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Synthesis and β-lactamase reactivity of α-substituted phenaceturates

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Abstract—β-Lactams with 6α (penicillins) or 7α (cephalosporins) substituents are often β-lactamase inhibitors. This paper assesses the effect of such substituents on acyclic β-lactamase substrates. Thus, a series of *m*-carboxyphenyl phenaceturates, substituted at the glycyl α-carbon by –OMe, $-CH_2OH$, $-CO_2^-$, and $-CH_2NH_3^+$, have been prepared, and tested for their reactivity against serine β-lactamases. The latter two are novel substituents in β-lactamase substrates. The methoxy and hydroxymethyl compounds were found to be poor to moderately good substrates, depending on the enzyme. The aminomethyl compound gave rise to a transiently stable ($t_{1/2} = 4.6$ s) complex on its reaction with a class C β-lactamase. The reactivity of the compounds against three low molecular weight DD-peptidases was also tested. Again, the methoxy and hydroxymethyl compounds proved to be quite good substrates with no sign of inhibitory complexes. The DD-peptidases reacted with one enantiomer (the compounds were prepared as racemates), presumably the D compound. The class C β-lactamase reacted with both D and L enantiomers although it preferred the latter. The structural bases of these stereo-preferences were explored by reference to the crystal structure of the enzyme by molecular modeling studies. The aminomethyl compound was unreactive with the DD-peptidases, whereas the carboxy compound did not react with any of the above-mentioned enzymes. The inhibitory effects of the –OMe and $-CH_2OH$ substituents in β-lactams apparently require a combination of the substituent and the pendant leaving group of the β-lactam at the acyl-enzyme stage.

1. Introduction

The major source of natural resistance to β-lactam antibiotics arises from bacterial production of β-lactamases, enzymes that hydrolytically destroy the antibiotic molecules.[1] β-Lactams that are not thus destroyed have a good chance of inhibiting D-alanyl-D-alanine (DD-) peptidases, which leads to cessation of bacterial cell wall biosynthesis and ultimately to bacterial cell death. The resistance provided by β-lactamases may be overcome by β-lactams that are poor β-lactamase substrates or by application of β-lactamase inhibitors. The latter molecules may also be antibiotics in their own right or be supplied as an appropriate mixture with an effective β-lactam.

With few exceptions, the potent β-lactamase inhibitors known to date interact covalently with β-lactamases, either as mechanism-based inhibitors or as transition state analogues.[2] Examples of the former include the commercially available β-lactamase inhibitors, clavulanic acid, sulbactam, and tazobactam, which as β-lactams themselves react initially with the enzymes as normal substrates to form an acyl-enzyme intermediate. Diversion of this initially formed acyl-enzyme into chemically and/or conformationally inert forms then leads to enzyme inhibition. The best known transition state analogue-generating molecules are the phosphonates and boronates.[2] Since the chemical transition states of the normal reaction are covalently bound to the enzyme, so too are good transition state analogues.

A variety of β-lactams, other than those mentioned above, are also known to form slowly hydrolyzing acyl-enzymes with particular β-lactamases. These are

Keywords: β-Lactamase; DD-peptidase; Bacterial enzymes; β-Lactam antibiotics; Steady state enzyme kinetics; Depsipeptide.
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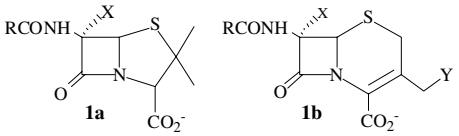
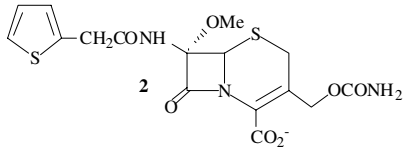
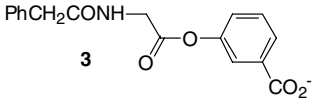
often referred to as inhibitory substrates since they interact with the enzyme as substrates but turn over slowly. One particular group of these has methoxy or hydroxymethyl substituents α to the β-lactam ring at the C-6 (penicillin) or C-7 (cephalosporin) position (**1a** and **1b**, respectively).



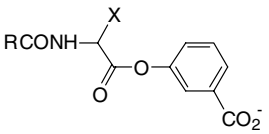
The first characterized of these were natural products, the cephamycins (**1b**, X = OMe).[3] A semisynthetic variant is the well-known cefoxitin **2**[4] which is still in clinical use as an injectable β-lactam (indicated, for example, for treatment of lower respiratory tract, urinary tract, and intra-abdominal infections). It has been shown to form transiently stable complexes with class A[5,6] and class C[7] β-lactamases. Temocillin, a 6-α-methoxypenicillin (**1a**, X = OMe), also forms inert complexes with β-lactamases.[5,8] Similarly, α-hydroxyethyl β-lactams are known to be more stable toward β-lactamases.[9,10]



For several years, we have used acylic depsipeptides such as **3** as convenient substrates of both β-lactamases and DD-peptidases.[11,12] These compounds combine the amido side chain of good β-lactamase and DD-peptidase substrates, a hydrolytically susceptible/torsionally flexible ester linkage,[12] and a carboxylate in the leaving group well placed to interact with polar residues of the active sites of these enzymes.[13]



In view of the above, we were interested to find whether the α-substituents of **1** would have inhibitory effects on the turnover of **3** by a variety of serine β-lactamases of class A and C, and by some representative low molecular weight DD-peptidases. Consequently, we have prepared **4**–**7** and studied the kinetics of their reaction with these enzymes. β-Lactams **1a** and **1b**, analogous to **6** and **7**, have not been prepared, to our knowledge. We hoped, however, that the $-CH_2NH_3^+$ and $-CO_2^-$ substituents in these molecules would interact with the polar residues of the β-lactamase active site or with hydrolytic water molecules and thereby disrupt catalysis.



4 R = $PhCH_2$, X = OMe

5 R = $PhCH_2$, X = CH_2OH

6 R = $PhCH_2$, X = $CH_2NH_3^+$

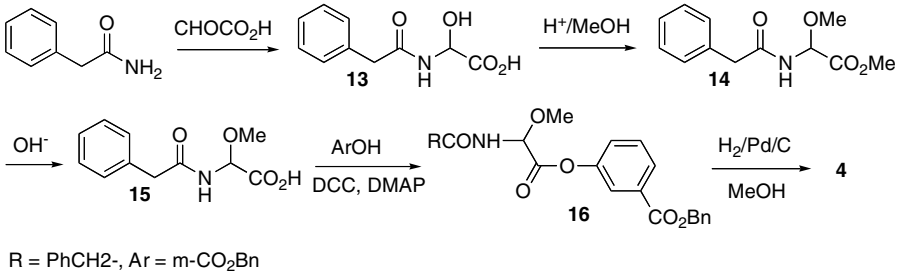
7 R = Ph, X = CO_2^-

Although many of these compounds were substrates of representative β-lactamases and DD-peptidases, no strong inhibitory complexes were detected. The structural basis of this result is discussed.

