On the Chemistry of β-Lactamase Inhibition by 6β-Bromopenicillanic Acid

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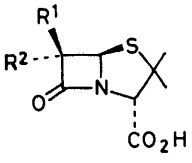
6β-Bromopenicillanic acid, a powerful inhibitor of β-lactamase I from *Bacillus cereus*, reacts with a serine residue in the enzyme and is bound, *via* an ester linkage, as the dihydrothiazine (2a). Spectroscopic and chemical evidence is presented for this assignment and the evidence compared to that obtained from the related model dihydrothiazine (2b). Under certain conditions the bound species underwent further chemical changes caused by an autoxidation reaction; the model dihydrothiazine (2b) undergoes similar autoxidation reactions.

6β-BROMOPENICILLANIC ACID (1a) is a powerful β-lactamase inhibitor.[1,2] By use of a tritiated derivative of the acid it has been shown [3] that it binds to β-lactamase I from *Bacillus cereus* by reaction with the serine unit at position 44 in the amino-acid sequence (position 70 according to the numbering of Ambler [4]). The bound form involves an ester linkage and spectroscopic evidence suggested that the bound species was the dihydrothiazine (2a).[3,5] It was found that, under certain conditions, the bound species underwent further chemical modifications.



(1) a; R^1 = Br, R^2 = H
b; R^1 = H, R^2 = Br
c; R^1 = R^2 = Br
d; R^1 = R^2 = H

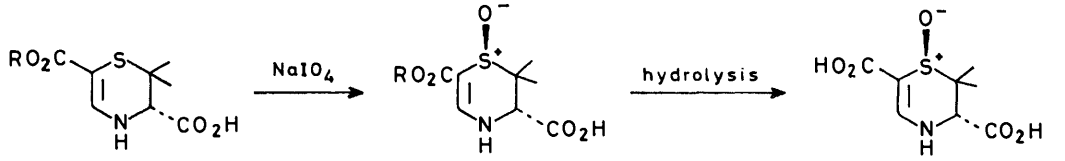
We now record the chemical evidence in support of the dihydrothiazine structure for the bound species and demonstrate that this derivative is readily autoxidised to form, as the major product, the alcohol (8).

DISCUSSION

Two principal routes for preparing 6β-bromopenicillanic acid were explored. Epimerisation, at pH 9.2, of 6α-bromopenicillanic acid (1b), itself prepared using a modification of the method of Cignarella *et al.*,[6] followed by purification by an acid–base extraction procedure, afforded a mixture of 5% of the 6β-isomer and 95% of the enzymically inactive 6α-compound. The equilibrium ratio of the two isomers approaches 1 : 8 but the free acids are unstable in solution and the mixture was converted into the potassium salt. A similar procedure reported by Pratt [7] afforded a product (isolated by lyophilisation of the crude reaction mixture) containing 12% of the 6β-isomer. A higher yield of the 6β-isomer could be obtained by the selective reduction of 6,6-dibromopenicillanic acid (1c) using tributyltin hydride. The reaction product was analysed by ^{1}H n.m.r. spectroscopy as a mixture containing the 6β-isomer (54%) together with the 6α-isomer (1b) (13%) and penicillanic acid (1d) (27%). Subsequent to our work two reports on the use of tributyltin hydride to reduce esters of bromopenicillanic acids have appeared.[8]

The reaction between 6β-bromopenicillanic acid and the β-lactamase I from *B. cereus* resulted in the complete inactivation of the enzyme [9] with concomitant appearance of a new u.v. absorbance at $\lambda_{max.}$ 326 nm. When the protein was treated with 3M-guanidinium chloride, which promotes unfolding of the native enzyme, the absorbance shifted to 314 nm, a value which matches the $\lambda_{max.}$ of the dihydrothiazine (2b). The latter dihydrothiazine, (2b), could be prepared by treatment of 6α-bromopenicillanic acid with 2 equivalents of sodium methoxide in methanol; it was identical to the product obtained by Stoodley [10] from the reaction of 6α-chloropenicillanic acid and sodium methoxide.

