Synthesis and Reactivity with β-Lactamases of "Penicillin-like" Cyclic Depsipeptides

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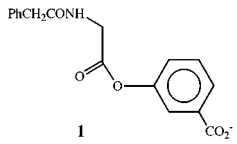
Several 7-carboxy-3-amido-3,4-dihydro-2*H*-1-benzopyran-2-ones have been synthesized as potential β-lactamase substrates and/or mechanism-based inhibitors. Substituted *o*-tyrosine precursors were prepared by the Sörensen method and then heated in vacuo to give the lactones. These compounds are cyclic analogues of aryl phenaceturates which are known to be β-lactamase substrates. The goal of incorporating the scissile ester group into a lactone was to retain the leaving group tethered to the acyl moiety at the acyl-enzyme stage of turnover by serine β-lactamases, in a manner similar to that during penicillin turnover. Further, in two cases, a functionalized methylene group *para* to the leaving group phenoxide oxygen was incorporated. These molecules possess a latent *p*-quinone methide electrophile which could, in principle, be unmasked during enzymic turnover and react with an active site nucleophile. All of these compounds were found to be substrates of class A and C β-lactamases, the first δ-lactones with such activity. Generally, k_{cat} values were smaller than for the analogous acyclic depsipeptides, which suggests that the tethered leaving group may obstruct the attack of water on the acyl-enzymes. Further exploration of this structural theme might lead to quite inert acyl-enzymes and thus to significant inhibitors. Despite the apparent advantage offered by the longer-lived acyl-enzymes, the functionalized compounds were no better as irreversible inhibitors than comparable acyclic compounds [Cabaret, D.; Liu, J.; Wakselman, M.; Pratt, R. F.; Xu, Y. *Bioorg. Med. Chem.* **1994**, *2*, 757−771]. Thus, even tethered quinone methides, at least when placed as dictated by the structures of the present compounds, were unable to efficiently trap a nucleophile at serine β-lactamase active sites.

Introduction

The β-lactamase enzymes and their evolutionary parents, the bacterial DD-peptidases, remain at the center of β-lactam research.[1,2] The search for new classes of molecules that interact with these enzymes is therefore an important part of the quest for new antibiotics. Demand for the latter has increased in response to the growing resistance of known pathogens and the emergence of new ones.[3]

Acyclic depsipeptides have been shown to be substrates of both β-lactamases and DD-peptidases and much has been learned about these enzymes from studies with these substrates.[4−11] In particular, the aryl phenaceturate **1** has been extensively employed as a β-lactamase substrate in mechanistic studies.[5,6,10,11] One offshoot of these studies has been the design of functionalized analogues of **1**, molecules such as **2**,[12] as potential mechanism-based inhibitors of β-lactam-recognizing enzymes. Acylation of the active site serine residue by **2** would yield a *p*-(bromomethyl) phenol. This should



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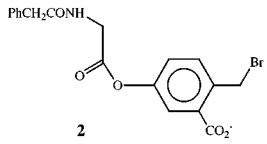

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eliminate bromide in a facile fashion to yield an electrophilic quinone methide (Scheme 1). Reaction of the latter with an active site nucleophile (Nu) could then potentially inactivate the enzyme. This theme has been effective with other serine hydrolases.[13−17]

Competition between reaction with an active site nucleophile and diffusion of the quinone methide from

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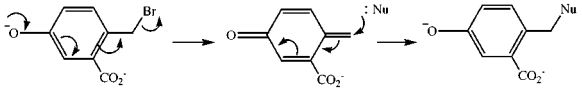
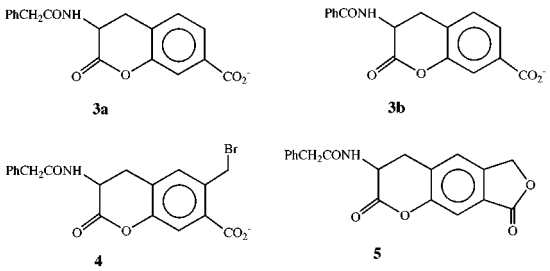
Scheme 1



the active site and subsequent hydrolysis (Nu = H_2O) would be expected to limit the effectiveness of **2** as an inhibitor, and, in fact, molecules such as **2** were not efficient inhibitors of β-lactamases.[12] If the quinone methide were tethered to the acyl group, however, one might expect greater efficiency, and, indeed, molecules incorporating this feature have effectively inhibited other serine hydrolases.[13−17] Consequently, we have constructed molecules analogous to **2**, but where the acyl group is tethered to the leaving group, and describe in this paper the interactions of one group of these molecules, dihydrobenzopyranones, with representative β-lactamases and a DD-peptidase. The specific molecules involved are the parent compounds **3a** and **3b** and two functionalized variants **4** and **5**. These compounds were found to be the first examples of δ-lactone substrates of β-lactamases.

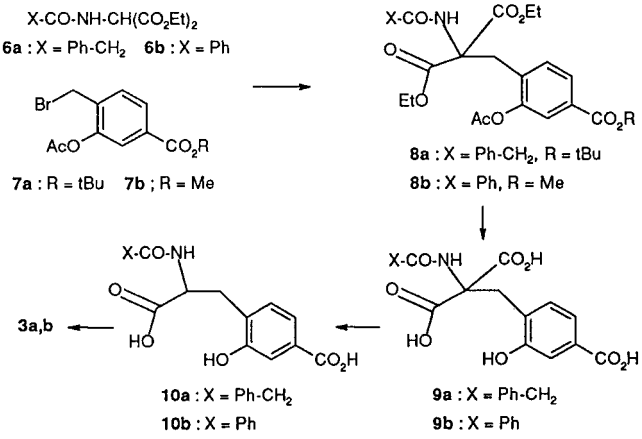


3a **3b** **4** **5**

Results

Synthesis. The dihydrobenzopyranones **3a**, **3b**, and **5** were synthesized by thermal lactonization of the corresponding substituted *o*-tyrosine precursors. These amino acids were prepared by the Sörensen method.[18] Alkylation of the anion of diethyl phenylacetamido- or benzamido-malonates **6a** or **6b** [19] with the substituted benzyl bromides **7a**[12] or **7b** led to the malonic esters **8a** and **8b**, respectively (Scheme 2). Alkaline hydrolysis and then acidification of these compounds under mild conditions were incomplete (NMR) and therefore were repeated twice. Decarboxylation of the triacids **9a** or **9b** furnished the racemic *N*-(phenylacetyl)-4-carboxy-2-hydroxyphenylalanine **10a** or its benzoyl analogue **10b**.

Scheme 2



Finally, the action of heat on these compounds in vacuo for a few minutes gave the substituted dihydrocoumarins **3a** or **3b**, also, in each case, in racemic form.