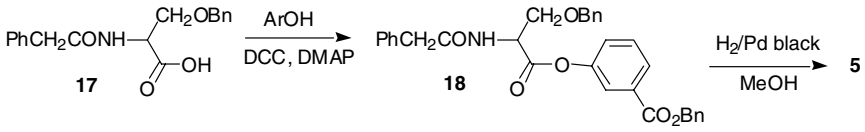
2. Results and discussion

The synthetic routes employed to obtain **4**–**7** are presented in outline in Schemes 1–4. The synthesis of **4** (Scheme 1) involved condensation of phenylacetamide with glyoxylic acid to form the α-hydroxyglycine derivative **13** which was converted to the α-methoxy analogue **14**, both steps by means of previously reported procedures.[14,15] Alkaline hydrolysis of **14**[16] yielded the corresponding acid **15** which was condensed with benzyl 3-hydroxybenzoate[17] to form **16**, the benzyl ester of **4**. Catalytic hydrogenation[12] of **16** then afforded the required product **4**. The synthesis of **5** (Scheme 2) required condensation of *O*-benzyl-*N*-(phenylacetyl)serine with benzyl 3-hydroxybenzoate, followed by catalytic hydrogenation. Preparation of **6** involved a similar sequence (Scheme 3). Only the final hydrogenation step of and subsequent isolation of **6** proved difficult. This compound was found to decompose readily during manipulation and could only be isolated in pure form as a zwitterion after hydrogenation under weakly acidic conditions.

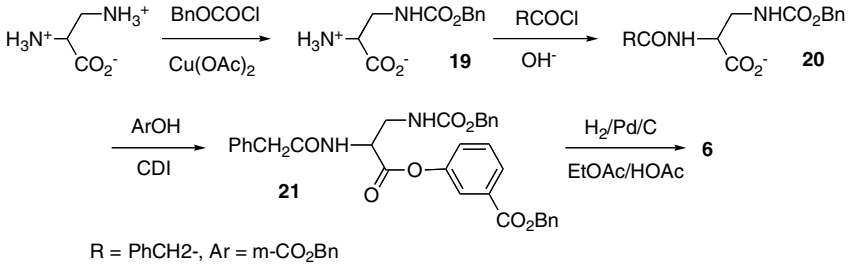
The preparation of **7** was a considerable challenge. The direct condensation of a malonic acid monoester with benzyl 3-hydroxybenzoate by means of DCC, DCC/DMAP or DCC/*p*-TSA/pyridine[18,19] failed. This was probably due to the poor reactivity of the phenol with the intermediate 5-(4*H*)-oxazolone, which exists predominantly in the enol form.[20] We therefore turned our attention to another route to the target molecule **7**: acylation of a hippurate dianion[21] with the chloroformate derived from benzyl 3-hydroxybenzoate (Scheme 4). To avoid the observed hippurate N-aryloxycarbonylation, we prepared an N-alkylated derivative having an acid-labile 2,4-dimethoxybenzyl substituent.[22] Thus, reductive alkylation ($NaBH_3CN$) of benzyl glycinate with 2,4-dimethoxybenzaldehyde gave the known[23] *N*-2,4-dimethoxybenzyl glycinate **22**. Acylation of **22** then furnished the required substituted hippurate **23**. Formation of the anion of **23** (LiHMDS) and its reaction with the chloroformate obtained by treatment of benzyl 3-hydroxybenzoate with triphosgene[24] led to the malonate **24**. Selective removal of the *N*-2,4-dimethoxybenzyl-protecting group



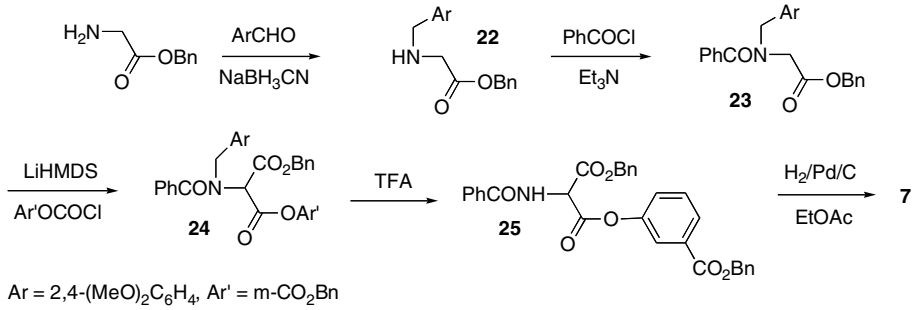
Scheme 1.



Scheme 2.



Scheme 3.



Scheme 4.

with trifluoroacetic acid afforded the dibenzyl ester **25**. Simultaneous hydrogenolysis of both remaining benzyl ester protecting groups gave the target molecule **7**. This malonic half ester, however, was not very stable and readily decarboxylated at room temperature to give the unsubstituted hippurate. The malonate anion, however, was more stable, thus enabling the kinetics studies described below.

The background hydrolysis rates of **4**–**7** were determined to establish the baseline against which to measure the enzyme-catalyzed reaction rates. In 100 mM MOPS, pH 7.5, 25 °C, the pseudo-first order rate constants for hydrolysis of **4**–**7** were 2.5×10^{-4}, 4.2×10^{-5}, 6.5×10^{-4}, and $1.7 \times 10^{-4}\ s^{-1}$, respectively. These appear to reflect the expected electronic effects of the substituents, although the value for **7** may also include a small contribution from intramolecular general base catalysis.

The hydrolyses of **4** and **5** were obviously enhanced and thus catalyzed by the class C P99 β-lactamase. The total progress curve for reaction of **4** was biphasic (Fig. 1), where both phases were faster than the background rate. The syntheses of both **4** and **5** yielded racemic products. Thus, it was evident that the enzyme catalyzed the hydrolysis of both the D and L-enantiomers of **4**. The rates of the two phases were sufficiently different that initial rates of each phase could be obtained. Thus, steady state rate parameters for each phase were obtained (Table 1). The less reactive enantiomer was assigned the D-configuration since this was the form that reacted with DD-peptidases (see below). Compound **5** was a poorer substrate than **4**

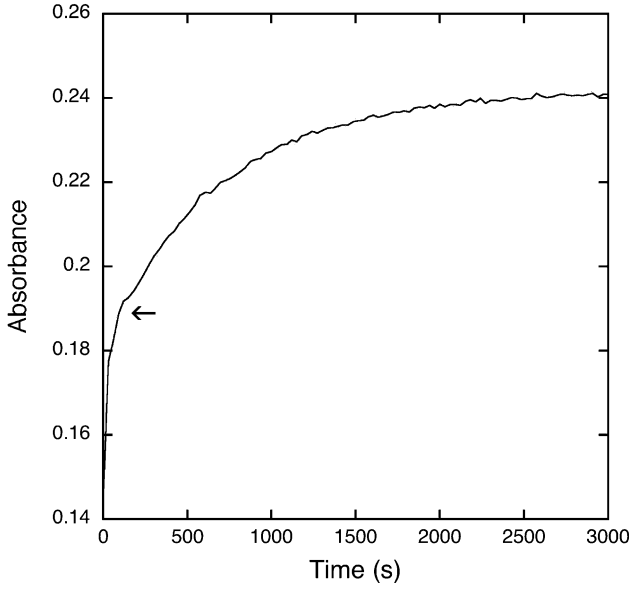
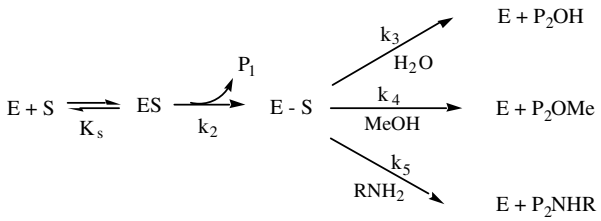


Figure 1. The hydrolysis of L-**4** (faster phase) and D-**4** (slower phase), each 0.1 mM, monitored spectrophotometrically at 290 nm and catalyzed by the P99 β-lactamase (0.2 μM). The arrow indicates the break between phases. In the absence of enzyme, **4** would have a half-life of 2800 s under these conditions.

but here also two phases, corresponding to the two enantiomers, could be distinguished (Table 1).

