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Chromogenic depsipeptide substrates for β-lactamases and penicillin-sensitive DD-peptidases

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Various ester and thioester derivatives of hippuric acid have been prepared which were substrates of both β-lactamases and DD-peptidases. The thioesters were more rapidly hydrolysed by nearly all the enzymes. Surprisingly, the enzymes acted rather efficiently on substrates which did not contain any chiral centre.

INTRODUCTION

The study of the kinetic properties of penicillin-sensitive DD-peptidases has been complicated by the absence of simple spectrophotometric assays for monitoring their activity. In the best cases, the enzymes catalyse hydrolysis or transfer reaction with simple peptides of general structure acyl-D-Ala-D-Ala, but the release of D-alanine cannot be followed continuously. The progress of the reaction must be monitored by a point-by-point assay of D-alanine (Frère *et al.*, 1976). However, Faraci & Pratt (1986) have assayed the activity and concentration of the *Streptomyces* R61 DD-peptidase (Frère *et al.*, 1973) spectrophotometrically at 254 nm with hippuryl DL-phenyl-lactate as substrate, but did not further analyse the interaction.

In the present study, we have synthesized various esters and thioesters of hippuric acid and related compounds and determined the characteristic parameters of their interaction with the penicillin-sensitive active-serine *Streptomyces* R61 and *Actinomadura* R39 DD-peptidases and with the penicillin-resistant Zn^{2+}-containing DD-peptidase of *Streptomyces albus* G. In addition, since Pratt and his co-workers demonstrated that various esters could also be hydrolysed by β-lactamases (Chandrika *et al.*, 1987), we tested the new compounds with the β-lactamases of *Enterobacter cloacae* P99 (class C), *Streptomyces albus* G (class A) and *Bacillus licheniformis* (class A).

MATERIALS AND METHODS

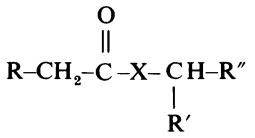
Enzymes

The various enzymes were produced and purified as described by Matagne *et al.* (1990) for the β-lactamases and by Fossati *et al.* (1978), Duez *et al.* (1978) and Frère *et al.* (1974) for the DD-peptidases of *Streptomyces* R61, *Streptomyces albus* G and *Actinomadura* R39 respectively.

Synthesis of substrates

Analytical methods. N.m.r. spectra were recorded on a 60 MHz Varian EM 360L or a 400 MHz Bruker AM spectrometer. I.r. spectra were recorded in KBr discs on a Perkin–Elmer 1320 and mass spectra on a Varian Mat 112 spectrometer. G.l.c. was performed on a gas chromatograph Varian 3300 equipped with an SE-30 5% column, and t.l.c. on Kieselgel 60 F254, D.C. Alufolien (Merck) plates with the following solvents (all proportions by vol.): A, ethyl acetate/benzene (1:4); B, chloroform/methanol/acetic acid (48:1:1); C, propan-2-ol/toluene (1:9); D, propan-2-ol/ethyl acetate/cyclohexane (2:1:7); E, hexane/ethyl acetate (2:1). For preparative chromatography Kieselgel 60 0.06–0.2 nm thick (Merck) was used.

Synthesis.



The following compounds were synthesized, the detailed structures of which are given in Table 1: carboxymethyl benzoylaminoacetate (S1a); carboxymethyl 4-nitrobenzoylaminoacetate (S1b); DL-lactyl benzoylaminoacetate (S1c); DL-mandelyl benzoylaminoacetate (S1d); DL-phenyl-lactyl benzoylaminoacetate (S1e); carboxymethyl benzoylaminothioacetate (S2a); carboxymethyl 3-(fur-2-yl)acryloylaminothioacetate (S2b); DL-lactyl benzoylaminothioacetate (S2c); DL-1-phenylbutyl benzoylaminoacetate (S3a); DL-phenyl(propionyl)methyl benzoylaminoacetate (S3b); DL-mandelyl 3-benzoylaminopropionate (S3c); DL-mandelyl benzenesulphonylaminoacetate (S3d).