2,4-Dimethyl-5-hydroxybenzoic acid[20] was prepared from 2,4-dimethylbenzoic acid by a more convenient route than the literature method: nitration, catalytic transfer hydrogenation, diazotization, and hydroxy-de-diazotization[21,22] (see the Experimental Section). Esterification of the carboxyl group and acetylation of the hydroxyl functional group of this acid gave acetate **11** (Scheme 3). Then, radical bromination of the methyl substituents, yielded methyl 2,4-bis-(bromomethyl)-5-acetoxybenzoate **12**. This dibromide possessed two reactive bromomethyl groups. To discriminate between these groups, the molecule was cyclized[23,24] to give the bromomethyl phthalide **13** with only one bromomethyl substituent. Moreover, the formation of the phthalide ring provided mutual protection of both the latent carboxylic and hydroxyl functions of the benzyl bromide **13** in the next step of the reaction sequence. Alkylation of the anion of compound **6a** with monobromide **13** led to the malonic ester **14**. Repeated hydrolysis and decarboxylation, as above for compound **8a**, led in this case to a mixture of diacid **10c** and phthalide **15**. On being heated in vacuo, this mixture gave the pure dilactone **5**. By analogy with Olah's method of cleavage of phthalide to 2-(iodomethyl)benzoic acid,[25] treatment of the dilactone **5** with BBr_3, sodium bromide, and tetrabutylammonium bromide in acetonitrile led to the bromomethylated dihydrobenzopyranone **4**. Despite the presence of a large excess of bromide anion and of several variations of the experimental procedure, the reaction did not go to completion, and a small amount of the parent dilactone **5** was always present in the product. Crystallization and chromatography were ineffective in separating these fragile molecules.

Stability in Aqueous Solution. Compounds **3a**, **3b**, **4**, and **5** reacted spontaneously in aqueous buffer solutions. ^{1}H NMR spectra of **3** in $NaHCO_3$ buffer showed clean conversion to a single product with general upfield movement of resonance peaks, as anticipated for δ-lac-

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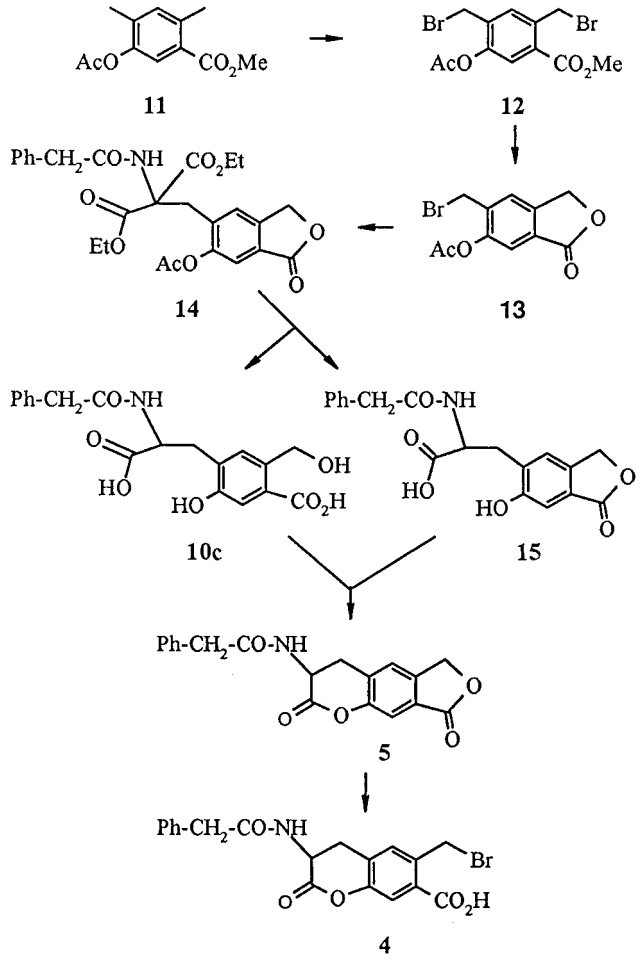
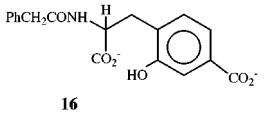
Scheme 3



tone hydrolysis at neutral pH. The final NMR spectrum from **3a** [(^2H_2O, HCO_3^-), 2.86 (dd, J = 13.2, 10.3 Hz, 1H), 3.20 (dd, J = 13.2, 4.8 Hz, 1H), 3.37, 3.48 (ABq, J = 14.4 Hz, 2H), 4.53 (dd, J = 10.3, 4.8 Hz, 1H), 6.7−7.3 (m, 8H)] was consistent with that expected of the hydrolysis product **16**. Absorption spectral changes leading from **3a** (λ_{max} 278 nm, log ϵ = 3.11) to **16** (λ_{max} 292 nm, log ϵ =



16

3.54) were consistent with production of a *m*-hydroxybenzoate (see also Figure 1, upper panel). First-order rate constants for the spontaneous hydrolysis of **3a**, **3b**, **4**, and **5** in 100 mM MOPS buffer (5% acetonitrile) at pH 7.5 and 25 °C were 5.6×10^{-4} s^{-1}, 5.0×10^{-4} s^{-1}, 1.4×10^{-3} s^{-1}, and 1.3×10^{-3} s^{-1}, respectively. These values can be compared with those of **1** and **2**, the acyclic analogues of **3a** and **4**, respectively, and of benzylpenicillin under comparable conditions, viz. 1.0×10^{-5} s^{-1}, 8.0×10^{-5} s^{-1}, and 1.5×10^{-5} s^{-1}, respectively.

***β*-Lactamase-Catalyzed Hydrolysis.** Addition of β-lactamase to solutions of **3a**, **3b**, **4**, and **5** led, as monitored by product absorption, to a two-phased reaction. Figure 1, upper panel, shows, for example, such data for **3a** (0.07 mM) and the P99 β-lactamase (0.2 μM). The first phase of reaction was enzyme-catalyzed; the second, of equal amplitude and having the same rate constant

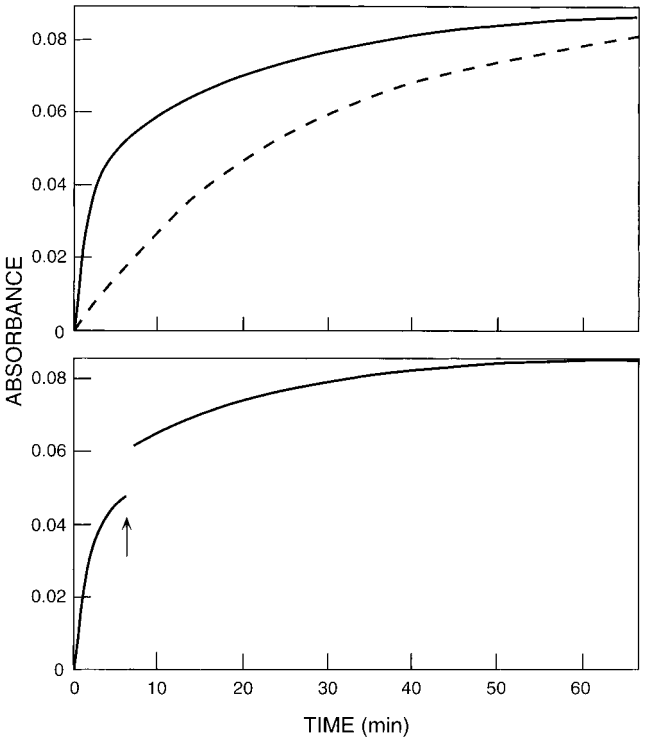
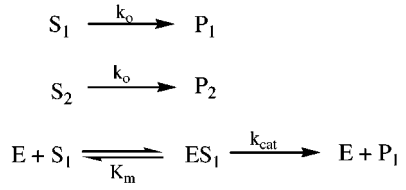
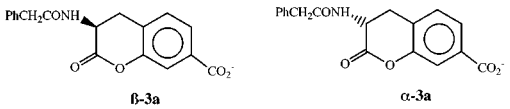