Comparison of the steady state parameters of **4** with those of the unsubstituted analogue **3** shows that the L-enantiomer of the former is comparable to **3** as a substrate of the P99 β-lactamase. Enzyme deacylation is known to be rate-determining under substrate saturation conditions for **3**,[12,25] that is, $k_{cat} = k_3$ (Scheme 5).

This was also found to be true for both L-**4** and D-**4** by application of the alternative nucleophiles methanol and D-phenylalanine at high substrate ($>K_m$) conditions.[25,26] Both of these nucleophiles enhanced the observed rate of reaction under these conditions, leading to the conclusion that $k_{cat} = k_3$ for **4** also. The partition ratios



Scheme 5.

k_4/k_3 of L-**4** and D-**4**, determined from kinetics experiments as described in Section 4, were comparable to that of **3** (Table 2), indicating that methanol had access to the acyl-enzyme comparable to that of water. The larger nucleophile D-Phe had much less access to the acyl-enzyme, with respect to water, for both L-**4** and D-**4** than for **3** (see k_5/k_3 values, Table 2). This presumably reflects the greater steric hindrance to nucleophile attack, and/or conformational rigidity leading to the same, in the former acyl-enzymes (see below for discussion of the structural aspects).

Both enantiomers of the hydroxymethyl analogue **5** were considerably poorer substrates of the P99 enzyme than **3** (and **4**). This may reflect electronic effects to some degree—hydroxymethyl less electron-withdrawing than methoxy—but must also reflect steric issues since the D-alanine analogue of **4** (X = Me) is more reactive than **5**.[26]

With neither **4** nor **5**, however, is there evidence of a significantly stable/inert acyl-enzyme. The –OMe and $-CH_2OH$ substituents do not have the effect here that they do with β-lactams.

The compounds **4** and **5** are very poor substrates of the class A TEM and PC1 enzymes (Table 1). Incubation of either **4** or **5** with these enzymes, followed by dilution into a solution of a good substrate under saturating conditions, gave no indication of an inert acyl-enzyme in any particular case. Thus, even in the case of the PC1 enzyme, which is more prone than the TEM enzyme to generate relatively stable acyl-enzymes, including

Table 1. Steady state kinetics parameters for the hydrolysis of depsipeptides catalyzed by β-lactamases

Substrate	Enzyme[a]				
	P99			TEM	PC1
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
4[b]	(L) 310 ± 20	0.81 ± 0.12	3.8×10^5	35	104
	(D) 13.7 ± 2.5	1.3 ± 0.4	1.1×10^4	($K_m \geqslant 0.4$ mM)	($K_m \geqslant 0.4$ mM)
5[b]	(L) ⩾ 1.0	⩾0.5	2.0×10^3	47	66
	(D) ⩾0.5	⩾0.5	1.0×10^3	($K_m \geqslant 0.5$)	($K_m \geqslant 0.5$)
6	0.15 ± 0.01	0.05 ± 0.01	3.0×10^3	n[c]	n
7		n[d]		n	n
3[e]	125	0.23	5.4×10^5	1.2×10^4	⩾420
				(K_m = 2.2 mM)	($K_m \leqslant 0.1$ mM)

[a] Enzymes: P99, the class C β-lactamase of *Enterobacter cloacae* P99; TEM, the class A β-lactamase of the TEM-2 plasmid; PC1, the class A β-lactamase of *Staphylococcus aureus* PC1.

[b] The separate reactivities of D and L-enantiomers were distinguished as described in the text. The activity of the TEM and PC1 enzymes against **4** and **5** was too low to allow the distinction to be made.

[c] n, no detectable enzyme catalysis.

[d] Competitive inhibition observed, K_i = 0.16 ± 0.03 mM.

[e] Taken from Ref. 39.

Table 2. Rate Constants and partition ratios for depsipeptide methanolysis and aminolysis (D-Phe) catalyzed by the P99 β-lactamase (Scheme 1)

Substrate	k_3 (s^{-1})	k_4 (s^{-1} M^{-1})	$k_4/k_{3'}$[a]	k_5 (s^{-1} M^{-1})	$k_5/k_{3'}$[a]
4 (D)	310	200 ± 17	36 ± 3	$(7.4 \pm 0.6) \times 10^4$	240 ± 18
4 (L)	13.7	3.0 ± 0.2	12 ± 1	$(3.3 \pm 0.4) \times 10^3$	240 ± 26
3[b]	125	59	26	1.8×10^5	8.0×10^4

[a] $k_{3'} = k_3/[H_2O] = k_3/55.5$.
[b] Taken from Ref. 26.

with **3**,[12] no evidence of an accumulating transient intermediate was obtained.

The results with **6** and **7** were rather unexpected. It had been anticipated that either the positive charge on **6** or the negative charge on **7** would interact with the active site of either a class A or a class C enzyme to generate an inert acyl-enzyme. Only in the case of **6** with the P99 enzyme was this hope realized. Deacylation was slow in this case, with an acyl-enzyme half-life of 4.6 s, but the rate of acylation was modest—$k_{cat}/K_m = k_2/K_s = 3.0 \times 10^3$ s^{-1} M^{-1}. It is possible, however, that the combination of an α-methylammonium substituent and, say, a third-generation cephalosporin side chain might generate an inhibitory substrate of class C β-lactamases. In most of the present experiments, however, **6** and **7** produced no evidence of covalent interaction; return of activity experiments, described above, gave no indication of transiently stable or inert acyl-enzymes formed between **6** and the class A enzymes or between **7** and any enzyme. It was surprising that neither enantiomer of **7** showed any reactivity at all. Non-covalent interaction, however, probably did take place. For example, **7** was a fast reversible inhibitor of the P99 enzyme with a K_i value, assuming competitive inhibition, of 0.16 mM.

Acyclic depsipeptides have also proven to be substrates of bacterial DD-peptidases, the target of β-lactam antibiotics.[26,27] Consequently, the reactivity of **4**–**7** against some typical low molecular mass DD-peptidases[28] was examined. The charged species **6** and **7** appeared to have no covalent reaction with these enzymes, they were neither substrates nor inhibitors. The compounds with the neutral substituents, **4** and **5**, were more reactive. These compounds reacted with the *Streptomyces* R61 DD-peptidase, for example, in what appeared to be a two-phased fashion. The slower phase, however, comprising half of the total material, reflected the rate of the background hydrolysis. The rate of the faster phase was enzyme concentration dependent. This showed that one enantiomer each of **4** and **5** was a DD-peptidase substrate, while the other was not. In view of the well-known stereochemical preference of these enzymes for the D-configuration at the penultimate amino acid residue of a substrate,[29] it seemed likely that the reactive enantiomers of **4** and **5** had the D-configuration. The residual compound after the DD-peptidase reaction, presumably the L-enantiomer, could then be hydrolyzed rapidly by addition of a β-lactamase (Fig. 2), thereby demonstrating the rate of reaction of the L-enantiomer with that enzyme (see above).