The dihydrothiazine structure (2a) is strongly supported by the ^{1}H n.m.r. spectrum of the labelled enzyme at 470 MHz in ^2H_2O [Figure (b)] (these experiments were carried out in collaboration with Dr. I. D. Campbell). The spectrum showed a resonance in the aromatic region at approximately 5.5 p.p.m. downfield from acetone (the signal from acetone is at 2.21 p.p.m. downfield from 2,2-



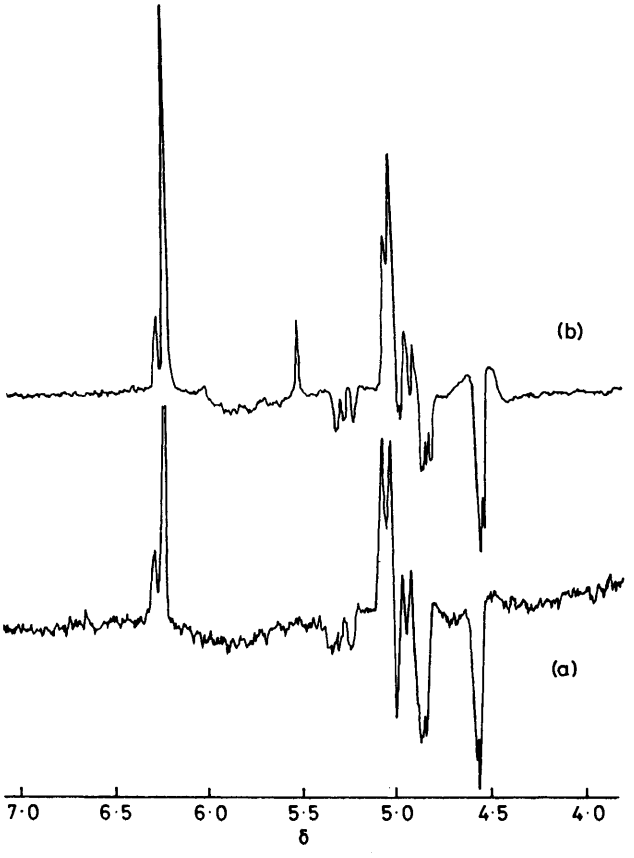
(2) a; R = enzyme
b; R = Me

(3) a; R = enzyme
b; R = Me

(4)

SCHEME 1

dimethyl-2-silapentane-5-sulphonate under these conditions [11]). The unlabelled enzyme lacked this resonance [Figure (a)], whereas the spectrum of the dihydrothiazine (2b) in ^2H_2O showed a resonance at this frequency assigned to H-5. The spin-echo spectra, with different pulse sequences,[12] suggest that resonance in the

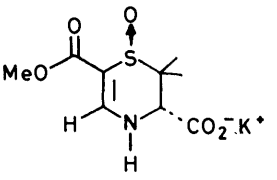


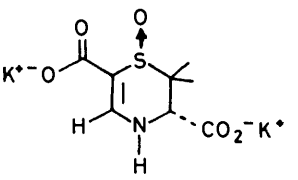
470 MHz N.m.r. spectra of β-lactamase I. The protein (100 mg ml^{-1}) was dissolved in 6M-guanidinium chloride, 0.5M-NaCl, and 1mM-EDTA in ^2H_2O; the apparent pH (glass electrode, meter reading) was 6.0. Spectra were recorded at 37 °C on the Oxford Enzyme Group 470 MHz spectrometer (P. Styles, I. D. Campbell, D. T. Hoult, R. Porteous, R. E. Richards, and N. F. Soffi, in ' Proceedings of the 4th E.E.N.C. Meeting, 1979). The separation between the 90° and 180° pulses was 60 msec; p.p.m. measured downfield from acetone. (a) Unlabelled enzyme. (b) Labelled enzyme.

labelled enzyme is a singlet. The difference between the labelled and unlabelled enzymes was most clearly seen under the conditions of the Figure but could also be seen in normal (not spin-echo) spectra. All these spectra were carried out with denatured enzymes in guanidinium chloride; with undenatured samples the spectra were too complex to discern the difference between labelled and unlabelled enzyme.

Further evidence in support of the dihydrothiazine structure (2a) was obtained by comparing the chemical behaviour of the bound enzyme with that of the model dihydrothiazine compound (2b). Treatment of the bound enzyme with sodium periodate resulted in a shift of the absorbance at 314 nm to a new position at 276 nm (denatured conditions). The dihydrothiazine (2b) also gave rise to a new u.v. absorbance at $\lambda_{max.}$ 274 nm with sodium periodate and the product of this reaction was the sulphoxide (3b), which had previously been prepared [13] by oxidation of (2b) with *m*-chloroperbenzoic acid. It is interesting that the rates of oxidation of the enzyme and of the model dihydrothiazine (2b) were very similar; this suggests that the sulphur atom in (2a) is accessible to solvent. These results suggested that the product resulting from periodate oxidation of the bound enzyme was the sulphoxide (3a). Additional support for this assertion was obtained by examining the hydrolysis product of the oxidised bound enzyme. It was found that under either neutral or mildly alkaline conditions the bound enzyme gradually liberated a compound with electrophoretic properties identical to that of the diacid (4), itself obtained by hydrolysis of the ester sulphoxide