Figure 1. Upper panel: The hydrolysis of **3a** (0.07 mM) followed spectrophotometrically at 300 nm in the absence (dashed line) and presence (solid line) of the P99 β-lactamase (0.2 μM). Lower panel: The hydrolysis of **3a** (0.07 mM) in the presence of the P99 β-lactamase (0.2 μM), followed (arrow) by addition of the TEM-2 β-lactamase (2.4 μM).

Scheme 4



as spontaneous hydrolysis, was not. These data were fitted to Scheme 4, where S_1 and S_2 represent the enantiomers of **3a**, and P_1 and P_2 their corresponding hydrolysis products. The scheme assumes that the enantiomers of **3a**, β-**3a**, and α-**3a**, were present in the reaction mixture in equal amounts and hydrolyzed



ß-3a α-3a

spontaneously with the same rate constant, k_o. One of the enantiomers is also a β-lactamase substrate. It was also assumed that the nonreactive enantiomer was not a significant competitive inhibitor. The data for reaction of all β-lactamases with **3** fitted well, quantitatively, to Scheme 4, by methods described in the Experimental Section, leading to values of k_{cat} and K_m (Table 1) for the reactive enantiomer.

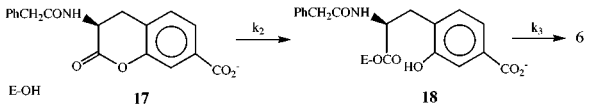
The TEM β-lactamase was shown to catalyze the hydrolysis of the same enantiomer of **3a** as did the P99 enzyme. Figure 1, lower panel, shows the effect of addition of the TEM enzyme after consumption by the P99 β-lactamase of its preferred enantiomer. The rate of

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

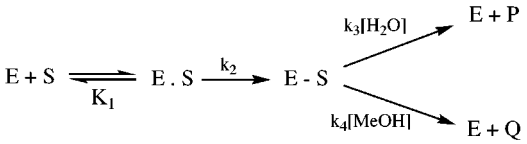
substrate		enzyme P99	enzyme TEM	enzyme PCl
3a	k_{cat} (s^{-1})	5.6 ± 0.4[e]	>1.2[f]	0.024 ± 0.002
	K_m (mM)[d]	0.29 ± 0.01	>1	0.015 ± 0.004
	k_{cat}/K_m ($s^{-1}M^{-1}$)	1.93×10^4	$(1.2 \pm 0.2) \times 10^3$	1.6×10^3
3b	k_{cat} (s^{-1})	0.40 ± 0.01	2.0 ± 0.2	>0.32[f]
	K_m (mM)[d]	0.031 ± 0.001	0.066 ± 0.002	>1
	k_{cat}/K_m ($s^{-1}M^{-1}$)	1.28×10^4	3.0×10^4	$(3.2 \pm 0.1) \times 10^2$
4	k_{cat} (s^{-1})	2.3 ± 0.1	>0.62[f]	–
	K_m (mM)[d]	0.10 ± 0.04	>1	–
	k_{cat}/K_m ($s^{-1}M^{-1}$)	2.3×10^4	$(6.2 \pm 0.1) \times 10^2$	–
5	k_{cat} (s^{-1})	7.3 ± 0.1	>2.7[f]	–
	K_m (mM)[d]	0.89 ± 0.01	>1	–
	k_{cat}/K_m ($s^{-1}M^{-1}$)	8.2×10^3	$(2.7 \pm 0.1) \times 10^3$	–
1[a]	k_{cat} (s^{-1})	125	25.4	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	$\geq 3 \times 10^6$
2[c]	k_{cat}/K_m ($s^{-1}M^{-1}$)	4.2×10^4	1.7×10^3	2.2×10^2

[a] The P99 and TEM data (20 mM MOPS, pH 7.5, 25 °C) are from ref 11. [b] Benzylpenicillin: The P99 data (0.1 M phosphate, pH 7.5, 25 °C) are from ref 4, the TEM data (0.1 M phosphate, pH 7.0, 30 °C) from ref 50, and the PC1 data (0.1 M phosphate, pH 7.5, 25 °C) from ref 5. [c] The data for this compound are from ref 12. [d] K_m values are calculated on the basis of the concentration of the reactive enantiomer, i.e., one-half of the total concentration. [e] Experimentally determined parameters from the present work are given as means with standard deviations. [f] Lower limits on K_m were taken to be the highest substrate concentrations employed.

Scheme 5



Scheme 6



the subsequent reaction is that of the spontaneous hydrolysis, indicating that the TEM β-lactamase also did not catalyze the hydrolysis of the remaining enantiomer to any significant extent. Qualitatively, **3b** behaved identically to **3a** in its interaction with the above enzymes.

The R61 DD-peptidase did not catalyze the hydrolysis of **3a** or **3b**, nor did these compounds inhibit the enzyme.

Reactions of β-Lactamases with the Potential Inhibitors, 4 and 5. Absorption spectra showed that the hydrolysis of one enantiomer of both **4** and **5** was also catalyzed by the P99 and TEM β-lactamases. Quantitative rate measurements led to the steady-state parameters of Table 1. Although the final sample of **4** contained ca. 15% of **5**, it is clear that both β-lactamases catalyzed the hydrolysis of **4** rather than only the contaminating **5** since the absorption amplitude of the enzyme-catalyzed reaction in each case corresponded to that expected for the hydrolysis of both components. These compounds did not appear to be particularly effective inhibitors however. For example, 1 mM **4** produced about 40% inhibition of the P99 β-lactamase (1.0 μM) in 3 h and a similar degree of inhibition of the TEM enzyme (2.0 μM) in 2 h; essentially all **4** would have disappeared from solution through a combination of enzyme-catalyzed and spontaneous hydrolysis in these time periods. The dilactone **5** was no more effective than **4** as an inhibitor of these enzymes.