Depsipeptides **4** and **5** (D-enantiomers) were quite effective substrates of the representative DD-peptidases

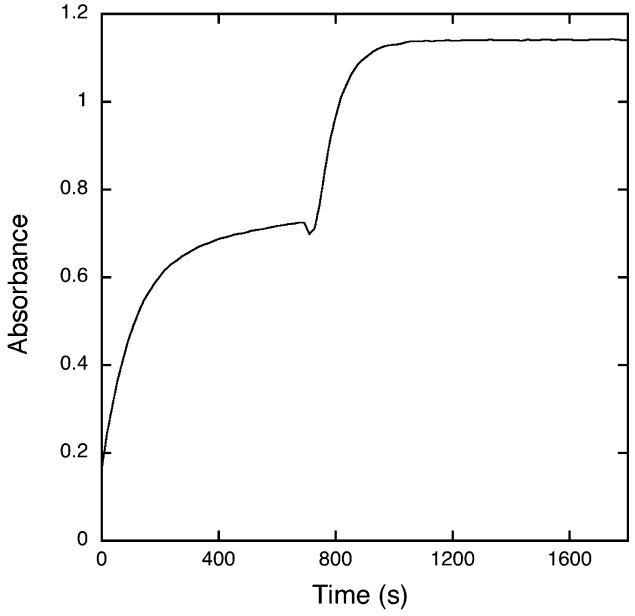


Figure 2. The hydrolysis of **4** (1.0 mM) monitored spectrophotometrically at 300 nm and catalyzed by the R61 DD-peptidase (0.21 μM). Only one enantiomer, presumably D, is hydrolyzed by the enzyme. At the break point, the P99 β-lactamase (0.05 μM), which catalyzes hydrolysis of the L enantiomer, was added.

(Table 3), and in fact, in the case of the R61 DD-peptidase, were more effective than **3**. Previous research has shown, it might be noted, that, as might be expected for a D-alanyl-D-alanine peptidase, the D-methyl analogue of **4** (X = Me) is also a better substrate of the R61 DD-peptidase than **3**.[26] The polarity, greater size, and hydrogen bonding potential of the α-substituents in **5**–**7**, therefore, did not lead to inhibitory behavior with the DD-peptidases.

Finally, it might be noted that the α-disubstituted species **8** and **9** (synthesis: D. Cabaret and M. Wakselman, unpublished) were not substrates of any of the above-mentioned enzymes except for the P99 β-lactamase where very slow turnover was observed (**8**: $k_{cat} = 0.02$ s^{-1}, $K_m = 0.83$ mM, $k_{cat}/K_m = 25$ s^{-1} M^{-1}; **9**: $k_{cat} = 0.009$ s^{-1}, $K_m = 0.43$ mM, $k_{cat}/K_m = 20$ s^{-1} M^{-1}).

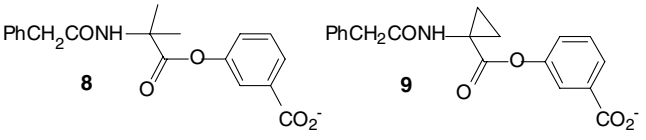


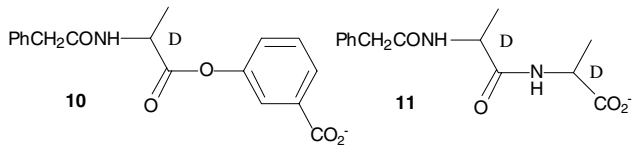
Table 3. Steady state kinetics parameters for the hydrolysis of depsipeptides catalyzed by DD-peptidases[a]

Enzyme[a]		Substrate[b]		
		4	**5**	**3**[c]
R61	k_{cat} (s^{-1})	44 ± 9	2.2 ± 0.3	1.51
	K_m (mM)	0.45 ± 0.03	0.35 ± 0.08	0.76
	k_{cat}/K_m (s^{-1} M^{-1})	9.8×10^4	6.3×10^3	2.0×10^3
R39	k_{cat} (s^{-1})	114 ± 7	0.65 ± 0.03	ND[d]
	K_m (mM)	0.20 ± 0.03	0.85 ± 0.06	
	k_{cat}/K_m (s^{-1} M^{-1})	5.7×10^5	7.7×10^2	
PBP3	k_{cat} (s^{-1})	106 ± 6	22 ± 1	ND
	K_m (mM)	0.20 ± 0.03	1.8 ± 0.1	
	k_{cat}/K_m (s^{-1} M^{-1})	5.3×10^5	1.2×10^4	

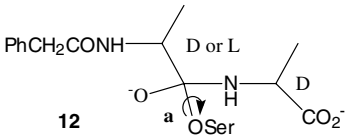
[a] Enzymes: R61, the soluble DD-peptidase of *Streptomyces* R61; R39, the soluble DD-peptidase of *Actinomadura* R39; PBP3, penicillin binding protein 3 of *Neisseria gonorrhoeae*.
[b] Only one enantiomer, assumed to be D, was a substrate—see text.
[c] Taken from Ref. 39.
[d] ND, not determined.

2.1. Structural considerations

Previous studies have shown that D-alanyl depsipeptides such as **10** and analogous D-alanyl peptides such as **11** are poorer substrates of the P99 β-lactamase than the analogous glycyl derivatives; the reverse preference is shown by the R61 DD-peptidase.[26,30] It was suggested that these observations reflect the natural substrate preference of the latter enzyme, the evolution of a class C β-lactamase from an enzyme resembling the R61 DD-peptidase, and the subsequent role of the former as a β-lactamase. Structural models demonstrated the presence of a D-methyl binding pocket at the active site of the DD-peptidase and its absence in the β-lactamase;[26,30] the crystal structure of a complex of the R61 DD-peptidase with a specific D-alanyl-D-alanine substrate subsequently confirmed the existence of this site.[31] Models suggested that the phenylacetylglycyl/alanyl moiety of these substrates may bind to the active sites of these enzymes in two general conformations, A or B. In conformation A, characterized, in particular, by a dihedral angle **a** (Ser C_β–Ser O_γ–C_{tet}–C_α) of ca. −70° (see **12**), the α-carbon is directed into the protein, while in B, characterized by the dihedral angle **a** of ca. −170°, it is directed outwards. D-alanyl derivatives bind to the R61 DD-peptidase in conformation A. This conformation is also favored by glycyl substrates of the P99 β-lactamase but is precluded for D-alanyl substrates which thus must bind to the β-lactamase in conformation B.



Similar modeling methods have now been employed to understand the structural basis of the results described above for the preferences displayed by the P99 β-lactamase and the R61 DD-peptidase for D versus L substituents at the penultimate residue of a substrate. In order to reduce the conformational complexity, and to complement the previous studies, models were made of D versus L methyl substituents in the tetrahedral intermediates D-**12** and L-**12**, which would be derived from enzymatic attack on the peptides D-**11** and L-**11**, respectively.



A combination of molecular dynamics and mechanics computations, performed as described in Section 4, led to the following results. The structure L-**12** is not dynamically stable in the A conformation at the R61 DD-peptidase active site because of unfavorable steric interaction between the L-methyl group and the side chain of Asn 161 and Tyr 159. In conformation B (an energy-minimized structure is shown in Fig. 3), the steric problems are lessened but the ligand interacts with the active site much more weakly than does D-**12** in the A conformation[26] (the E_{int} value[32] for the latter is 28.5 kcal/mole more negative). Further, as demonstrated by 200 ns molecular dynamics runs, L-**12** in the B conformation is much more mobile than D-**12** in the A conformation. As noted previously[33], the mobility of a substrate at the R61 DD-peptidase active site appears to inversely correlate with its reactivity. These models, therefore, do appear in accord with the observed substrate preferences of the R61 DD-peptidase. One would expect that similar considerations would lead to the same preferences in other DD-peptidases, such as the R39 enzyme and pbp3 (Table 3).

