass (FAB) m/z 221 (MH^+). Anal. calcd for $C_{11}H_{12}N_2O_3$: C, 59.99; H, 5.49; N, 12.72. Found: C, 59.86; H, 5.41 ; N, 12.61.

2-Phenylacetamido-(2′-methoxy-4′-carboxy)phenylacetic acid (25). The α-aminonitrile **23** (62 mg) was dissolved in 6N HCl (1 mL) and heated in a stoppered flask for 16 h at 115 °C. Evaporation to dryness gave 85 mg (95%) of the pure α-aminoacid hydrochloride **24**. ^{1}H NMR ($CDCl_3$) δ: 3.96 (s, 3H, CH_3), 5.27 (s, 1H, CH), 7.52 (d, $J=8.64$ Hz, 1H, H_5), 7.71 (d, $J=1.66$ Hz, 1H, H_2), 7.72 (dd, 1H, H_6).

The 2-amino-(2′-methoxy-4′-carboxy)phenylacetic acid **24** (85 mg, 0.5 mmol) was dissolved in 3M NaOH (4 mL) and the solution was cooled to 0 °C. A solution of 0.25 mL (2 mmol) of phenylacetyl chloride in ethyl acetate (3 mL) was then added. The mixture was stirred at room temperature for 16 h, then acidified with concd hydrochloric acid. The solution was saturated with sodium chloride and extracted with ethyl acetate. The organic layer was dried over Na_2SO_4 and the solvent evaporated. The residue was purified by chromatography (ethyl acetate:acetic acid 95:5), affording 97 mg (75%) of the title product **25**; R_f 0.64; mp 189 °C; ^{1}H NMR (CD_3COCD_3) δ: 3.60 (s, 2H, CH_2Ph), 3.85 (s, 3H, OCH_3), 5.87 (d, $J=8.0$, 1H, CHAr), 7.19–7.35 (m, 5H, Ar), 7.46 (d, $J=8.0$, 1H, H_5), 7.57 (d, $J=1.5$, 1H, H_2), 7.62 (dd, 1H, H_6), 7.69 (d 1H, NH); ^{13}C NMR ($CDCl_3/CD_3OD$) δ: 42.54 (CH_2Ph), 52.80 (CH Ar), 54.99 (OCH_3), 111.54–134.04 (C Ar), 156.54 (C phenol), 167.93, 171.21 and 171.62 (COO and CON); MS ES:m/z (%) 366 (100) MNa^+. Anal. calcd for $C_{18}H_{17}NO_6$: C, 62.97; H, 4.99; N, 4.08. Found: C, 62.88; H, 4.81; N, 3.96.

2-Phenylacetamido-(2′-hydroxy-4′-carboxy)phenylacetic acid (8m). 3 mL of a 1 M solution of BBr_3 in dichloromethane was added under argon to a solution of 86.3 mg (0.25 mmol) of **25** in 3 mL of dichloromethane. The mixture was stirred at room temperature for 10 days, after which water (1 mL) was added and the product extracted with ethyl acetate. The organic layer was dried over sodium sulfate and the solvent evaporated. The residue was purified by chromatography (ethyl acetate:acetic acid 95:5), affording 62.4 mg (72%) of the title acid **8m**; R_f 0.45; mp 205–208 °C; ^{1}H NMR (CD_3COCD_3) δ: 3.65 (s, 2H, $ArCH_2$), 5.86 (d, $J=7.72$, 1H, CH), 7.19–7.41 (m, 6H), 7.52 (dd, $J=8.00$ and 1.56, 1H, H_6), 7.56 (d, $J=1.56$, 1H, H_2), 7.89 (d, 1H, NH); ^{13}C NMR (CD_3COCD_3) δ: 43.12 (CH_2Ar), 52.80 (CH), 118.03–l36.77 (C Ar), 156.20 (C phenol), 167.36, 171.66 and 171.96 (CO_2 and CON); MS $C_{17}H_{15}NO_6$: 330, MH^+ and 352, MNa^+.

6-Carboxy-3-phenylacetamido-3*H*-1-benzofuran-2-one (7). The acid **8m** (47.4 mg) was heated in vacuo at 200 °C for 20 min in a GKR 50 Büchi apparatus. Then the

product was dissolved in acetone and precipitated by addition of pentane. The collected solid was then dissolved in ether, the solution filtered on a hydrophobic filter (0.45 μm) and the solvent evaporated to give the title lactone **7**; mp 226–229 °C; ^{1}H NMR (CD_3COCD_3) δ: 3.61 (s, 2H, $ArCH_2$), 5.46 (d, $J=6.44$, 1H, CH), 7.18 to 7.32 (m, 5H, Ar), 7.38 (d, $J=7.72$, 1H, H_5), 7.64 (d, $J=1.29$, 1H, H_2), 7.83 (dd, 1H, H_6), 8.48 (d, 1H, NH). ^{13}C NMR (CD_3COCD_3) δ: 42.71 (CH_2Ar), 52.09 (CH), 111.86–l36.24 (C Ar), 155.11 (C phenol), 166.81, 171.53 and 173.26 (CO_2 and CON). MS (ES): m/z 312 (MH^+), 334 ($M+Na^+$), and 350 ($M+K^+$). HRMS (ES^-) calcd for $[C_{17}H_{12}NO_5]^-$: 310.0715. Found: 310.0719.