***β*-Lactamase-Catalyzed Methanolysis and Aminolysis.** It is assumed that the hydrolysis of these δ-lactones by serine β-lactamases proceeds by way of a covalent acyl-enzyme intermediate (Scheme 5) in the same way as does the hydrolysis of β-lactams and acyclic depsipeptides.[5] Analysis of the steady-state parameters of Table 1 is therefore advanced by knowledge of whether k_{cat} represents the rate constant for acylation (k_2) or deacylation (k_3) of the enzyme. This can be conveniently determined for the P99 and PCl β-lactamases from the effect of alternative nucleophiles on k_{cat}. If deacylation were rate-determining at saturation, k_{cat} would be increased by the presence of alternative nucleophiles, provided, as is true for the above-mentioned enzymes, that the enzyme can generally accommodate these nucleophiles.[5,6] In the present case, methanol was observed to increase k_{cat} for the hydrolysis of **3a** by the P99 enzyme (data not shown) to an extent expected from a k_4/k_3 ratio (Scheme 6) of 29. This is very similar to the value of this parameter for **1**, viz. 27.[10] Methanol has no effect however on the value of k_{cat}/K_m, as expected for the mechanism of Scheme 6, and as is observed for **1**.[10] ^{1}H NMR spectra of a reaction mixture containing 3.0 M d_4-methanol showed approximately equal amounts of two products, one the hydrolysis product, the other, with a similar spectrum, presumably the methyl ester.

Methanol also accelerated turnover of **3a** by the PCl β-lactamase (data not shown). The effect on k_{cat} reflected a k_4/k_3 ratio of 6.7. The comparable value for **1** was determined from previous data[5] (when eq 1 was applied, see Experimental Section) to be 13.3. Methanol had no effect on turnover of **3a** by the TEM β-lactamase. That enzyme, however, has not previously been found to catalyze methanolysis of substrates.[5]

The rate of reaction of **3a** in the presence of the P99 β-lactamase was not increased by D-phenylalanine (up to 30 mM). No product other than that of hydrolysis was observed in the ^{1}H NMR spectra of reaction mixtures. This result contrasts sharply with the results of similar experiments with **1**, where the aminolysis by D-amino acids was observed to be efficiently catalyzed by this

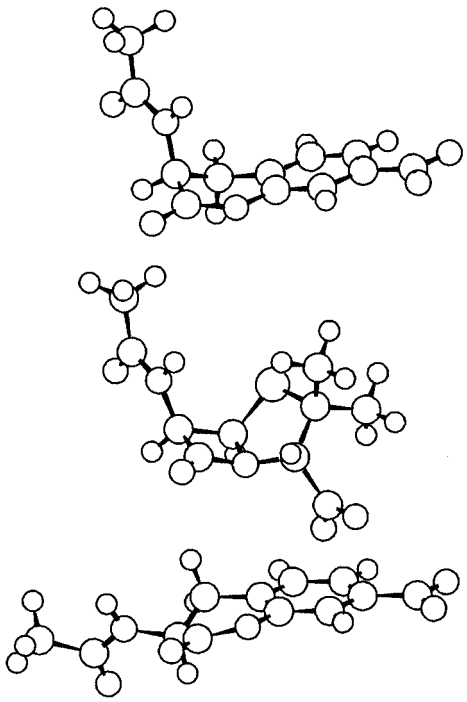


Figure 2. AM1 structures of, from the top, the methyl analogue of β-**3a** (axial 3-amido conformation), methyl penicillin, and the methyl analogue of β-**3a** (equatorial 3-amido conformation).

enzyme.[6] D-Phenylalanine also had no effect on the rate of turnover of **3a** by the TEM β-lactamase, a result in accord with precedent.[4]

Discussion

The cyclic esters **3**−**5** were significantly more labile in aqueous solution than their acyclic analogue **1** and the β-lactam antibiotic benzylpenicillin. This reflects the identity of the former compounds as δ-lactones, which are known to be particularly sensitive to nucleophilic cleavage.[26]

Despite the lability of **3**−**5** in aqueous solution, we were able to demonstrate that they were, in general, β-lactamase substrates. They seem to be the first-reported δ-lactones whose hydrolysis is catalyzed by these enzymes, although monocyclic β-lactones, direct analogues of monocyclic β-lactams, have been shown to be substrates.[27,28] Only one-half of each compound **3**−**5** was a β-lactamase substrate. We assume that this means that only one enantiomer—each compound is racemic at the 3-position—is a β-lactamase substrate. It would then seem likely, by analogy to the specificity of β-lactamases for β-lactams bearing β-amido substituents, that the reactive enantiomer was β-**3a**. Figure 2 shows AM1 energy-minimized structures of the acetamido analogues of β-**3a** along with that of methylpenicillin. Both axial and equatorial conformers of β-**3a**, which have very similar heats of formation at the AM1 level (the axial conformer is favored by 0.4 kcal/mol) are shown. Clearly the conformer with the amido substituent in the axial position most closely resembles the penicillin. Only the carboxyl group seems out of place. It has been previously observed, however, that the position of carboxyl substitution on acyclic carboxyphenyl phenaceturates does not have a very strong influence on the ability of these compounds to be β-lactamase substrates;[10] the position of the carboxyl group of **3** and **4**, when bound to the enzyme active site, would, however, be much more restricted than that of **1**.

It can be safely assumed from all precedent that the β-lactamase-catalyzed hydrolysis of **3**−**5** proceeds by way of the usual double displacement mechanism of serine β-lactamase catalysis (Scheme 5). Assuming the structural analogy with benzylpenicillin shown in Figure 2, it seems likely that the amido substituent of **3**, in both the Michaelis complex **17** and the acyl-enzyme intermediate **18**, would occupy the usual side-chain specificity pocket.[29,30] In the acyl-enzyme, the tethered leaving group may have, by virtue of the methylene group, not present in penicilloates, greater freedom of motion than in the latter, and therefore a greater opportunity to conform to the specificity of the active site.

In aqueous methanol solutions, the P99 and PC1 β-lactamases commonly catalyze methanolysis of substrates as well as hydrolysis, as shown in Scheme 6. Thus, methanol and water compete for acyl-enzyme E−S in the deacylation step of turnover.

As described in the Results section, the partition ratio k_4/k_3 for **3a** with both the above-mentioned β-lactamases is not greatly different from that for **1** with the same enzymes. This suggests that methanol experiences no greater a steric barrier (vs water) to nucleophilic attack on the acyl-enzyme of either the P99 or the PC1 β-lactamase generated from **3a** than from the acyclic analogue **1**. It should be noted that these enzymes represent a class C and a class A β-lactamase, respectively. These enzymes probably differ in the details of proton transfer during catalysis by virtue of different active site functional groups,[29,30] and perhaps also in the face of the carbonyl group of the acyl-enzyme to which the nucleophile approaches.[31,32] It is striking, however, that the P99 β-lactamase does not catalyze the aminolysis of **3a** by D-phenylalanine as it does, quite effectively, the aminolysis of **1**. On the other hand, the P99 enzyme does *not* catalyze the aminolysis of benzylpenicillin by D-phenylalanine.[33] The tethered leaving groups derived from **3a** and benzylpenicillin in their respective acyl-enzymes may impede approach of the bulky nucleophile D-phenylalanine.

These results therefore indicate that the δ-lactones **3**−**5** react with β-lactamases in a manner that has features of the reactivity of their acyclic analogue **1** on one hand, and of β-lactams on the other.