E_{int} values of energy-minimized complexes of D-**12** and L-**12** with the P99 β-lactamase were also computed. L-**12** in conformation B (Fig. 4) was more stable than that in conformation A by 21.2 kcal/mole and more stable than D-**12** in conformation B by 23.0 kcal/mole. These results are in agreement with the experimental observations that L-**4** and L-**5** are better substrates of the P99 β-lactamase than the corresponding D-enantiomers. Frere et al.[34,35] have

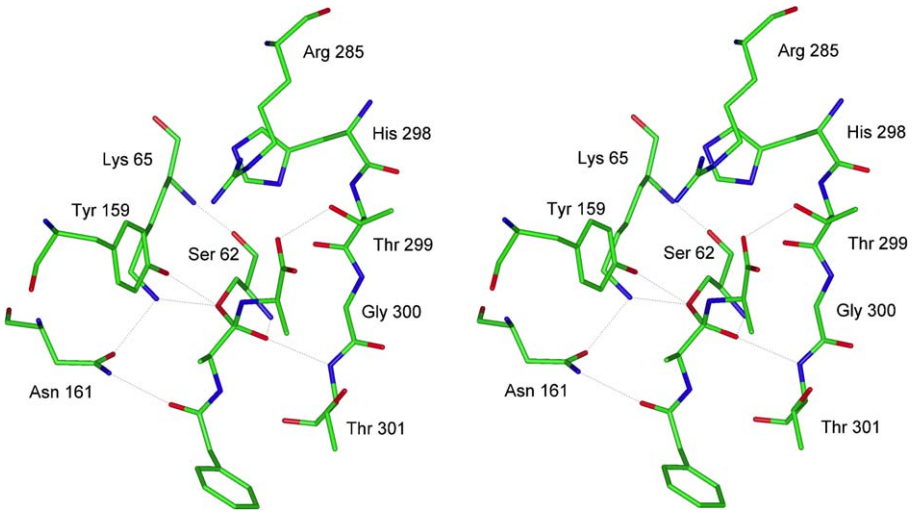


Figure 3. Stereoview of an energy-minimized tetrahedral intermediate structure **11** formed on reaction of the peptide L-**10** with the P99 β-lactamase. The ligand is shown in the (reactive) B conformation (see text); only heavy atoms are displayed.

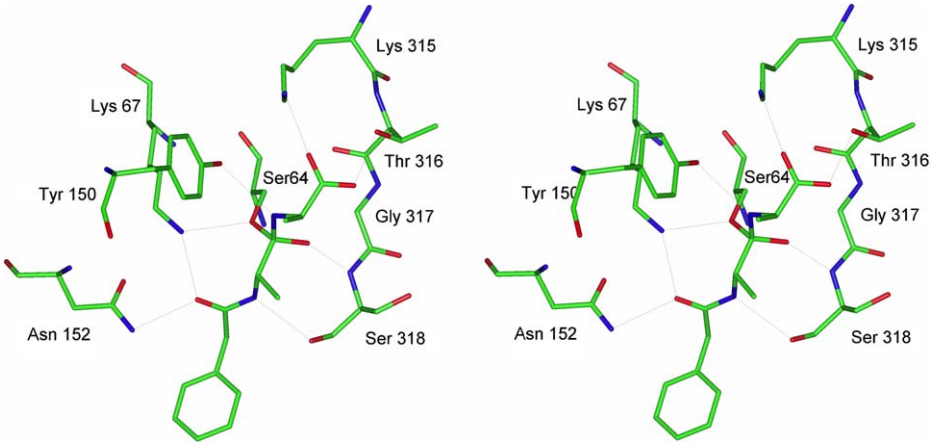


Figure 4. Stereoview of an energy-minimized tetrahedral intermediate structure **11** formed on reaction of the peptide L-**10** with the R61 DD-peptidase. The ligand is shown in the B conformation (see text); only heavy atoms are displayed.

previously observed that L-stereochemistry at the penultimate position may produce a substrate of class C β-lactamase of comparable or greater reactivity than the D-analogue. Thus, with both the P99 β-lactamase and the R61 DD-peptidase, the L enantiomers of **4**, **5**, and **10** probably react preferentially in the B conformation when bound to the enzyme (Figs. 3 and 4).

Inspection of the models suggested that attempts at productive binding by the α-disubstituted analogues **8** and **9** to these active sites would likely lead to disruption of the catalytic AsnLysTyrSer combination by the additional methyl group.

3. Conclusions

The methoxy and hydroxymethyl compounds **4** and **5**, respectively, generally were substrates of representative class A and C β-lactamases and of the low MW DD-peptidases tested. No sign of transiently stable acyl-enzyme intermediates was seen, in contrast with the β-lactams **1a** and **1b**. It seems likely that a combination of the substituent and the pendant leaving group of a β-lactam is needed to extend the acyl-enzyme lifetime. The acyl enzyme intermediates derived from acyclic substrates are presumably more conformationally mobile than those from β-lactams and can thus access reactive conformations such as B (Fig. 4). Very similar conclusions were previously reached with respect to third-generation cephalosporin side chains where again both the side chain and the pendant leaving group were required to achieve an inert acyl-enzyme.[36,37] The aminomethyl and carboxylate substituents of **6** and **7**, respectively, did not interact productively with the enzymes examined except with the class C β-lactamase where reaction with **6** generated a transiently stable acyl-enzyme.

4. Experimental

4.1. Enzyme kinetics

The *Enterobacter cloacae* P99, TEM-2, and *Staphylococcus aureus* PC1 β-lactamases were purchased from the Centre for Applied Microbiology and Research, Porton

Down, Wilts., UK, and used as received. Samples of the *Streptomyces* R61 and R39 DD-peptidases and penicillin binding protein 3 of *Neisseria gonorrhoeae* were generous gifts of Dr. Jean-Marie Frère, Dr. Paulette Charlier, both of the University of Liège, Liège, Belgium, and Dr. Robert A. Nicholas of the University of North Carolina, Chapel Hill, NC, respectively.

Solution conditions for the kinetics runs were as follows: 0.1 M MOPS buffer, pH 7.5, at 25 °C (P99, PC1, and TEM β-lactamases), 0.1 M MOPS buffer, pH 7.5, at 37 °C (R61 DD-peptidase), 0.1 M pyrophosphate buffer, pH 8.5, at 37 °C (penicillin binding protein 3), and 0.1 M Tris buffer, pH 7.7, at 37 °C (R39 DD-peptidase). Stock solutions of the depsipeptides were prepared in DMSO. The DMSO concentration in reaction mixtures after addition of substrate was 5% v/v. Reactions of **4**–**9** were monitored spectrophotometrically at 290 nm ($\Delta\varepsilon = 1760\ M^{-1}\ cm^{-1}$) or 300 nm ($\Delta\varepsilon = 950\ M^{-1}\ cm^{-1}$). Steady state kinetics parameters for the enzyme-catalyzed reactions were obtained from initial rates by non-linear least squares fitting of the data to the Henri–Michaelis–Menten equation. In some cases, where the reaction progress curve was obviously biphasic, initial rates as a function of substrate concentration could be determined separately for the fast and slow phases. Enzyme-catalyzed methanolysis experiments were performed as described previously;[26] methanol concentrations were varied from zero to 2.5 M. Substrate aminolysis kinetics were also obtained as described in a previous paper;[30] D-phenylalanine concentrations ranging up to 40 mM were employed.