Carboxymethyl benzoylaminoacetate (S1a) was prepared as described by Schwyzer *et al.* (1955) but ethyl bromide was replaced by t-butyl chloroacetate. t-Butyloxycarbonylmethyl benzoylaminoacetate was purified by chromatography on silica gel in solvent A. ^1H n.m.r. (60 MHz): δ (p.p.m.) HMDSO ([^2H]chloroform) 1.6 (s, 9H), 4.3 (d, 2H), 4.7 (s, 2H), 7.15 (s, broad, 1H) and 7.3–8 (m, 5H). The acid function was deprotected as described by Bryan *et al.* (1977). The final white crystalline powder (m.p. 165–170 °C) was shown to be homogeneous by t.l.c. in solvent B. ^1H n.m.r. (400 MHz): δ (p.p.m.) ([$^2H_6$]acetone) 4.4 (d, 2H), 4.8 (s, 2H), 7.6–85 (m, 5H) and 8.3 (s, broad, 1H).

Carboxymethyl 4-nitrobenzoylaminoacetate (S1b) was prepared following the same method as for S1a but t-butyl bromoacetate and *p*-nitrohippuric acid were used. Yield: 45%; m.p.: 178–180 °C; m.s.: *m/z* 282, 207, 179, 150, 132; ^1H n.m.r. (400 MHz): δ (p.p.m.) ([$^2H_6$]dimethyl sulphoxide), 4.2 (d, 2H), 4.7 (s, 2H), 8.2–8.4 (q, 4H) and 9.4 (*s*, 1H); ^{13}C n.m.r.: δ (p.p.m.) 50 (C6), 70 (C8), 133 (C3), 139 (C2), 148 (C4), 159 (C1) and 174–180 (3CO).

DL-Lactyl benzoylaminoacetate (S1c) was prepared as described by Armstrong *et al.* (1988) and the deprotection of the acid function was performed as above for S1a.

(*a*) t-Butyl 2,2,2-trichloroacetamidate: b.p.: 62 °C (20 mmHg);

Abbreviation used: HMDSO, hexamethyldisiloxane.

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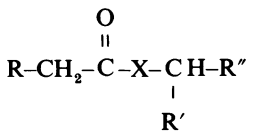
Table 1. Structures of the tested esters and thioesters



X = O			X = S			X = O			
	R	R′		R′	R′		R	R′	R″
S1a	C_6H_5–CO–NH–	H–	S2a	C_6H_5–CO–NH–	H–	S3a*	C_6H_5–CO–NH–	C_6H_5–	–CH_2–CH_2–CH_3
S1b	O_2N–C_6H_5–CO–NH–	H–	S2b		H–	S3b*	C_6H_5–CO–NH–	C_6H_5–	
S1c*	C_6H_5–CO–NH–	CH_3–	S2c*	C_6H_5–CO–NH–	CH_3–	S3c*	C_6H_5–CO–NH 	C_6H_5–	–CO_2H
S1d*	C_6H_5–CO–NH–	C_6H_5–				S3d*	C_6H_5–SO_2–NH–	C_6H_5–	–CO_2H
S1e*	C_6H_5–CO–NH–	–CH_2–C_6H_5							

R″ was –CO_2H in all cases

* These substrates were racemic mixtures.

^{1}H n.m.r. (60 MHz): δ (p.p.m.) HMDSO ([^{2}H]chloroform) 1.7 (s, 9H) and 8.2 (s, broad, 1H).

(*b*) t-Butyl 2-chloropropanoate: ^{1}H n.m.r. (400 MHz): δ (p.p.m.) HMDSO ([^{2}H]chloroform) 1.4 (s, 9H), 1.5 (d, 3H) and 4.2 (q, 1H).