Methyl 3-benzyloxy-4-formyl benzoate (27). Methyl 3-hydroxy-4-formyl benzoate **26** (178 mg, 0.99 mmol) was dissolved in anhydrous DMF (6 mL). Dry K_2CO_3 (400 mg), then benzyl bromide, 0.12 mL (1 equiv) in DMF (2 mL), were added. The mixture was stirred at room temperature for 2 h. The product was extracted into ethyl acetate and the organic phase washed with water (3×), dried over $MgSO_4$ and evaporated. The crude product was purified by chromatography (pentane:ether 3:1), affording 257 mg (96%) of the title product **27**; R_f 0.36; oil; ^{1}H NMR ($CDCl_3$) δ: 3.96 (s, 3H, CH_3), 5.26 (s, 2H, CH_2), 7.35–7.50 (m, 5H, Ar), 7.71 (dd, 1H, $J=1.3$ Hz, $J=8.09$ Hz, H_6), 7.77 (d, 1H, H_2), 7.91 (d, 1H, H_5), 10.59 (s, 1H, CHO); ^{13}C NMR ($CDCl_3$) δ: 52.84 ($COOCH_3$), 70.97 (OCH_2), 114.26–136.56 (Ar), 160.81 (C phenol), 166.21 (CO), 189.52 (CHO). Anal. calcd for $C_{16}H_{14}O_4$: C, 71.10; H, 5.22. Found: C, 70.97; H, 5.26.

2-Amino-2-(2′-benzyloxy-4′-methoxycarbonyl)phenylacetonitrile (28). The aldehyde **27** (162 mg, 0.6 mmol) was dissolved in methanol (4 mL) and the solution was added at 0 °C to a mixture of 45 mg of NaCN and 75 mg of NH_4Cl in a 33% aqueous solution of ammonia (1.5 mL). After the mixture was stirred overnight at room temperature, the methanol was evaporated, water was added, and the product extracted with dichloromethane. The solution was dried over $MgSO_4$ and evaporated. Chromatography (ether:pentane 3:1) gave 120 mg (73%) of the α-aminonitrile **28**; R_f 0.38; mp 76 °C; ^{1}H NMR ($CDCl_3$) δ: 2.08 (s, 2H, NH_2), 3.94 (s, 3H, CH_3), 5.10 (s, CH), 5.23 (s, CH_2), 7.35–7.50 (m, 5H, Ar), 7.53 (d, 1H, $J=6.07$, H_5), 7.70 (s, 1H, H_2), 7.72 (d, 1H, H_6); ^{13}C NMR ($CDCl_3$) δ: 43.60 (CH), 52.63 ($COOCH_3$), 70.99 (CH_2), 120.37 (CN), 113.20–135.83 (Ar), 155.82 (C phenol), 166.47 (CO). Anal. calcd for $C_{17}H_{16}N_2O_3$: C, 68.90; H, 5.44; N, 9.45. Found: C, 68.92; H, 5.41; N, 9.24,

2-Phenylacetyl-(2′-hydroxy-4′-carboxy)phenylacetic acid (8m). The α-aminonitrile **28** was dissolved in 6 M HCl (2 mL) and heated in a stoppered flask for 16 h at 115 °C. Evaporation to dryness gave 61.5 mg of the α-amino acid hydrochloride **29**, pure by TLC, R_f 0.56 (ethyl acetate:acetic acid 1:1). ^{1}H NMR (CD_3OD) δ: 5.11 (s, 1H, CH), 7.36 (d, $J=7.54$ Hz, 1H, H_5), 7.54 (d, $J=1.47$ Hz, 1H, H_2), 7.55 (dd, 1H, H_6). ^{13}C NMR (CD_3OD) δ: 53.68 (CH), 117.33–134.40 (C Ar), 156.17 (C phenol), 168.68 and 169.84 (COO). The crude product was directly acylated by phenylacetyl chloride as previously described to give the title acid **8m** which was purified by chromatography (ethyl acetate:acetic acid 95:5), affording 97 mg (75%) of the title product.

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19. Scheme 6 and the discussion surrounding it is supported by the observations that the C_3-H of 5-nitro-coumaran-2-one[20] and 3-phenyl-coumaran-2-one[21] are rapidly exchanged in D_2O. The carbon acid pK_as of these compounds are reported to be 9.7[20,22] and 8.4,[21] respectively; the pK_a of the parent coumaran-2-one is 12.3.[21]
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