4.2. Molecular modeling

The structures of Figures 1 and 2 were derived from computational models of enzyme-substrate complexes that were set up essentially as previously described[26] and run on an SGI Octane 2 computer with INSIGHT II 2005 (Accelrys, San Diego, CA). In these models of the P99 β-lactamase, Lys 67 and Lys 315 were cationic, Tyr 150 was neutral, and the tetrahedral intermediate **11** was dianionic. The corresponding residues of the R61 DD-peptidase, Lys 62 and Tyr 159, were also treated as cationic and neutral, respectively. Lys 315 of the β-lactamase corresponds to His 298 of the DD-peptidase. The latter residue was treated as neutral in the simulations; there is no direct evidence for this but a histidine residue is likely to be neutral at pH 7.5, particularly when directly adjacent (closest heavy atom contact $\leqslant 4$ Å) to an arginine side-chain nitrogen atom (Arg 285). Initially, the ligands were placed in conformations A and B[26] and the stability of these conformations explored by molecular dynamics (200 ps runs, where the entire protein together with solvating water molecules was unrestricted). Typical snapshots of dominant conformations were selected for energy minimization. The minimized structures were then used to calculate interaction energies, E_{int},[32] between ligand and protein. The residues included in these calculations were Ser 64, Lys 67, Tyr 150, Asn 152, Lys 315, Thr 316, Gly 317, and Ser 318 of the P99 β-lactamase and Ser 62, Lys 65, Tyr 159, Asn 161, Arg 285, His 298, Thr 299, Gly 300, and Thr 301 of the R61 DD-peptidase.

4.3. Syntheses

4.3.1. 3-Carboxyphenyl *N*-(phenylacetyl)-α-methoxyglycinate (4) (Scheme 1). *N*-(Phenylacetyl)-α-hydroxyglycine (**13**) was prepared by the method of Zoller and Ben-Ishai.[14] Thus, phenylacetamide (26.1 g, 0.2 mol) and glyoxylic acid (23.0 g, 0.22 mol) were heated under reflux in acetone (150 ml) for 17 h. The solvent was then removed by evaporation, yielding a residual oil that crystallized on standing overnight. The colorless product was recrystallized from chloroform/dioxane (1:1) in 60% yield. Mp 80–81 °C. ^{1}H NMR (DMSO-d_6) δ 3.49 (2H, s), 5.41 (1H, d, $J = 8.7$ Hz), 7.23–7.35 (5H, m), 8.89 (1H, d, $J = 8.4$ Hz).

The α-hydroxyacid (19.3 g, 92 mmol) was dissolved in methanol (225 ml), and the solution was ice cooled.[15] Concd sulfuric acid (3 ml) was added to the chilled mixture, and the resulting solution was stirred for 2 days at room temperature. The final cloudy mixture was poured into ice/saturated sodium bicarbonate and extracted with ethyl acetate (3× 125 ml). The combined ethyl acetate extracts were then dried over magnesium sulfate, filtered, and evaporated. The resulting colorless solid, the methyl ester **14**, was then recrystallized from methanol/water (1:1) in 80% yield. Mp 68–71 °C. ^{1}H NMR ($CDCl_3$) δ 3.43 (3H, s), 3.69 (2H, s), 3.81 (3H, s), 5.56 (1H, d, $J = 9.2$ Hz), 6.41 (1H, d, $J = 9.1$ Hz), 7.29–7.43 (5H, m).

Alkaline hydrolysis of **14** then produced the corresponding acid **15**.[16] Thus, the methyl ester **14** (10 g, 42 mmol) was dissolved in potassium hydroxide/methanol (2.0 g/150 ml) and left at room temperature overnight. The solvent was then evaporated and the residue dissolved in water (100 ml). The solution was extracted with diethyl ether (2× 50 ml) and then acidified with 5% phosphoric acid (50 ml) to pH 2. The resulting acidic solution of the product was extracted with ethyl acetate (2× 100 ml), and the extracts were dried over magnesium sulfate. The solvent was removed by evaporation and the colorless residue recrystallized from acetonitrile to give the product (**15**) in 53% yield. Mp 200–202 °C. ^{1}H NMR (DMSO-d_6) δ 3.19 (3H, s), 3.52 (2H, s), 5.25 (1H, d, $J = 8.9$ Hz), 7.20–7.31 (5H, m), 9.41 (1H, d, $J = 8.9$ Hz).

The procedure employed for condensation of the acid **15** with benzyl 3-hydroxybenzoate was adapted from that of Gallo and Gellman.[17] The acid (2.0 g, 8.9 mmol), benzyl 3-hydroxybenzoate (2.02 g, 8.9 mmol), and 4-dimethylaminopyridine (100 mg, 0.82 mmol) were dissolved in methylene chloride (28 ml). The stirred solution was then cooled to 0 °C on ice, treated with dicyclohexylcarbodiimide (2.28 g, 11.0 mmol), and allowed to return to room temperature overnight. The resulting slurry was filtered, and the filtrate washed sequentially with water, 10% citric acid, water, saturated sodium bicarbonate, and water. The filtrate was then concentrated on a rotary evaporator, yielding a pale yellow-brown liquid that slowly solidified overnight under

oil pump vacuum (**16**, yield 94%). Mp 122 °C. R_f 0.75 (CH_2Cl_2/MeOH 99:1). 1H NMR (DMSO-d_6) δ 3.32 (3H, s), 3.57 (2H, s), 5.38 (2H, s), 5.54 (1H, d, J = 8.0 Hz), 7.29–7.83 (5H, m), 7.37–7.51 (6H, m), 7.62 (1H, t, J = 8.0 Hz), 7.74 (1H, s), 7.93 (1H, d, J = 8.1 Hz), 9.41 (1H, d, J = 8.3 Hz). ^{13}C NMR ($CDCl_3$) δ 43.59, 56.08, 67.56, 79.48, 118–137, 151.25, 164.80, 167.22, 171.97. Anal. Calcd for $C_{25}H_{23}NO_6$: C, 69.27; H, 5.35; N, 3.23. Found: C, 69.25; H, 5.56; N, 3.12.

The required compound, 3-carboxyphenyl *N*-(phenylacetyl)-α-methoxyglycinate, **4**, was then obtained from catalytic hydrogenation of **16**. Compound **16** (1.5 g), dissolved in methanol (75 ml), was hydrogenated (35 psi) overnight over 10% Pd/C (150 mg). The resulting mixture was then filtered through a Celite pad, washed with methanol, and evaporated. The resulting colorless solid, **4**, obtained in quantitative yield, was recrystallized from benzene/ethyl acetate (3:1). Mp 168–171 °C. ^{1}H NMR (DMSO-d_6) δ 3.34 (3H, s), 3.59 (2H, s), 5.55 (1H, d, J = 8.3 Hz), 7.29–7.39 (6H, m), 7.59 (1H, t, J = 8.0 Hz), 7.71 (1H, s), 7.87 (1H, d, J = 7.7 Hz), 9.42 (lH, d, J = 8.2 Hz). ^{13}C NMR (CD_3COCD_3) δ 43.57, 56.06, 79.55, 124–137, 151.50, 166.27, 171.97. Anal. Calcd. for $C_{18}H_{17}NO_6$: C, 62.96; H, 4.99; N, 4.08. Found: C, 62.88; H, 4.96; N, 4.12.