The rate constants of Table 1 merit some comment. First, there are some interesting differences between **3a** and **3b**. Deacylation (k_{cat}) of the P99 enzyme after its

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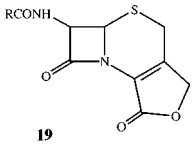
reaction with **3a** is more rapid than with **3b** while the opposite applies to the PC1 enzyme. Also remarkable too for the PC1 enzyme is the much less stable acyl-enzyme (K_m) from **3b** than from **3a** and the complementary higher deacylation rate constants (k_{cat}) for **3b**. The comparison of kinetic data between **3a** and its acyclic analogue **1** also raises interesting contrasts. Both the P99 and TEM enzymes prefer **1** to **3a**, but again PC1 differs. The latter result arises from a more stable acyl-enzyme formed from **3a** than from **1**. Apparently **3a** fits well into the PC1 active site at the acyl-enzyme stage. The significantly greater deacylation rate (k_{cat}) of **1** vs **3a** from the P99 active site may reflect steric hindrance by the tethered leaving group on the β-face of the acyl group. The PC1 deacylation rates (k_{cat}), where **1** and **3a** react equally well, indicate no hindrance to the occluded nucleophile on the α-face of the carbonyl group of the acyl-enzyme in a class A enzyme.

Acylation of all enzymes (k_{cat}/K_m) by benzylpenicillin is much more rapid than by **3a**. This presumably reflects the differences in structure between these substrates, and in particular perhaps in the different position of the carboxylate groups (Figure 2). The P99 and more particularly the PC1 enzyme also catalyze the hydrolysis of the acyl-enzyme derived from benzylpenicillin much more effectively than that from **3a**. The combination of a K_m of 15 μM and a k_{cat} of 0.024 s^{-1} (an acyl-enzyme half-life of 29 s) almost qualifies **3a** as an inhibitor of the PC1 β-lactamase. The tight binding of **3b** to the P99 enzyme is also noticeable.

Turnover rates of **4** and **5** (and **2**) by the P99 and TEM β-lactamases appear to be closely similar to that of **3a**. These compounds are therefore substrates to some degree, as required of mechanism-based inhibitors.[34] The substrate activity of the dilactone **5**, lacking a carboxylate, is of interest. A comparable situation would be that of cephalosporin lactone **19** which is a remarkably effective β-lactamase substrate.[35,36] Again the point is made that although a well-placed carboxylate can be very important in a β-lactamase substrate, it is certainly not essential.



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The absence of any reaction between the R61 DD-peptidase and the δ-lactones is very striking when compared with the rapid acylation ($k_2/K_s = 1070\ s^{-1}M^{-1}$)[9] of this enzyme by benzylpenicillin and by the acyclic depsipeptide **1** ($k_2/K_s = 3520\ s^{-1}\ M^{-1}$),[37] particularly when the strong similarity between the active sites of the DD-peptidase and the P99 β-lactamase is taken into account.[38] This difference presumably reflects a difficulty encountered by the more rigid (than **1**) and somewhat differently shaped (than penicillin: Figure 2) **3** in binding to the narrower, more rigid, and more specific active site cleft of the DD-peptidase. It is interesting to contrast this result with that obtained with *N*-(phenylacetyl)aziridine-2-carboxylate, a structurally closer analogue to penicillin.[39]

The extent of irreversible inhibition of β-lactamases caused by **4** and **5** was not significantly greater than that by the acyclic analogue **2**.[12] The modest inhibitory activity of the latter compound thus may have arisen as much from the absence of a suitable active site functional group in the vicinity of the electrophilic methylene (Scheme 1) as from rapid diffusion of the product or the quinone methide into solution. The lifetime of the acyl-enzyme formed by reaction between **4** and the P99 β-lactamase, ca. 0.4 s, should be long enough to allow the elimination reaction of Scheme 1 to occur and create the quinone methide at the active site.[40]

We have therefore shown that the δ-lactones **3**−**5** are β-lactamase but not DD-peptidase substrates. Further, in certain instances, quite inert acyl-enzymes were generated. Despite these acyl-enzyme lifetimes, the functionalized δ-lactones **4** and **5** were not significantly better irreversible inhibitors than the acyclic analogue **2**. A problem in the development of these compounds as enzyme inhibitors, either by way of their forming inert acyl-enzymes, or in more complex ways such as is indicated in Scheme 1, is their rapid spontaneous hydrolysis in neutral solution which competes very effectively with any reaction with an enzyme. Future inhibitor design based on these compounds would clearly require more hydrolytically stable species; more specific placing of the carboxylate group would also be worth further exploration.

Experimental Section

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

Analytical and Kinetic Methods. Stock solutions of the lactones were prepared in acetonitrile (Aldrich, Sureseal) rather than 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 acetonitrile concentration after addition of the substrate was 5% v/v. β-Lactamase reactions were monitored spectrophotometrically by means of a Hewlett-Packard HP 8452 spectrophotometer. Reactions of **3a**, **3b**, **4**, and **5** were monitored at 300 nm. Enzyme concentrations were 0.2 μM (P99, **3a**), 0.7 μM (P99, **4b** and **5**), 2 μM (P99, **3b**), 0.2 μM (TEM, **3b**), 2.65 μM (TEM, **3a**), 6.0 μM (TEM, **4** and **5**), and 2.65 μM (PC1, **3a** and **3b**). To obtain steady state parameters, total progress reaction curves were obtained at 0.5 mM and 1.0 mM substrate, and the data quantitatively fitted to Scheme 4 by means of the FITSIM program.[46]

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(40) There is only a limited amount of data in the literature on the rates of elimination of Br^- from *p*-(bromomethyl)phenols and on the lifetimes of quinone methides in aqueous solution. The information available[41−45] suggests that quinone methide formation may be faster than 2.3 s^{-1} (k_{cat} for **4** with the P99 β-lactamase) while its hydration is likely to be slower, i.e., the quinone methide should accumulate as an acyl-enzyme.
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The inhibitory activity of **4** and **5** against the β-lactamases was assessed by monitoring the loss of enzyme activity in incubation mixtures of enzyme and inhibitor; small aliquots were removed at appropriate times and assayed (initial rates) against benzylpenicillin. The activity of the R61 DD-peptidase in the presence of **4** and **5** was determined spectrophotometrically by means of the substrate *N*-(phenylacetyl)glycyl-D-thiollactate/4,4′-dipyridyl disulfide.[19]

To study the effects of methanol on the steady-state parameters, the latter were determined in the presence of methanol (0−3.0 M) as described above, and the partition ratio, k_4/k_3 (Scheme 6), obtained from eq 1 where k^{M}_{cat} is the observed k_{cat} in methanol solution, k^{o}_{cat} the value in the absence of

$$k^{M}_{cat} = k^{o}_{cat}\{1 + k_4[MeOH]/k_3(55.56 - 2.25[MeOH])\} \quad (1)$$

methanol, 55.56 M is taken as the concentration of water in the absence of methanol, and 2.25 is the ratio of molar volumes of methanol and water at 25 °C.