^{13}C N.m.r. chemical shifts and coupling constants [a]

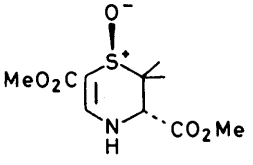




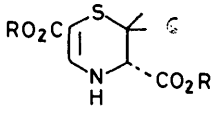
		Assignment
18.10, q, *J* 128.8	18.23, q, *J* 133.8	CH_3
23.70, q, *J* 125.9	23.83, q, *J* 130.8	CH_3
53.13, m	52.21, m	$S-C(CH_3)_2$
54.82, q, *J* 148.4		OCH_3
57.68, d, *J* 143.5	57.42, d, *J* 144.0	$CHCO_2^-$ K^+
96.48, d, *J* 2.97	101.56, d, *J* 3.9	S–C=
150.52, d, *J* 175.78	147.92, d, *J* 172.8	CH=
171.35, m	176.04, m	C=O
175.78, m	176.95, m	C=O

[a] Chemical shifts in p.p.m. relative to acetone referenced against tetramethylsilane. *J* Values in Hz. Values obtained in a JEOL FX 60 spectrometer, using D_2O as solvent.

(3b) with 1M-NaOH (3.5 h, 40 °C) (Scheme 1). The structure of (4) was indicated from its ^{13}C n.m.r. spectrum (Table) and was confirmed by methylation with ethereal diazomethane, which afforded the dimethyl ester (5), identical to the methylation product derived from compound (3b).



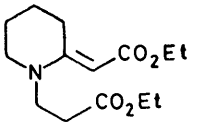
(5)



(6) a; R = H
b; R = Me

The finding that 6β-bromopenicillanic acid reacts with serine-44 of β-lactamase I to generate a dihydrothiazine species provides some insight into the inactivation process. The ester bond which links serine to the bound dihydrothiazine unit is part of a conjugated system and would be expected to be deactivated to nucleophilic

attack as a result of delocalisation of the nitrogen lone-pair. (The resistance of the model compound (2b) towards nucleophiles supports this point. The thiazine (2b) *was found* to be unreactive towards nucleophiles such as ammonia, benzylamine, and hydroxylamine.) Hydrolysis to give the unstable diacid (6a) was achieved, albeit under forcing conditions (1M-NaOH, 40—50 °C, 6 h). By contrast it has been shown [13] that the selective hydrolysis of the simple ester function at C-3 in the ester (6b) can be achieved at room temperature with one equivalent of NaOH in water. A similar result has been observed for the diester (7), in which the saturated ester group can be selectively hydrolysed by base.[14]

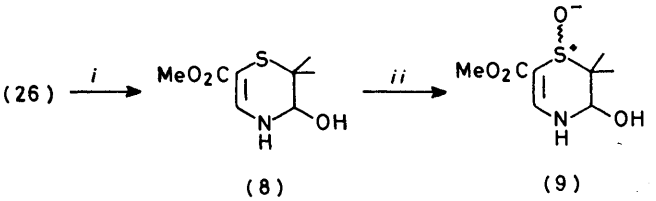


(7)

The conjugated ester at C-6 thus behaves as a vinylogous urethane. It is possible that the electronic properties of this function contributes to its resistance to hydrolysis, as well as the steric constraints imposed on the bound enzyme by formation of the dihydrothiazine ring compared to those imposed by normal substrates that are rapidly turned over by the enzyme. It is of interest that the formation of a vinylogous urethane has been invoked to explain the inactivation of the *E. coli* RTEM β-lactamase by clavulanic acid.[15]

During the course of degradation work on the bound enzyme to establish the active site further chemical changes involving the bound dihydrothiazine moiety were observed. The inactivated enzyme was first digested with trypsin which revealed that the inhibitor was bound to the tryptic peptide T7,[16] which has the structure Phe-Ala-Phe-Ala-Ser-Thr-Tyr-Lys. The parent T7 peptide behaves, electrophoretically, as a monoacidic base, owing to the presence of the lysine residue whilst the bound T7 fragment, which contains an additional carboxy-group contributed by the bound dihydrothiazine moiety, was neutral (zero charge at pH 6.5) when the tryptic digest was subjected directly to electrophoresis. However, when the T7 peptide was first purified by paper chromatography, using n-butanol–acetic acid–water–pyridine (15 : 3 : 12 : 10) as solvent and then subjected to electrophoresis it again behaved as a monoacidic base (charge +1 at pH 6.5). This change in overall charge which occurred during the paper purification step, appeared to be the result either of the loss of a carboxy-group or the unmasking of a new basic centre. In order to clarify this question the chemistry of the dihydrothiazine model compound (2b) was investigated.