(*c*) DL-2-Propionyl-t-butyl benzoylaminoacetate: m.p.: 85–90 °C; ^{1}H n.m.r. (400 MHz): δ (p.p.m.) ([^{2}H]chloroform) 1.48 (m, 9H), 1.6 (d, 3h), 4.3 (m, 2H), 5 (q, 1H), 6.7 (s, broad, 1H) and 7.2–7.8 (m, 5H).

(*d*) DL-Lactyl benzoylaminoacetate: m.p.: 175–180 °C; t.l.c.: homogeneous in solvent C; ^{1}H n.m.r. (400 MHz): δ (p.p.m.) ([$^2H_4$]methanol) 1.4 (d, 3H), 4.1 (q, 2H), 5 (q, 1H) and 7.3–7.7 (m, 5H); ^{13}C n.m.r.: δ (p.p.m.) 18.5 (3C), 42 (2C), 65 (1C), 128–134 [6C (C6, 128; C3–C5, 129; C4, 132; C1, 134], 169 (CO) and 173 (CO).

DL-Mandelyl benzoylaminoacetate (S1d) and DL-phenyl-lactyl benzoylaminoacetate (S1e) were prepared by the method of McClure (1964).

(*a*) S1c: m.p.: 166–168 °C; t.l.c.: homogeneous in solvent B; ^{1}H n.m.r. (400 MHz): δ (p.p.m.) ([$^2H_6$]acetone) 4.4 (m, 2H), 6.1 (s, 1H), 7.5 (m, 10H) and 8.4 (s, 1H); i.r.: 1690, 1740, 2400–3400 and 3300–3600 cm^{-1}.

(*b*) S1d: T.l.c.: homogeneous in solvent B; ^{1}H n.m.r.: δ (p.p.m.) HMDSO ([$^2H_6$]acetone) 3.2 (m, 2H), 4.2 (d, 2H), 5.2 (q, 1H), 7.2–7.9 (m, 10H) and 8.1 (s, 1H); m.s.: *m/z* 327, 310, 282, 226 and 209.

Carboxymethyl benzoylaminothioacetate (S2a) was prepared by the method of Schwyzer & Harlimann (1954) and finally purified by recrystallization in water. M.p.: 138–142 °C; i.r.: 1675, 1650–1700, 3000 and 3300 cm^{-1}; ^{1}H n.m.r. (400 MHz); δ (p.p.m.) ([$^2H_6$]acetone) 3.8 (s, 2H), 4.5 (m, 2H), 7.6–8.1 (m, 5H) and 8.6 (s, 1H).

Carboxymethyl 3-(fur-2-yl)acryloylaminothioacetate (S2b) was obtained in a three-step reaction.

(*a*) *N*-Succinimidyl 3-(fur-2-yl)acrylate was prepared by the method of Anderson *et al.* (1964). Yield: 76%; m.p.: 113–115 °C; ^{1}H n.m.r. (400 MHz): δ (p.p.m.) ([^{2}H]chloroform): 2.8 (s, 4H) and 6.3–7.9 (m, 5H).

(*b*) 3-(Fur-2-yl)acryloylglycine: 10 mmol of *N*-succinimidyl 3-(fur-2-yl)acrylate was dissolved in 20 ml of tetrahydrofuran, and a solution containing 0.01 mol of glycine and 1.82 g of $NaHCO_3$ in 15 ml of water was added. After 1 h, the aqueous phase was acidified with HCl (1 M) and extracted with dichloromethane. The solid was filtered and recrystallized. Yield: 64%; ^{1}H n.m.r.: δ (p.p.m.) ([$^2H_6$]dimethyl sulphoxide) 4 (s, 2H), 6.5 (s, 1H), 6.6 (m, 1H), 6.8 (d, 1H), 7.3 (d, 1H), 7.7 (s, 1H) and 8.5 (t, 1H); ^{13}C n.m.r.: δ (p.p.m.) 40 (C8), 112 (C3), 113 (C2), 119 (C6), 127 (C5), 145 (C4), 149 (C1), 149 (C1), 156 (C7) and 170 (C9).