To obtain the structures of Figure 2, molecules were constructed by means of the Builder Module of INSIGHT II, version 95.0 (Biosym/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. Analytical thin-layer chromatography (TLC) and preparative column chromatography were performed on Kieselgel F 254 and on Kieselgel 60 (0.063−0.200 mm), respectively. Unless otherwise stated, the eluent used was the same for TLC and for chromatography purification.

Methyl 3-Acetoxy-4-(bromomethyl)benzoate (7b). A mixture of of methyl 3-acetoxy-4-methyl benzoate (1.57 g, 7.55 mmol), NBS (1.48 g, 8.30 mmol) and benzoic peroxide (50 mg), in CCl_4(30 mL) was refluxed for 3h under argon. The reaction mixture was cooled to room temperature and filtered. The filtrate was evaporated and the residue was purified by chromatography (pentane/AcOEt: 9/1), affording product **7b** (1.39 g, 64%): R_f 0.24. mp 111 °C. ^{1}H NMR ($CDCl_3$) δ 2.40 (s, 3H), 3.92 (s, 3H), 4.43 (s, 2H), 7.50 (d, 1H), 7.80 (d, 1H), 7.89 (dd, 1H). ^{13}C NMR ($CDCl_3$) δ 20.92, 26.70, 52.61, 124.59, 127.59, 131.05, 131.95, 134.71, 149.00, 165.90, 168.87. Anal. Calcd for $C_{11}H_{11}O_4Br$: C, 46.01; H, 3.86. Found: C, 46.31; H, 4.01.

Diethyl (2-Acetoxy-4-(t-butoxycarbonyl)benzyl)phenylacetamidomalonate (8a). To a solution of 205 mg (0.7 mmol) of diethyl phenylacetamidomalonate **6a**[19] in dry DMF (3 mL) was added 28 mg (0.7 mmol) of NaH (60% in oil). The mixture was allowed to react for 15 min. Then a solution of 223 mg (0.66 mmol) of *tert*-butyl 3-acetoxy-4-(bromomethyl)benzoate **7a**[12] in THF (2 mL) was added. After being stirred for 30 min, the mixture was poured into a 10% HCl solution and extracted with ethyl acetate. The ethyl acetate solution was dried over Na_2SO_4 and the solvent evaporated. The residue was purified by column chromatography (dichloromethane/methanol: 97.5/2.5), affording 348 mg (95%) of a glassy product: R_f 0.59. ^{1}H NMR ($CDCl_3$) δ 1.26 (t, 7.0 Hz, 6H), 1.59 (s, 9H), 2.23 (s, 3H), 3.54 (s, 2H), 3.58 (s, 2H), 4.23 (q, 7.0 Hz, 4H), 6.46 (s, 1H), 6.93 (d, 8.0 Hz, 1H), 7.58 (d, 1.6 Hz, 1H), 7.67 (dd, 1H). ^{13}C NMR ($CDCl_3$) δ 20.4, 28.0, 32.3, 43.2, 66.4, 81.2, 116.8−136.4, 149.7, 166.9−170.0. Anal. Calcd for $C_{29}H_{35}NO_9$: C, 64.31; H, 6.51; N, 2.59. Found: C, 64.22; H, 6.32; N, 2.62.

Diethyl (2-Acetoxy-4-(methoxycarbonyl)benzyl)benzamidomalonate (8b). Compound **8b** was prepared in 79% yield in the same manner as compound **8a**, using, however, diethyl benzamidomalonate instead of diethyl phenylacetamidomalonate as starting material. R_f 0.32 (dichloromethane/methanol: 99/1). ^{1}H NMR ($CDCl_3$) δ 1.27 (t, 7.0 Hz, 6H), 2.05 (s, 3H), 3.70 (s, 2H), 3.86 (s, 3H), 3.87−4.41 (m, 4H), 7.14−7.53 (m, 9H). ^{13}C NMR ($CDCl_3$) δ 14.2, 20.6, 32.7, 52.3, 63.1, 67.2, 123.8−136.0, 150.1; 165.1, 166.9, 167.7, 169.7. Anal. Calcd for $C_{25}H_{27}NO_9$: C, 61.85; H, 5.61; N, 2.89. Found: C, 61.82; H, 5.48; N, 2.71.

7-Carboxy-3-(phenylacetamido)-3,4-dihydro-2*H*-1-benzopyran-2-one (3a). Malonate **8a** (130 mg, 0.24 mmol) was dissolved in ethanol (5 mL), 20% sodium hydroxide (5 mL) added, and the mixture stirred at room temperature for 6 h. The ethanol was evaporated, and the aqueous solution was washed with diethyl ether, acidified with 50% hydrochloric acid, and extracted with ethyl acetate. Evaporation of the solvent gave a product which was stirred in a solution of TFA (1 mL) in CH_2Cl_2 (1 mL) for 2 h. The solution was then evaporated to dryness, and the residue containing the substituted malonic acid **9a** was heated for a few minutes at 180 °C for decarboxylation. The saponification reaction was incomplete (presence of ethyl groups in the NMR spectrum) and was therefore repeated. Thus, the product was dissolved in a mixture of ethanol (5 mL) and 20% sodium hydroxide (5 mL) and stirred at room temperature for 2 h. Evaporation of the ethanol, acidification with hydrochloric acid, extraction with ethyl acetate, and evaporation of the solvent gave the racemic *N*-(phenylacetyl)-4-carboxy-2-hydroxyphenylalanine **10a**. ^{1}H NMR (CD_3OD) δ 2.91 (dd, 10.0 and 13.5 Hz, 1H), 3.32 (dd, 4.7 and 13.5 Hz, 1H), 3.44 (s, 2H), 4.78 (dd, 4.7 and 10.0 Hz, 1H), 7.05−7.39 (m, 8H). This acid was heated at 180 °C for 5 min in a GKR 50 Büchi apparatus to give the lactone **3a** (66 mg, 85%) which crystallized from acetone: mp 246−248 °C. R_f 0.50 (ethyl acetate/methanol: 7/3). ^{1}H NMR (CD_3COCD_3) δ 3.32 (dd, 12.8 and 15.6 Hz, 1H), 3.39 (dd, 7.5 and 15.6 Hz, 1H), 3.71 (s, 2H), 4.97 (td, 7.8 and 12.8 Hz, 1H), 7.49 (d, 7.8 Hz, 1H), 7.66 (d, 1.4 Hz, 1H), 7.84 (dd, 1H), 7.93 (d, 7.8 Hz, 1H). ^{13}C NMR (CD_3COCD_3) δ 32.2, 42.2, 48.1, 116.8−136.4, 152.0, 166.6, 167.6, 171.2. MS (ES, 30V) *m*/*z* 348 (MNa^+); 326 (MH^+), 208 (MH^+ − PhCH=C=O). Anal. Calcd for $C_{18}H_{15}NO_5$: C, 66.45; H, 4.65; N, 4.30. Found: C, 66.36; H, 4.76; N, 4.14.