The dihydrothiazine ester acid (2b) was stable above pH 7 but at lower pH values autoxidation reactions occurred, which were accompanied by marked changes in the u.v. spectrum. When compound (2b) was dissolved in a buffer of sodium acetate–acetic acid at pH 4.0 in the presence of air the absorbance at $\lambda_{max.}$ 314 nm associated with the thiazine (2b) disappeared and two new absorbances appeared at $\lambda_{max.}$ 303 and 275 nm. The major product (44%) was identified as the thiazine alcohol (8), $\lambda_{max.}$ 303 nm. Also isolated from the reaction were the



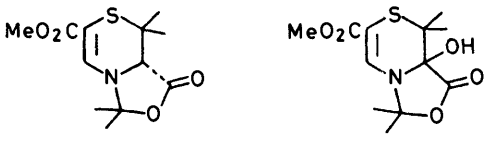
(8) (9)

SCHEME 2 *Reagents: i*, air–H^+; *ii*, $NaIO_4$ or H_2O_2

alcohol sulphoxides (9), $\lambda_{max.}$ 275 nm (15%), which were obtained as a 2 : 1 mixture of isomers. The alcohol sulphoxides (9) could also be prepared from the alcohol (8) by oxidation with either sodium periodate or hydrogen peroxide. A small amount of the dihydrothiazine sulphoxide (3b), $\lambda_{max.}$ 275 nm (6%), was also obtained from the reaction (Scheme 2). The same u.v. changes were also observed when the thiazine (2b) was agitated as a suspension in either 1% v/v aqueous acetic acid or water and in both instances the major product isolated was again the thiazine alcohol (8). These latter procedures were, however, less reliable and reproducible than the former reaction procedure. When the dihydrothiazine (2b) was treated with 1% aqueous acetic acid under oxygen- and peroxide-free conditions no change occurred, confirming that the reaction is an autoxidation process.

These results suggested that the change in the charge on the T7 peptide observed during the purification procedure might also be associated with an autoxidative decarboxylation of the bound dihydrothiazine to give a derivative related to the alcohol (8). In order to test this hypothesis the dihydrothiazine (2b) was subjected to the same purification procedure used for the T7 fragment. Its subsequent electrophoretic mobility indicated the formation of a neutral compound (zero charge at pH 6.5). Furthermore, the u.v. spectrum of this material consisted mainly of a band at $\lambda_{max.}$ 303 nm, corresponding to the absorbance of the alcohol (8).

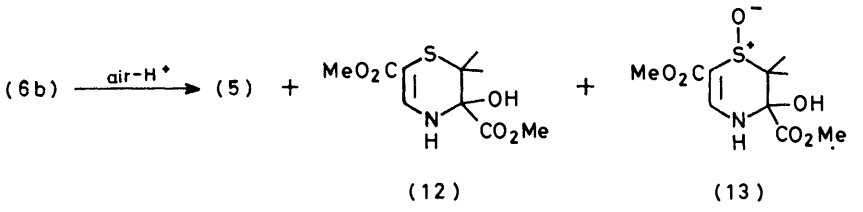
The autoxidation of compound (2b) to produce the dihydrothiazine alcohol (8) finds some precedent in the reports by Stoodley [17] that the dihydrothiazine (10) is converted into the hydroxy-derivative (11) in acetone



(10) (11)

containing toluene-*p*-sulphonic acid. The mechanism of the autoxidation reaction was briefly explored. The possibility that the sulphoxide (3b) was a precursor of the alcohol (8) was ruled out since it was stable in both dilute

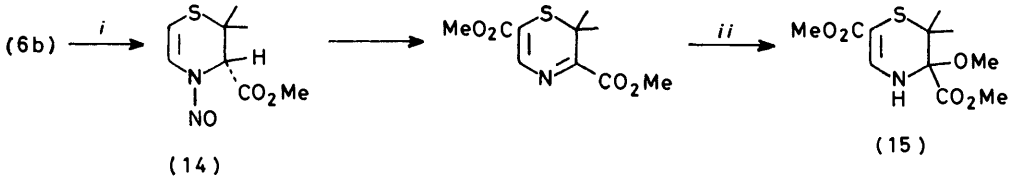
acetic and hydrochloric acids. In order to shed further light on the mechanism, the autoxidation of the diester (6b) was examined. It was found that the diester (6b) underwent a relatively slow autoxidation at room temperature in a mixture of acetonitrile and acetic acid. The rate of autoxidation was increased by warming the mixture in the presence of a small quantity of the radical initiator azobisisobutyronitrile and, under these conditions, the same products were formed. The least polar compound, isolated in 26% yield was identified as