(*c*) Carboxymethyl 3-(fur-2-yl)acryloylaminothioacetate was synthesized by the method used for carboxymethyl benzoylaminothioacetate. Yield: 31%; m.p.: 180–186 °C; ^{1}H n.m.r.: δ (p.p.m.) ([$^2H_6$]dimethyl sulphoxide) 3.7 (s, 2H), 4.2 (d, 2H), 6.5 (m, 1H, 1H), 6.7 (s, 1H), 7.3 (d, 1H), 7.8 (s, 1H) and 9 (s, 1H); ^{13}C n.m.r.: δ (p.p.m.) 40 (C8), 58 (C10), 122 (C3), 124 (C2), 127 (C6), 139 (C5), 154 (C4), 160 (C1), 176 (C7), 180 (C11) and 208 (C9).

DL-1-Phenylbutyl benzoylaminoacetate (S3a) was prepared by the method of Brook & Chan (1983). The compound was homogeneous on t.l.c. in solvent D. ^{1}H n.m.r. (60 MHz): δ (p.p.m.) HMDSO ([^{2}H]chloroform) 0.7–1.5 (m, 3H–7H), 4 (d, 2H), 4.8 (m, 1H), 6.4 (s, broad, 1H) and 7–7.5 (m, 5H).

DL-Phenyl(propionyl)methyl benzoylaminoacetate (S3b) was prepared as described by Hassner & Alexanian (1978), purified by chromatography on silica gel with solvent E and recrystallized in light petroleum (b.p. 60–80 °C): m.p.: 78 °C; t.l.c. homogeneous in solvent E. ^{1}H n.m.r. (60 MHz): δ (p.p.m.) HMDSO ([^{2}H]chloroform) 1.1 (d, 3H), 3.8–4.1 (m, 2H, 2H), 5.8 (s, 1H), 6.4 (s, 1H) and 7.2–7.6 (m, 5H).

DL-Mandelyl 3-benzoylaminopropionate (S3c) was prepared as was the pentyl ester, by coupling of 3-benzoylaminopropionic acid with benzyl DL-mandelate.

(*a*) 3-Benzoylaminopropionic acid was prepared by dissolving 0.25 mol of 3-aminopropionic acid in 200 ml of aq. 2.5 M-NaOH. An equimolar amount of benzoyl chloride was added in 70 ml of tetrahydrofuran and the mixture was stirred for 60 min at 20 °C. The acid was precipitated by the addition of HCl and was homogeneous in solvent D. M.p.: 122 °C; i.r.: 1625, 1720, 2600 and 3300 cm^{-1}; ^{1}H n.m.r. (60 MHz): δ (p.p.m.) ([$^2H_6$]chloroform) 2.4 (t, 2H), 3.5 (m, 2H) and 7.2–7.8 (m, 5H).

(*b*) Sodium benzyl DL-mandelate (0.1 equivalent of Na^+) was dissolved in 100 ml of dry ethanol and added with 0.1 mol of DL-mandelic acid. After evaporation of ethanol, the dry residue was

dissolved in 100 ml of dimethylformamide, and 0.1 mol of benzyl chloride was added. After reaction, the mixture was poured on ice and extracted with diethyl ether. The ether phase was dried over $MgSO_4$. The compound, crystallized in toluene, was homogeneous in solvent C. ^1H n.m.r. (60 MHz): δ (p.p.m.) ([^2H]chloroform) 4.9–5 (d, 2H, 1H) and 7.1 (m, 10H). This product was then condensed with 3-benzoylaminopropionic acid as above for S3a.