4.3.2. 3-Carboxyphenyl *N*-(phenylacetyl)-serinate (5) (Scheme 2). *N*-(Phenylacetyl)-D-serine(OBn) (**17**) was prepared from the reaction of D-serine(OBn) with phenylacetyl chloride in aqueous base.[12] It was recrystallized from ethanol/water (1:1) in 36% yield. Mp 106 −108 °C. ^{1}H NMR (DMSO-d_6) δ 3.54 (2H, s), 3.60 (1H, dd, J = 4.5, 10.0 Hz), 3.76 (1H, dd, J = 4.4, 10.0 Hz), 3.78 (1H, m), 4.48 (2H, s), 7.27–7.37 (10H, m), 8.46 (1H, d, J = 8.1 Hz), 12.8 (1H, s). The condensation of **12** with benzyl 3-hydroxybenzoate to obtain **18** (in 58% yield) was carried out as described above for **16**. ^{1}H NMR (DMSO-d_6) δ 3.58 (2H, s), 3.81 (1H, dd, J = 4.4, 9.9 Hz), 3.95 (1H, dd, J = 5.4, 9.9 Hz), 4.56, 4.62 (2H, ABq, J = 15 Hz), 4.82 (1H, dt, J = 4.4, 5.4, 6.9 Hz), 5.39 (2H, s), 7.2–7.5 (16H, m), 7.61 (1H, t, J = 7.8 Hz), 7.65 (1H, s), 7.92 (1H, d, J = 7.9 Hz), 8.88 (1H, d, J = 7.3 Hz).

To complete the synthesis of **5**, **18** (0.61 g) was dissolved in methanol (10 ml) and palladium black (0.65 g) added to the solution. The mixture was then shaken for 19 h under 35 psi of hydrogen gas. The reaction mixture was filtered through a Celite pad, which was washed with methanol; solvent from the pooled filtrate was evaporated. The resulting colorless solid was recrystallized from ethyl acetate/benzene (1:1) in 64% yield. Mp 163 −164 °C. ^{1}H NMR (DMSO-d_6) δ 3.52, 3.56 (2H, ABq, J = 14.2 Hz), 3.77 (1H, dd, J = 4.8, 10.9 Hz), 3.93 (1H, dd, J = 4.8, 10.9 Hz), 4.52, 4.56 (1H, br), 7.2–8.0 (9H, m), 8.85 (1H, d, J = 6.5 Hz).

4.3.3. 3-Carboxyphenyl 2-(phenylacetamido)-3-aminopropionate (6) (Scheme 3). The β-amino group of 2,3-diaminopropionic acid was first protected by benzyloxycarbonylation, essentially by the procedure of Folsch and Serck-Hanssen.[38] Thus, 2,3-diaminopropionic acid (2.0 g, 19.0 mmol) and cupric acetate (1.42 g, 20 mmol) were dissolved in water (35.6 ml). The pH of the solution was raised to 5 using 1 M NaOH solution. MgO (0.712 g, 176 mmol) was added followed by benzyl chloroformate (3.40 ml, 20 mmol). The reaction mixture was stirred for 5 h during which time a blue precipitate formed. The precipitate was collected by filtration on sintered glass and washed separately with water (50 ml), methanol, and diethyl ether. The solid was then suspended in boiling 10% aqueous acetic acid solution and thioacetamide (3.00 g, 20 mmol) added. The mixture was heated for 10 min where upon a black solid appeared. This was removed by filtration through Celite and washed with a small amount of boiling water. Colorless crystals formed in the filtrate after its cooling on ice for 3 h. The solid product (**19**) was collected by filtration and dried in vacuo over KOH; yield 2.25 g (49%). ^{1}H NMR (D_2O): δ 3.55 (1H, dd, J = 2.5, 4.0 Hz), 3.7 (1H, dd, J = 2.2, 3.6 Hz), 4.7 (1H, t, J = 2.3 Hz), 5.1 (2H, s), 7.4 (5H, br s).

Phenylacetylation of the α-amino group of **19** was achieved as follows. Compound **19** (2.23 g, 9.4 mmol) was dissolved in 3.5 M aqueous NaOH solution (5.4 ml). The solution was cooled to 0 °C on ice, and phenylacetyl chloride (1.25 ml, 10 mmol) was added in 0.25 ml portions over 1 h with stirring. The mixture was then stirred to room temperature over 2 h. The resulting white suspension was dissolved by addition of the minimum volume of hot water on a steam bath and 0.12 g decolorizing charcoal added. The charcoal was removed by filtration and the hot filtrate acidified with concd HCl to pH 1. The precipitate formed on cooling was recrystallized from water, yielding **20** in 77% yield. Mp 106–108 °C. ^{1}H NMR (DMSO-d_6): δ 3.35 (2H, m), 3.45 (2H, s), 4.30 (1H, m), 5.00 (2H, s), 7.25 (5H, br s), 7.35 (5H, br s), 8.3 (1H, d, J = 9.1 Hz).

The condensation of **20** with benzyl 3-hydroxybenzoate in the presence of carbonyldiimidazole was carried out as previously described.[12] The product, **21**, was purified by silica gel flash chromatography with ethyl acetate/hexane (2:3) as eluent; yield 30%. Mp 74–76 °C. ^{1}H NMR (DMSO-d_6) δ 3.55 (2H, s) 3.55 (2H, m), 4.55 (1H, m), 5.0 (2H, s), 5.4 (2H, s), 7.15–7.40 (15H, m), 7.45 (1H, d, J = 8.4 Hz), 7.55 (1H, t, J = 8.3 Hz), 7.7 (1H, s), 7.9 (1H, d, J = 8.2 Hz), 8.7 (1H, d, J = 8.6 Hz).

The catalytic hydrogenation of **21** to obtain **6** required much attention to detail because of the instability of the product. The following procedure was the most effective of several tried. Compound **21** (100 mg, 0.18 mmol) was dissolved in ethyl acetate (15 ml) containing a little acetic acid (100 μl). Palladium (10%) on activated carbon (150 mg) was added and the reaction mixture was shaken under 35 psi hydrogen gas for 12 h. At that stage, an additional 50 mg of palladium catalyst was added and the mixture was hydrogenated for an additional 6 h. The catalyst and a white precipitate were then collected by filtration and washed with TFA. Excess TFA was then removed by rotary evaporation, isolating the TFA salt of the product. This was suspended in chloroform (10 ml) and 2,6-lutidine (2 ml) was added. The mixture was stirred at 20 °C overnight and

then placed in the freezer for an additional night to aid precipitation of the product. The product was collected by centrifugation, washed with chloroform, and dried in vacuo, yielding 22 mg (37%) of **6** as an off-white powder. Mp 115 °C (d). ^{1}H NMR (D_2O): δ 3.5 (1H, dd, J = 8.3, 13.8 Hz), 3.65 (1H, dd, J = 5.0, 14.9 Hz), 3.80 (2H, s), 4.95 (1H, dd, J = 5.9, 8.6 Hz), 7.09 (1H, d, J = 7.8 Hz), 7.37 (5H, br s), 7.46 (1H, s), 7.49 (1H, t, J = 7.8 Hz), 7.80 (1H, d, J = 7.8 Hz). ESMS(−) *m/e* 343.

4.3.4. 3-Carboxyphenyl phenylacetamidomalonate (7) (Scheme 4).