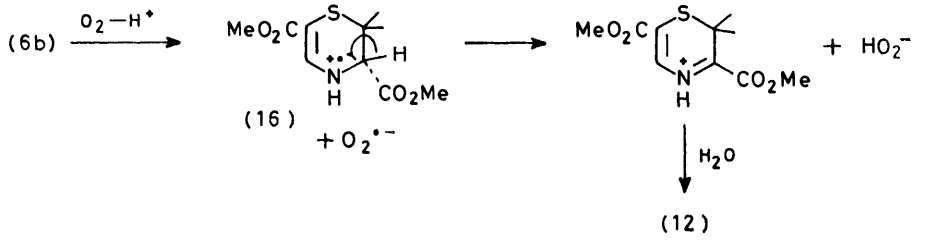
SCHEME 3

(6b) to the hydroxy-compound (12) also has precedent in the oxidation of the compound (6b) to (15) by nitrous acid in methanol.[18] The mechanism postulated for this reaction (Scheme 4) involved the nitroso-intermediate (14).

The autoxidation of the diester (6b) is best explained *via* a radical intermediate (16) (Scheme 5). In the case of the monomethyl ester (2b), formation of the alcohol (8) could proceed either by an initial oxidation to (17) followed by decarboxylation, or by a concerted oxidative

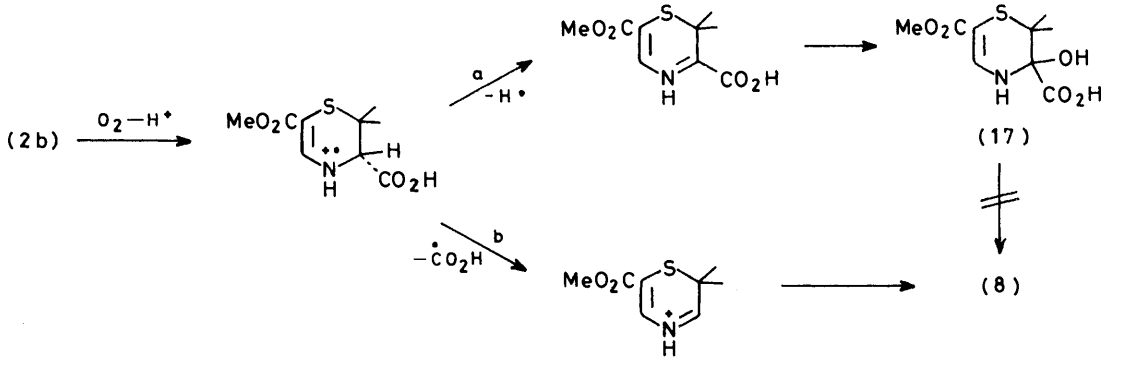


SCHEME 4 *Reagents: i*, HNO_2–MeOH; *ii*, MeOH



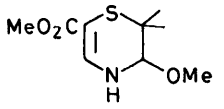
SCHEME 5

the hydroxy-ester (12), which was previously described by Stoodley [18] and formed by acidification of the methoxy-derivative (15). In addition, the sulphoxides (5) and (13) were obtained in yields of 16 and 26% respectively (Scheme 3). The oxidation of the diester decarboxylation. The former process could be ruled out since the postulated intermediate (17) is not transformed into the alcohol (8) under acidic conditions. The most likely reaction path therefore proceeds *via* path b, Scheme 6.



SCHEME 6

Autoxidation of the acid (2b) in methanol gave the methoxy-derivative (18), which is consistent with the intermediacy of an imine species. Finally, oxidation of the acid (2b) to the alcohol (8) could also be accomplished



(18)

(82% yield) by use of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) in aqueous acetone at pH 4, whilst the ester (6b) with DDQ afforded the hydroxy-ester (12).

EXPERIMENTAL

M.p.s were recorded with a Kofler hot-stage apparatus. I.r. spectra were recorded with a Perkin-Elmer 157G spectrometer and u.v. spectra with a Unicam SP800 instrument for solutions in ethanol. ^{1}H N.m.r. spectra were obtained with a JEOL MH100 instrument, with deuteriochloroform as solvent, unless otherwise stated, and tetramethylsilane as internal reference. Mass spectra were obtained with a Kratos–A.E.I. MS30 instrument. T.l.c. was carried out on Merck silica gel GF_{254} using the solvent system indicated. Light petroleum refers to the fraction of boiling range 40—60 °C. Solutions were dried over $MgSO_4$.