(*c*) After esterification, the protective group was removed by H_2 (1.3 MPa) in methanol and in the presence of Pd/C (10%) as described by Govardhan & Pratt (1987). The acid was purified by h.p.l.c. on an ET 250 18/4 Nucleosil-5 C_{18} column (Macherey–Nagel). Solvent 1 was 0.1% (v/v) trifluoroacetic acid in water, and solvent 2 was 0.1% (v/v) trifluoroacetic acid in acetonitrile. ^1H n.m.r. (400 MHz) δ (p.p.m.): ([$^2H_6$]chloroform) 2.7 (m, 2H), 3.6 (m, 2H), 5.8 (s, 1H) and 7.2–7.7 (m, 10H).

DL-Mandelyl benzenesulphonylaminoacetate (S3d) was prepared by coupling DL-mandelic acid with benzenesulphonylglycyl chloride.

(*a*) Benzene sulphonylaminoacetic acid was synthesized by the method used for benzoylaminopropionic acid. The compound (m.p.: 180 °C) was homogeneous by t.l.c. in solvent D. ^1H n.m.r. (60 MHz) δ (p.p.m.) ([^2H]chloroform) 3.4 (s, 2H) and 7 (m, 5H).

(*b*) Benzenesulphonylacetyl chloride was obtained by refluxing 10 g of benzenesulphonylaminoacetic acid in 100 ml of $SOCl_2$ for 2 h. After evaporation of the excess of $SOCl_2$, brownish crystals were obtained at 20 °C. M.p.: 70–80 °C; i.r.: 1790 and 3250 cm^{-1}.

(*c*) DL-Mandelyl benzenesulphonylaminoacetate: 0.05 mol of benzene sulphonylacetyl chloride was dissolved in dry tetrahydrofuran, and to it was added 0.05 mol of mandelic acid in the same solvent containing 0.1 mol of pyridine. The mixture was stirred for 16 h. After evaporation of the tetrahydrofuran, the residue was dissolved in dichloromethane and washed with 1 M-HCl. The acid was extracted with $NaHCO_3$ solution and precipitated with HCl. The precipitate was dissolved in ethyl acetate, dried on $MgSO_4$ and recrystallized in dichloromethane/light petroleum (b.p. 60–80 °C). The compound (m.p.: 140 °C) was homogeneous by t.l.c. in solvent A. i.r.: 1720, 1740 and 3250 cm^{-1}; ^1H n.m.r. (400 MHz): δ (p.p.m.) ([$^2H_6$]acetone) 4.1 (d, 2H), 6 (s, 1H) and 7.56–8 (m, 10H).

Kinetic data

All kinetic measurements with β-lactamases were performed in 50 mM-sodium phosphate buffer, pH 7.0, at 30 °C. Those with the *Streptomyces* R61 DD-peptidases were performed in 50 mM-Tris/HCl buffer, pH 7.5, at 37 °C, and those with the DD-peptidases of *Actinomadura* R39 and *S. albus* G were performed in 50 mM-Tris/HCl buffer, pH 7.5, containing 4 mM-$MgCl_2$ at 37 °C.

All spectrophotometric measurements were made with a Beckman DU8 spectrophotometer linked to an Apple II microcomputer via a RS 232 interface. Kinetic parameters were usually determined by monitoring the complete time course of substrated hydrolysis under the following conditions: S1a, 254 nm, $\Delta\epsilon = 620\ M^{-1}\cdot cm^{-1}$; S1b, 283 nm, $\Delta\epsilon = 1100\ M^{-1}\cdot cm^{-1}$; S1c, 250 nm, $\Delta\epsilon = 540\ M^{-1}\cdot cm^{-1}$; S1d, 254 nm, $\Delta\epsilon = 600\ M^{-1}\cdot cm^{-1}$; S1e, 254 nm, $\Delta\epsilon = 550\ M^{-1}\cdot cm^{-1}$; S2a, 250 nm, $\Delta\epsilon = 1800\ M^{-1}\cdot cm^{-1}$; S2b, 322 nm, $\Delta\epsilon = 4700\ M^{-1}\cdot cm^{-1}$; S2c, 250 nm, $\Delta\epsilon = 1000\ M^{-1}\cdot cm^{-1}$.