7-Carboxy-3-benzamido-3,4-dihydro-2*H*-1-benzopyran-2-one (3b). Compound **3b** was prepared by the same experimental procedure as employed for compound **3a**. First, racemic *N*-benzoyl-4-carboxy-2-hydroxyphenylalanine **10b** (85%) was obtained. ^{1}H NMR (CD_3COCD_3) δ 3.21 (dd, 13.7 and 9.5 Hz, 1H), 3.44 (dd, 13.7 and 4.9 Hz, 1H), 4.97 (m, 1H), 7.37−7.83 (m, 9H). This acid was heated at 200 °C for 40 min in a GKR 50 Büchi apparatus to give the lactone **3b**: mp 258 °C (dec). R_f 0.62 (ethyl acetate/methanol: 7/3). ^{1}H NMR (CD_3COCD_3) δ 3.46 (m, 2H), 5.19 (m, 1H), 7.42−7.57(m, 8H). ^{13}C NMR (CD_3SOCD_3) δ 29.2, 47.0, 116.8−133.5, 150.8, 166.3, 166.4, 167.2. Anal. Calcd for $C_{17}H_{13}NO_5$, 0.25 H_2O: C, 61.20; H, 4.67; N, 4.20. Found: C, 61.27; H, 4.69; N, 4.29.

Methyl 2,4-Dimethyl-5-acetoxybenzoate (11). 2,4-Dimethylbenzoic acid (485 mg, 3.23 mmol) was dissolved in concentrated H_2SO_4 (2 mL). The solution was cooled to 0 °C and stirred. Then, a mixture of concentrated sulfuric acid (1 mL) and concentrated nitric acid (1 mL) was added gradually. Stirring was continued for 1 h, the mixture was poured on ice, and the solid precipitate was filtered, washed with water, and dried. The nitration reaction gave a mixture of 2,4-dimethyl-5-nitro-, 2,4-dimethyl-3-nitro-, and 2,4-dimethyl-3,5-dinitrobenzoic acid. A first purification was performed by column chromatography (dichloromethane/methanol: 95/5). Then, crystallization from ethanol gave the pure 2,4-dimethyl-5-nitrobenzoic acid (236 mg, 35%): mp 196 °C (lit.[47] mp 197.5−198.5 °C). R_f 0.28. ^{1}H NMR ($CDCl_3$) δ 2.54 (s, 3H), 2.59 (s, 3H), 7.31 (s, 1H), 8.47 (s, 1H).

A solution of this acid (310 mg, 0.63 mmol) in methanol (10 mL) and 3 drops of concentrated sulfuric acid was refluxed overnight. The solvent was evaporated and the product purified by chromatography (pentane/ether: 3/1), affording 315 mg (95%) of methyl 2,4-dimethyl-5-nitrobenzoate: R_f 0.69. ^{1}HNMR ($CDCl_3$) δ 2.64 (s, 3H), 2.66 (s, 3H), 3.93 (s, 3H), 7.24 (s, 1H), 8.61 (s, 1H).

The nitrobenzoate (265 mg, 1.27 mmol) was dissolved in absolute ethanol (10 mL). Cyclohexene (1 mL) and 10% Pd−C

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(20 mg) were added, and the mixture was refluxed vigorously for 16 h. After filtration and washing of the catalyst with ethanol, the solution was evaporated and the product purified by column chromatography (pentane/ether: 1/1), affording 193 mg (85%) of methyl 2,4-dimethyl-5-aminobenzoate:[48] R_f 0.55. mp 68 °C. ^{1}H NMR ($CDCl_3$) δ 2.17 (s, 3H), 2.46 (s, 3H), 3.85 (s, 3H), 6.92 (s, 1H), 7.26 (s, 1H).

A mixture of 112 mg (0.63 mmol) of the aminobenzoate and 20% sulfuric acid (4 mL) was treated at 0 °C with a solution of 53 mg of sodium nitrite in water (2 mL). To the resulting solution of the diazonium salt, concentrated sulfuric acid (4 mL) was added slowly with an efficient cooling. The final solution was added to boiling 50% sulfuric acid (20 mL). The temperature was maintained at 100 °C for 5 min then the solution was poured on ice and extracted with ethyl acetate. After being dried over sodium sulfate, the solvent was evaporated and the product purified by chromatography (dichloromethane/methanol: 9/1) to give the 2,4-dimethyl-5-hydroxybenzoic acid (89 mg, 86%): mp 185 °C (lit.[49] mp 187−188 °C). R_f 0.32. ^{1}H NMR (CD_3OD) δ 2.25 (s, 3H), 2.50 (s, 3H), 7.01 (s, 1H), 7.38 (s, 1H).

This acid (564 mg, 3.40 mmol) was esterified by refluxing in methanol (20 mL) with sulfuric acid as catalyst to give methyl 2,4-dimethyl-5-hydroxybenzoate (522 mg, 86%): mp 89−90 °C (lit.[49] mp 88−89 °C). R_f 0.50 (pentane/ether: 1/1). ^{1}H NMR ($CDCl_3$) δ 2.26 (s, 3H), 2.50 (s, 3H), 3.88 (s, 3H), 7.27 (s, 1H), 7.40 (s, 1H).

A solution of 198 mg (1.1 mmol) of the hydroxybenzoate in a mixture of acetic anhydride (0.12 mL, 1.2 mmol), triethylamine (0.17 mL, 1.2 mmol), DMAP (20 mg), and dichloromethane (10 mL) was stirred at room temperature for 2 h. The reaction was quenched with methanol (0.5 mL), and the mixture was washed twice with water, dried over sodium sulfate, and evaporated. The product was purified by chromatography (dichloromethane), affording 225 mg (92%) of methyl 2,4-dimethyl-5-acetoxybenzoate **11**: R_f 0.33. mp 54 °C. ^{1}H NMR ($CDCl_3$) δ 2.18 (s, 3H), 2.56 (s, 3H), 2.32 (s, 3H), 3.86 (s, 3H), 7.11 (s, 1H), 7.61 (s, 1H). Anal. Calcd for $C_{12}H_{14}O_4$: C, 64.85; H, 6.35. Found: C, 64.81; H, 6.34.

Methyl 2,4-Bis-(bromomethyl)-5-acetoxybenzoate (12). A solution of 605 mg (2.72 mmol) of acetoxybenzoate **11** in carbon tetrachloride (15 mL) was refluxed for 1 h in the presence of *N*-bromosuccinimide (1.07 g, 6.0 mmol) and benzoyl peroxide (20 mg). After removal of the succinimide by filtration, the solvent was evaporated and the product purified by column chromatography (dichloromethane), giving 385 mg (38%) of methyl 2,4-bis-(bromomethyl)-5-acetoxybenzoate **12** which was used directly in the following step. R_f 0.68. ^{1}H NMR ($CDCl_3$) δ 2.40 (s, 3H), 3.94 (s, 3H), 4.39 (s, 2H), 4.93 (s, 2H), 7.53 (s, 1H), 7.77 (s, 1H).