6α-Bromopenicillanic acid (1b).—6-Aminopenicillanic acid (10.8 g, 0.05 mol) was added to water (225 ml) containing sodium bromide (25.73 g, 0.25 mol), concentrated H_2SO_4 (25 ml), and methanol (250 ml) cooled to 0 °C. The resulting solution was treated with sodium nitrite (5.18 g, 0.075 mol) whilst maintaining the temperature of the solution at 0—2 °C. After 30 min the solution was warmed to 5 °C over 15 min and then extracted with chloroform (400 ml). The organic extract was dried and then concentrated to a pale yellow foam (10.02 g, 72%), δ 1.56 (3 H, s, CH_3), 1.64 (3 H, s, CH_3), 4.57 (1 H, s, H-3), 4.82 (1 H, d, *J* 1.5 Hz, H-6), and 5.40 (1 H, d, *J* 1.5 Hz, H-5), $\nu_{max.}$ (KBr) 1 780 and 1 740br cm^{-1}. The *NN'*-dibenzylethylenediamine salt was prepared by treatment of the acid (1.11 g) with the amine (0.48 g) in ether at 0 °C. The precipitate (1.29 g, 81%) was recrystallised from chloroform–ethanol, m.p. 163—165 °C (decomp.) (lit.,[6] 164—165°), $\nu_{max.}$ (KBr) 1 765 and 1 605 cm^{-1} (Found: C, 47.8; H, 5.0; N, 6.7. $C_{32}H_{40}Br_2N_6O_6S_2$ requires C, 48.0; H, 5.0; N, 7.0%). The pure, free acid was regenerated by acidification (H_3PO_4) of a solution of the dibenzylethylenediamine salt (3.00 g) in water (350 ml) and methanol (470 ml) at 0 °C to pH 2.5. The solution was extracted with dichloromethane and the extracts washed with saturated brine followed by water, dried, and concentrated to a colourless foam (1.98 g, 94%). The potassium salt was prepared by treatment of an ice-cold solution of the free acid (300 mg) in dry ether with potassium 2-ethylhexanoate (195 mg) dissolved in dry ether containing a small amount of absolute ethanol. The *potassium salt*, which precipitated from solution, was collected by centrifugation, washed with ether, and dried (282 mg, 79%). Recrystallisation from dry ether–ethanol afforded needles, m.p. 214 °C (decomp.) (vacuum-sealed tube, preheated to 190 °C), $[\alpha]_D^{25}$ + 191° (*c* 0.58 in MeOH), $\nu_{max.}$ (KBr) 1 770 and 1 600 cm^{-1}, δ (CD_3OD) 1.55 (3 H, s, CH_3), 1.60 (3 H, s, CH_3), 4.34 (1 H, s, H-3), 4.97 (1 H, d, *J* 1.5 Hz, H-6), and 5.47 (1 H, d, *J* 1.5 Hz, H-5) (Found: C, 30.1; H, 2.7; N, 4.4. $C_8H_9NO_3BrSK$ requires C, 30.2; H, 2.85; N, 4.4%).

6β-Bromopenicillanic Acid (1a).—(a) *Epimerisation of 6α-bromopenicillanic acid.* A solution of 6α-bromopenicillanic (1b) (100 mg) in buffer (0.025M-borax solution, adjusted to pH 9.2 with NaOH) was stirred at room temperature for 89 h. The solution was cooled in ice, acidified to pH 2 with 10% H_3PO_4, and extracted into ether. The ether layers were washed with saturated sodium sulphate solution, dried, and concentrated to a foam (74 mg, 74%) which contained 5% (n.m.r.) of the 6α-isomer, δ 1.57 (s, overlapping 3α-CH_3), 1.71 (s, 3β-CH_3), 4.59 (s, H-3), 5.37 (d, *J* 4 Hz, H-6), and 5.59 (d, *J* 4 Hz, H-5). The mixture of the free acids was converted into the corresponding potassium salts (68%), using potassium 2-ethylhexanoate by the above-described procedure.