***N*-Benzoyl-*N*-(2,4-dimethoxybenzyl)glycine benzyl ester (23).** To a solution of benzyl glycinate (1.41 g, 7 mmol), in acetic acid (15 ml), were added sodium acetate (574 mg, 7 mmol) and then 2,4-dimethoxybenzaldehyde (1.16 g, 7 mmol). The reaction mixture was stirred for 30 min at room temperature before addition in several portions of $NaBH_3CN$ (440 mg, 7 mmol). The reaction mixture was stirred overnight and the acetic acid was evaporated. The residue was dissolved in dichloromethane and the solution washed with 0.5 N NaOH. The organic layer was dried over $MgSO_4$ and then the solvent was evaporated. The crude benzyl *N*-2,4-dimethoxybenzylglycinate **22**[23] was dissolved in dichloromethane (20 ml), then were added successively triethylamine (0.974 ml, 7 mmol) and, dropwise, a solution of benzoyl chloride (0.810 ml, 7 mmol) in dichloromethane (20 ml). Stirring was continued for 2 h, the mixture was poured into a $KHSO_4$ solution, the organic layer was washed with saturated $NaHCO_3$, dried over $MgSO_4$, and the solvent evaporated. The residue was purified by silica gel column chromatography (CH_2Cl_2/AcOEt 90:10), affording 2.30 g (78%) of the title ester **23**. R_f 0.53. Mp 83 °C. ^{1}H NMR δ ($CDCl_3$): 2 isomers: 3.67 and 3.72 (2s, 3H), 3.80 (s, 3H), 3.94 and 4.21 (s, 2H), 4.51 and 4.78 (2s, 2H), 5.13 and 5.20 (2s, 2H), 6.3–6.5 (m, 2H), 6.97 (d, 1H), 7.27–7.52 (m, 10H). ^{13}C NMR δ ($CDCl_3$): 2 isomers: 43.6 and 46.3, 49.2 and 50.4, 53.6 and 55.3, 55.4 and 55.6, 67.0–67.5, 98.6–136.1, 158.8 and 161.0, 169.4 and 169.6, 172.3 and 172.8. Anal. Calcd for $C_{25}H_{25}NO_5$: C, 71.58; H, 6.01; N, 3.34. Found: C, 71.49; H, 6.11; N, 3.27.

3-(Benzyloxycarbonyl)phenyl chloroformate. A solution of benzyl 3-hydroxybenzoate (912 mg, 4 mmol) and of *N*,*N*-dimethylaniline (912 mg, 4 mmol) in THF (6 ml) was slowly added with cooling to a solution of 396 mg (1.33 mmol) of bis (trichloromethyl) carbonate in THF (6 ml). The reaction mixture was stirred at room temperature overnight then poured into 0.1 N HCl at 0 °C and extracted with ethyl acetate. The organic layer was dried over $MgSO_4$ and the solvent evaporated to give a mixture of the starting benzyl 3-hydroxybenzoate and the chloroformate, in the ratio 27:73, which was directly used in the next step. ^{1}H NMR δ ($CDCl_3$): 5.39 (2s, 2H), 7.34–7.48 (m, 6H), 7.52 (t, 1H), 7.93 (s, 1H), 8.05 (dd, 1H). ^{13}C NMR δ ($CDCl_3$): 67.5, 116.6 to 135.7, 149.7, 151.5, 165.2.

2-Benzoyl-(2,4-dimethoxybenzyl)-aminomalonic acid benzyl ester 3-(benzyloxycarbonyl)phenyl ester (24). At −78 °C, a solution of **23** (276 mg, 0.66 mmol) in THF (3 ml) was slowly added to a 1.0 N solution of LiHMDS in hexane (2 ml). The mixture was stirred at −78 °C for 1 h, then a solution of 191 mg (about 0.66 mmol) of the crude 3-(benzyloxycarbonyl)phenyl chloroformate in THF (3 ml) was added dropwise. The reaction mixture was stirred at room temperature for a further 3 h and then poured into a 0.1 N $KHSO_4$ solution. After extraction with ethyl acetate, the organic layer was washed with water, dried over $MgSO_4$, and the solvent evaporated. The product was purified by two successive chromatographic steps on silica gel: the first eluent was CH_2Cl_2/AcOEt 95:5, the second cyclohexane/AcOEt 70:30; the yield of **24** was 142 mg (43%). ^{1}H NMR δ ($CDCl_3$): 3.67 and 3.79 (2s, 2× 3H), 4.58 (dd, 2H), 4.88 (s, 1H), 5.23 and 5.37 (2s, 2× 2H), 6.38–6.46 (m, 2H), 7.23 (d, 1H), 7.33–7.56 (m, 16H), 7.74 (s, 1H), 7.94 (m, 1H). ^{13}C NMR δ ($CDCl_3$): 49.8, 55.3–55.6, 62.3, 67.1–68.2, 98.8–136.1, 151.8, 164.3, 165.3, 165.7 and 172.6. Anal. Calcd for $C_{40}H_{35}NO_9$: C, 71.31; H, 5.24; N, 2.08. Found: C, 71.10; H, 5.32; N, 2.04.

2-Benzoylaminomalonic acid benzyl ester 3-(benzyloxycarbonyl)phenyl ester (25). To a solution of the *N*-2,4-dimethoxybenzylhippurate **24** (119 mg, 0.23 mmol) in dichloromethane (1 ml) was slowly added 1 ml TFA at 0 °C. The mixture was stirred for 1 h at room temperature and the solvents were then removed by evaporation to give a residue, which was purified by silica gel chromatography (eluent: CH_2Cl_2/AcOEt 95:5), to give 81 mg (87%) of the title product **25**. R_f 0.71. The product was recrystallized from diethyl ether. Mp 126 °C. ^{1}H NMR δ ($CDCl_3$): 5.36 (dd, 2H), 5.38 (s, 2H), 5.66 (d, 1H), 7.18 (m, 1H), 7.27 (d, 1H), 7.34–7.57 (m, 14H), 7.77 (s, 1H), 7.85–7.89 (m, 2H), 7.98 (m, 1H). ^{13}C NMR δ ($CDCl_3$): 57.3, 67.0, 68.9, 122.3–136.0, 150.5, 165.1, 165.9, 167.3. Anal. Calcd for $C_{31}H_{25}NO_7$: C, 71.12; H, 4.81; N, 2.68. Found: C, 70.93; H, 4.85; N, 2.62.

3-Carboxyphenyl phenylacetamidomalonate (7). The hydrogenolysis was performed at 0 °C in a 50 ml round-bottomed flask equipped with a three-way tap and magnetic stirring. The recrystallized dibenzylester **25** (20 mg) was dissolved in ethyl acetate (6 ml) then 10 mg of 10% Pd/C was added. A vacuum was applied to the flask until the solvent began to bubble. Then the flask was filled with hydrogen. After 30 min, the reaction was complete as shown by TLC (silica gel, eluent: ethyl acetate/acetic acid 9:1. R_f **7**: 0.40) and (silica gel, eluent: CH_2Cl_2/ethyl acetate 95:5. R_f **25**: 0.51). The solution was then filtered and the solvent removed under vacuum at 0 °C. The NMR spectra of the crude product showed no notable impurities. The compound, however, is not very stable toward decarboxylation at room temperature. ^{1}H NMR δ [$(CD_3)_2CO$]: 5.68 (d, 1H), 7.35–7.64 (m, 5H), 7.97 (m, 1H), 7.98–8.03 (m, 2H), 8.39 (d, 1H). ^{13}C NMR δ [$(CD_3)_2CO$]: 58.0, 123.5–134.3, 151.8, 166.6, 166.7, 167.6, 167.7. ESMS(+), (CH_3CN–H_2O), *m/e* 344 $[M+H]^+$.

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