When the substrate was a racemic mixture, the $\Delta\epsilon$ value was computed for the pure enantiomer.

The K_m values were often larger than 1 mM and only the $k_{cat.}/K_m$ values were determined. In the few cases where K_m was smaller than 0.5 mM, the individual values of $k_{cat.}$ and K_m were computed (De Meester *et al.*, 1987).

When K_m was higher than 2 mM, and the enzymic activity was low, initial rates were measured and the kinetic parameters derived from Hanes plots.

In some cases, complex time courses (bursts) were observed, and when possible first-order rate constants were derived for inactivation (k_i) and re-activation (k_r) as described by De Meester *et al.* (1987).

Stability of the carboxymethyl benzoylaminothioacetate substrate

The carboxymethyl benzoylaminothioacetate substrate was much more unstable in the presence of nucleophilic buffers such as Tris. For that reason, the experiments were always performed in phosphate buffer for most enzymes and in Hepes buffer for the *Actinomadura* R39 and *S. albus* G DD-peptidases. Moreover, non-specific hydrolysis was also observed by benzylpenicillin-inactivated *Streptomyces* R61 and *Actinomedura* R39 DD-peptidases. This non-specific hydrolysis amounted to 1% and 10% of the specific reactions for the R61 and R39 enzymes respectively. Conversely, the substrate was stable in the presence of the β-lactamases after inactivation of those enzymes by β-iodopenicillanate.

RESULTS

Commercially available DL-phenyl-actyl benzoylaminoacetate (S1e) was a substrate for several of the serine β-lactamases and DD-peptidases. As shown by Fig. 1, a β-lactamase hydrolysed only 50% of the racemic mixture, and the other half was hydrolysed by Pronase, which indicated that the D-isomer was very probably the substrate for the first enzyme. Similar results were obtained with all racemic mixtures of series 1 and 2: Pronase hydrolysed one isomer, and the β-lactamases and DD-peptidases the other.

As expected on the basis of the known specificity of DD-carboxypeptidases and β-lactamases (see the Discussion section), substrates S3a, S3b and S3c were not significantly hydrolysed by the various enzymes. A very slow hydrolysis of S3c and S3b was, however, recorded with the P99 and R61 enzymes respectively. Surprisingly, Pronase hydrolysed only S3c and extremely slowly. The sulphonyl substrate S3d was not hydrolysed by any of the enzymes tested. It exhibited slight inhibitory properties on the *E. cloacae* P99 β-lactamase (7.5% inhibition at 2.2 mM).

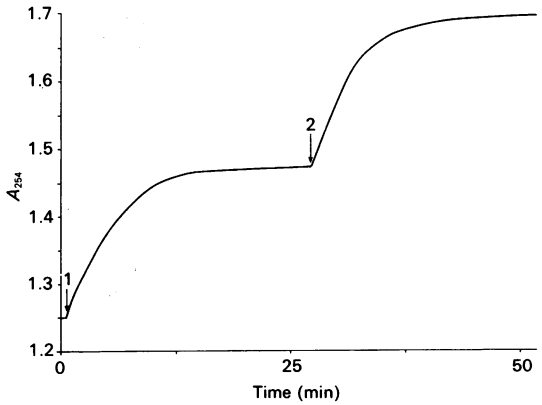


Fig. 1. Hydrolysis of substrate S1e by Pronase and the *E. cloacae* P99 β-lactamase

To a 0.45 ml of 0.76 mM-DL-phenyl-lactyl benzoylaminoacetate solution in 50 mM-cacodylate buffer, pH 5.5, was added 10 μg of Pronase in a volume of 2 μl (arrow 1). After 25 min the absorbance stabilized ($\Delta A_1 = 0.210$) and 2 μg of the *E. cloacae* β-lactamase was added (arrow 2). After stabilization of the absorbance a further increase (ΔA_2) of 0.211 was measured. Identical results were obtained when the order of addition of the enzymes was reversed.