(b) *Reduction of 6,6-dibromopenicillanic acid.* Tributyltin hydride (214 mg, 0.73 mmol) was added in portions over 35 h to a solution of the dibromo-acid (220 mg, 0.61 mmol) in ether (10 ml), containing a few mg of azobisisobutyronitrile, under reflux. After removal of the solvent the residue was triturated with light petroleum and then dried *in vacuo* to yield a foam (157 mg), consisting mainly (^{1}H n.m.r.) of the 6β-bromopenicillanic acid (54%), δ 1.57 (3 H, s, CH_3), 1.71 (3 H, s, CH_3), 4.59 (1 H, s, H-3), 5.37 (1 H, d, *J* 4 Hz, H-6), 5.59 (1 H, d, *J* 4 Hz, H-5), as well as the 6α-isomer (13%), penicillanic acid (27%), and the starting 6,6-dibromopenicillanic acid (6%).

(3S)-3,4-*Dihydro*-6-*methoxycarbonyl*-2,2-*dimethyl*-2H-1,4-*thiazine*-3-*carboxylic Acid* (2b).—6α-Bromopenicillanic acid (19.3 g, 0.07 mol) in methanol (150 ml) at 0 °C was treated with sodium methoxide [from Na (3.3 g)] in methanol (75 ml) and the mixture allowed to warm to room temperature over 14 h before the precipitate was collected (14.3 g, 82%), $\nu_{max.}$ (KBr) 3 360, 1 650, and 1 610 cm^{-1}. The salt (10 g) was dissolved in water (200 ml) and acidified to pH 1 with 2M-HCl, and the free acid (2b), which precipitated, was collected, washed with a little water, and dried. The acid (8.01 g, 88%) was purified by reprecipitation, m.p. 195 °C (decomp.; vacuum sealed tube preheated to 160 °C) [lit.,[18] 176—178 °C (decomp.)]; $[\alpha]_D^{25}$ −127° (*c* 0.4 in MeOH), $\nu_{max.}$ (KBr) 3 330, 1 750, 1 700, 1 660, and 1 600 cm^{-1}; $\lambda_{max.}$ (EtOH) 314 nm (ε 12 000) (Found: C, 46.6; H, 5.6; N, 6.0. Calc. for $C_9H_{13}NO_4S$: C, 46.75; H, 5.65; N, 6.0%).

The thiazine was also produced by similar treatment of the mixture of 6α- and 6β-bromopenicillanic acids.

(1S,3S)-3-*Carboxy*-3,4-*dihydro*-6-*methoxycarbonyl*-2,2-*dimethyl*-2H-1,4-*thiazine* 1-*Oxide* (3b).—The dihydrothiazine (2b) (0.23 g, 1 mmol) was dissolved in 1% w/v ammonium hydrogencarbonate solution (7.5 ml) and treated with sodium periodate (0.21 g, 1 mmol) in water (2 ml). After 10 min the solution was saturated with NaCl and washed with ethyl acetate. The aqueous phase was adjusted to pH 1 with 2M-HCl and exhaustively extracted with ethyl acetate. Concentration of the dried extracts afforded the sulphoxide (3b) (0.23 g, 98%), $[\alpha]_D^{25}$ −92° (*c* 0.26 in H_2O). Upon recrystallisation from acetone–ethyl acetate some loss of optical activity occurred, the crystals obtained having $[\alpha]_D^{25}$ −39° (*c* 0.4 in H_2O), m.p. 135—136 °C (lit.,[13] 140—142 °C for racemate), $\nu_{max.}$ (KBr) 3 240, 1 695, and 1 595 cm^{-1}, $\lambda_{max.}$ (EtOH) 275 nm (ε 12 700), δ [$(CD_3)_2SO$] 0.82 (3 H, s, CH_3), 1.36 (3 H, s, CH_3), 3.68 (3 H, s, OCH_3), 3.96 (1 H, d, *J* 1 Hz, H-3), 7.78 (1 H, d, *J* 8 Hz, H-5), and 8.87br (1 H, d, *J* 8 Hz, NH) (addition of D_2O caused the doublet at δ 8.87 to disappear and the signals at δ 3.96 and 7.78 to collapse to

singlets) (Found: C, 43.8; H, 5.4; N, 5.8. Calc. for $C_9H_{13}NO_5S$: C, 43.7; H, 5.3; N, 5.7%).