6-Acetoxy-5-(bromomethyl)-3*H*-isobenzofuran-1-one (13). A flask containing the methyl 2,4-bis-(bromomethyl)-5-acetoxybenzoate **12** (85 mg, 0.22 mmol) was immersed in an oil bath at 160−180 °C for 15 min. The cyclized product was purified by chromatography (dichloromethane/methanol: 99/1). A yield of 61 mg (95%) of 6-acetoxy-5-(bromomethyl)-phthalide **13** was obtained as a powder: R_f 0.21 (dichloromethane). mp 152 °C. ^{1}H NMR ($CDCl_3$) δ 2.43 (s, 3H), 4.48 (s, 2H), 5.27 (s, 2H), 7.57 (s, 1H), 7.70 (s, 1H). ^{13}C NMR ($CDCl_3$) δ 21.0, 26.6, 69.4, 120.3−149.6, 168.5, 169.7. Anal. Calcd for $C_{11}H_9O_4Br$: C, 46.34; H, 3.18; O, 22.45. Found: C, 46.35; H, 3.36; O, 22.28.

5-(2,2-(Bis(ethoxycarbonyl)-2-phenylacetamido)ethyl-6-acetoxy-3*H*-isobenzofuran-1-one (14). To a solution of 120 mg (0.42 mmol) of (bromomethyl)phthalide **13** in THF (5 mL) was added a mixture prepared by adding 17 mg (0.42 mmol) of NaH (60% in oil) to a solution of 124 mg (0.42 mmol) of diethyl phenylacetamidomalonate **6a** in dry DMF (3 mL) and stirring the mixture for 45 min. The whole mixture was then poured into a solution of 10% HCl and extracted with ethyl acetate. The organic layer was washed with water, dried over Na_2SO_4, and then evaporated. The residue was purified by chromatography (pentane/ethyl acetate: 1/1), affording 151 mg (72%) of the 5-(2,2-bis(ethoxycarbonyl)-2-phenylacetamido)-ethyl-6-acetoxyphthalide **14**: R_f 0.52. mp 73 °C. ^{1}H NMR ($CDCl_3$) δ 1.24 (t, 7.0 Hz, 6H), 2.27 (s, 3H), 3.54 (s, 2H), 3.66 (s, 2H), 4.18 (q, 7.0 Hz, 4H), 5.13 (s, 1H), 6.53 (s, 1H), 7.20 (s, 1H), 7.54 (s, 1H). ^{13}C NMR ($CDCl_3$) δ 20.7, 33.0, 43.5; 66.4, 69.3; 119.7−143.4, 150.6, 167.1−170.3. Anal. Calcd for $C_{26}H_{27}NO_9$: C, 62.76; H, 5.47; N, 2.87. Found: C, 62.66; H, 5.51; N, 2.76.

3,4-Dihydro-3-(phenylacetamido)2,5-dihydro-2-oxofuro-[3,4-d]-2*H*-1-benzopyran-2-one (*5*). To a solution of malonate **14** (114 mg, 0.23 mmol) in ethanol (10 mL) was added 20% sodium hydroxide (10 mL), and the mixture was stirred at room temperature for 16 h. The ethanol was evaporated, and the aqueous solution was washed with diethyl ether and then acidified with 50% hydrochloric acid. Extraction of the aqueous solution with ethyl acetate and then evaporation of the solvent gave a product which was heated for a few minutes at 180 °C for decarboxylation. The product was again dissolved in a mixture of ethanol (5 mL) and 20% sodium hydroxide (5 mL) and stirred at room temperature for 2 h. Evaporation of the ethanol, acidification with hydrochloric acid, extraction with ethyl acetate, and evaporation of the solvent gave a mixture of acid **10c** and phthalide **15**. Compound **10c**: ^{1}H NMR (CD_3COCD_3) δ 3.02 (dd, 7.0 and 12.6 Hz, 1H), 3.29 (dd, 4.6 and 13.6 Hz, 1H), 3.51 (s, 2H), 4.74 (s, 2H), 4.80 (m, 1H), 7.17−7.38 (m,7H), 7.42 (d, 7.7 Hz, 1H). Compound **15**: ^{1}H NMR (CD_3COCD_3) δ 3.05 (dd, 7.0 and 13.8 Hz, 1H), 3.30 (dd, 5.2 and 13.8 Hz, 1H), 3.48 (s, 2H), 4.85 (m, 1H), 5.14 (s, 2H), 7.17 to 7.29 (m, 7H), 7.80 (d, 7.8 Hz, 1H). This mixture was heated at 180 °C for 15 min to give the dilactone **5** (64 mg, 83%). ^{1}H NMR (CD_3COCD_3) δ 3.34 (dd, 12.4 and 15.8 Hz, 1H), 3.41 (dd, 7.6 and 15.8 Hz, 1H), 3.66 (s, 2H), 4.94 (dt, 7.7 and 12.5 Hz, 1H), 5.37 (s, 2H), 7.44 (s, 1H), 7.62 (s, 1H), 7.82 (d, 7.7 Hz, 1H).^{13}C NMR (CD_3COCD_3) δ 31.6, 43.6, 48.2, 70.5, 123.7−143.7, 167.4−171.6. Anal. Calcd for $C_{19}H_{15}NO_5$, 0.25 H_2O: C, 66.75; H, 4.57; N, 4.10. Found: C, 66.69; H, 4.63; N, 4.11. MS (ES, 70 V) *m*/*z* 360 (MNa^+), 338 (MH^+), 242, (MHNa − $PhCH_2CO^+$), 142 ($PhCH_2CONa^+$), 120 ($PhCH_2COH^+$).

6-(Bromomethyl)-7-carboxy-3-(phenylacetamido)-3,4-dihydro-2*H*-1-benzopyran-2-one (4). To a solution of 40 mg (0.12 mmol) of dilactone **5** in acetonitrile (2 mL) were added 40 mg (0.37 mmol) of sodium bromide and 3 mg of tetrabutylammonium bromide. The mixture was cooled to 0 °C in an ice bath under a dry argon atmosphere before the addition of a solution of boron tribromide in dichloromethane (1 mL, 1 mmol). The mixture was stirred for 24 h at room-temperature The reaction mixture was quenched with ice/water, and dichloromethane was added. The dichloromethane layer was washed with water and dried over sodium sulfate. Evaporation of the solvent left a pale yellow solid (35 mg). The NMR spectra showed the presence of a mixture of the bromo derivative **4** (85% yield) and the starting dilactone **5** (15% yield). Compound **4**: ^{1}H NMR (CD_3COCD_3) δ 3.37−3.51(m, 2H), 3.66 (s, 2H), 4.90−4.99 (m, 1H), 5.06 (s, 2H), 7.51 (s, 1H), 7.60 (s, 1H), 7.87 (d, 7.4 Hz, 1H). ^{13}C NMR (CD_3COCD_3) δ 30.4, 31.5, 43.2, 47.9, 119.8−136.7, 167.5−172.9. MS (ES, 70 V) *m*/*z* 417 and 419; 416 to 420 (MH^+).

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