Table 2. $k_{cat.}/K_m$ values for the enzymic hydrolysis of substrates of series 1 and 2

	$k_{cat.}/K_m$ ($M^{-1} \cdot s^{-1}$)					
	β-Lactamases			DD-Peptidases		
Substrate	*E. cloacae* P99	*S. albus* G	*B. licheniformis*	*Streptomyces* R61	*Actinomadura* R39	*S. albus* G (Zn^{2+})
S1a	310±20	90±15	Complex kinetics	540±20	3400±200	< 20
S1b	Complex kinetics	100±20	100±20	350±40	6600±400	0
S1c	135±25	0	0	1500±100	Complex kinetics	220±25
S1d	4600±250	120±15	290±20	2400±600	Complex kinetics	2740±400
S1e	20000±1000	310±20	80±10	2600±200	Complex kinetics	0
S2a	2400±150	15600±1000	4500±200	100000±10000	11500±2000	N.D.*
S2b	1000±100	8300±700	4700±500	4700±400	15400±400	N.D.*
S2c	Complex kinetics	11400±600	6200±200	Complex kinetics	1800±150	N.D.*

* Strong inhibition by the thiol product.

Table 3. Individual k_{cat} and K_m values for some interactions

Substrate	Enzyme	$k_{cat.}$ (s^{-1})	K_m (mM)
S1d	*Streptomyces* R61 DD-peptidase	5±1	2±0.2
S2a	*Streptomyces* R61 DD-peptidase	5±1	0.050±0.01
S2a	*Actinomadura* R39 DD-peptidase	0.9±0.2	0.08±0.015
S2b	*Actinomadura* R39 DD-peptidase	0.3±0.2	0.02±0.005

Compounds of series 1 and 2 were all and to various degrees substrates of the DD-peptidases and β-lactamases (Tables 2 and 3). In most cases, the K_m values were high and only $k_{cat.}/K_m$ values were computed. Surprisingly, if Pronase hydrolysed the L-isomer of the racemic mixtures, it did not hydrolyse the non-chiral substrates S1a and S2a and acted extremely slowly on S2b. Complex kinetics were observed in several cases. In the interactions between 0.5 mM-Sle and the *Actinomadura* R39 DD-peptidase and 0.2 mM-S2c and the *E. cloacae* β-lactamases the time courses were characterized by a burst followed by a linear hydrolysis. Under those conditions the ratio of the initial rate (v_0) to that at the stationary state (v_{ss}) was 6±0.8 in the first case and 8.5±0.8 in the second. The respective half-transition times were 12±3 and 32±2 s. In both cases and during the first 60 s of the reaction, the amount of substrate hydrolysed was similar to that utilized under the same conditions with substrates S1a and S1b for the *Actinomadura* R39 DD-peptidase and with substrate S2a with the *E. cloacae* β-lactamase. In the other cases the time courses were more complex and the phenomena were not further analysed.

The possible inhibition of the enzymes by the L-isomer and/or the reaction products was also tested by determining initial rates in their presence. First, no inhibition was recorded when hippuric acid, mercaptoacetic acid and DL-mercaptopropionic acid were added to reaction mixtures containing the *E. cloacae* β-lactamase and the *Streptomyces* R61 DD-peptidase. The possible influence of the L-isomer was tested by comparing the initial rates of the reaction with or without a preliminary hydrolysis of the L-isomer by Pronase. No differences were recorded.

DISCUSSION

The main goal of this work was to prepare potential chromogenic substrates for DD-peptidases which might also be substrates of β-lactamases. This goal seems to have been attained. Several esters and thioesters have been obtained whose hydrolysis by those enzymes can directly be monitored in the near u.v. Some of those compounds exhibited substrate properties similar to or even better than those of the classical peptide substrate of the DD-peptidases Ac_2-L-Lys-D-Ala-D-Ala ($k_{cat.}/K_m = 53000\ M^{-1} \cdot s^{-1}$ and $4600\ M^{-1} \cdot s^{-1}$ for the *Actinomadura* R39 and *Streptomyces* R61 DD-peptidases respectively). The most successful compound with the *Streptomyces* R61 DD-peptidase was the thioester S2a, and with the *Actinomadura* R39 enzyme another thioester, S2b. Moreover, both compounds exhibited rather large $\Delta\epsilon$ values upon hydrolysis (-1800 and $-4700\ M^{-1} \cdot cm^{-1}$ respectively). In the future it will be possible to utilize those compounds as reporter substrates in inactivation or inhibition experiments, which will greatly facilitate those studies. Many compounds were also substrates of the class A and C β-lactamases, but in those cases the $k_{cat.}/K_m$ values were at least two orders of magnitude lower than those observed with good β-lactam substrates of those enzymes. Nevertheless those compounds will be extremely valuable for comparative studies between the two families of penicillin-recognizing enzymes.

The few structural variations that have been performed on the general structure

certainly do not allow us to draw general conclusions. However, a few points deserve to be underlined.

(1) The fact that compounds of series 3 did not appear to be substrates of any of the enzymes confirmed various 'dogmas' concerning the specificity of DD-peptidases and β-lactamases: (i) the scissile bond must be in α to a free carboxylate (S3a and S3b); (ii) the penultimate residue on the *C*-terminal side must be an α-amino acid. Moreover, if the amino group of this penultimate amino acid was blocked as a sulphonyl derivative, the compound was very poorly recognized.

(2) In racemic mixtures Pronase always hydrolysed one isomer and our enzymes the other. The most likely interpretation was that the former was specific for the L-isomers and the latter for the D-isomers.

(3) In all cases when the structures were strictly comparable, the thioesters were better substrates of all the enzymes than the esters, which might be expected on the basis of the relative sensitivities of the two classes of compounds to nucleophilic attack. A remarkable property of the thioesters was that they were better substrates of class A β-lactamases than of class C, in sharp contrast with the behaviour of the esters.

(4) Surprisingly the β-lactamases and DD-peptidases were active on non-chiral substrates of general structure R–CO–NH–CH_2–CO–X–CH_2–CO_2^-. In some cases a *C*-terminal–CH_2–CO_2^- even yielded a better substrate than a –D-$CHCH_3$–CO_2^- group (compare compounds S1a with compound S1c and compound S2a with compound S2c). Moreover, several enzymes exhibited a strong preference for an aromatic side chain on the *C*-terminal residue (compare compounds S1d and S1e with compound S1a with the *E. cloacae* β-lactamase and with the *Streptomyces* R61 peptidase).

Finally, the activity of the Zn^{2+}-containing *S. albus* G DD-peptidase was rather weak with all substrates, the only exception being S1d, for which the $k_{cat.}/K_m$ value was not much lower than that observed with the classical peptide substrate (6000 $M^{-1} \cdot s^{-1}$).

In conclusion, we have shown that a variety of esters and thioesters can be recognized by β-lactamases and DD-peptidases. For the former enzymes this has little practical importance, since more sensitive methods are available that utilize much better substrates. On the other hand, for the latter enzymes our new substrates will be extremely valuable for direct assays and inhibition studies. Indeed, the hydrolysis of the new compounds can be directly detected in the near u.v., but, in addition, their $k_{cat.}/K_m$ values can be even larger than those observed with the classical peptide substrates. It will be interesting to study the possibility of using the new compounds to assay for the activity of various penicillin-binding proteins that are devoid of activity on short peptide analogues of their true nascent peptidoglycan substrates.

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