(1S,3S)-3,4-*Dihydro*-3,6-*bismethoxycarbonyl*-2,2-*dimethyl*-2H-1,4-*thiazine* 1-*Oxide* (5).—Esterification of (3b) with diazomethane, followed by trituration with ether, afforded the diester (5) as a solid, $[\alpha]_D^{25}$ −52° (*c* 1.5 in $CHCl_3$), $\nu_{max.}$ (KBr) 3 290, 3 180, 1 740, 1 670, and 1 585 cm^{-1}, δ 0.96 (3 H, s, CH_3), 1.52 (3 H, s, CH_3) 3.80 (3 H, s, OCH_3), 3.88 (3 H, s, OCH_3), 4.30 (1 H, s, H-3), 7.14br (1 H, NH), and 7.86 (1 H, d, *J* 8 Hz, H-5), δ_C ($CDCl_3$) 15.82 (q, *J* 128.8, CH_3) 21.77 (q, *J* 129.8, CH_3), 50.78 (m, C-2), 51.79 (q, *J* 147.5, OCH_3), 53.19 (q, 148.2, OCH_3), 53.19 (d, *J* 148.5, C-3), 99.83 (d, *J* 4.95, C-6), 145.66 (d, *J* 174.7, C-5), 166.21 (m, C=O), and 170.24 (m, C=O). Recrystallisation of (5) from dichloromethane–toluene afforded the racemate, $[\alpha]_D^{25}$ 0°, m.p. 120—122 °C (lit.,[13] 114—116 °C) (Found: C, 46.1; H, 5.9; N, 5.2. Calc. for $C_{10}H_{15}NO_5S$: C, 46.0; H, 5.8; N, 5.4%.)

(1S,3S)-3,6-*Dicarboxy*-3,4-*dihydro*-2,2-*dimethyl*-2H-1,4-*thiazine* 1-*Oxide* (4).—The sulphoxide (3b) (0.25 g, 1 mmol) was heated in 1M-NaOH) (10 ml) at 40 °C for 3.5 h. The solution was cooled in ice, saturated with NaCl, acidified to pH 1 with 6N-HCl and extracted with ethyl acetate to afford the *title compound* as a foam (77 mg, 33%), $\nu_{max.}$ (KBr) 3 310, 1 710br, 1 660, and 1 590 cm^{-1}, $\lambda_{max.}$ (EtOH) 277 nm, δ [$(CD_3)_2SO$] 0.80 (3 H, s, CH_3) 1.35 (3 H, s, CH_3), 3.91 (1 H, s, H-3), 7.69 (1 H, d, *J* 8 Hz, H-5), and 8.64 (1 H, d, *J* 8 Hz, NH). Methylation of this material with ethereal diazomethane produced the diester sulphoxide (5).

Attempted Reactions of the Dihydrothiazine (2b) *with Nucleophiles*.—(*a*) The thiazine (46 mg) was stirred with 1M-NH_3 solution (5 ml) at room temperature. After 6 days, no change had occurred and acidification gave starting material (53%).

(*b*) A suspension of the thiazine (46 mg) in water (5 ml) was treated with benzylamine (2 equiv.). After 6 days no change was apparent and starting material (59%) was recovered.

(*c*) A solution of the sodium salt of the thiazine (46 mg) in water (5 ml) was treated with hydroxylamine hydrochloride (13.9 mg, 1 equiv.) at 40 °C for 4 days No change was noted in this time and the reaction mixture gave a negative hydroxamic acid test.

(3S)-3,4-*Dihydro*-2,2-*dimethyl*-2H-1,4-*thiazine*-3,6-*dicarboxylic Acid* (6a).—The monoethyl ester (2b) (0.57 g, 2.5 mmol) was heated under nitrogen in 1N-NaOH (25 ml) at 40 °C for 6 h. The solution was cooled to 0 °C, acidified to pH 2.5 with phosphoric acid, and extracted with ethyl acetate (8 × 30 ml) at 0—5 °C. The extracts were washed with saturated brine, dried, and concentrated to give the unstable *diacid* (6a) (0.40 g, 75%) as a foam, $\nu_{max.}$ (KBr) 3 370, 1 715, 1 645, and 1 595 cm^{-1}, $\lambda_{max.}$ (EtOH) 313 and 270 (shoulder) nm, δ (D_2O, Na salt) 1.20 (3 H, s, CH_3), 1.46 (3 H, s, CH_3), 3.72 (1 H, s, H-3), and 7.43 (1 H, s, H-5). Methylation of the diacid with ethereal diazomethane produced the diester (6b) (80%), m.p. 165—167 °C (from MeOH) (lit.,[10] 165—167 °C), $[\alpha]_D^{25}$ −37° (*c* 0.44 in $CHCl_3$), identical to the sample produced by methylation of the monoester (2b).

Oxidation of the Diacid (6a) *with Periodate*.—The diacid (6a) (0.15 g, 0.7 mmol) and water (5 ml) containing ammonium hydrogencarbonate (0.16 g, 1.6 mmol) at 0 °C was treated with sodium periodate (0.16 g, 0.7 mmol) in water (2 ml) for 30 min. The solution was saturated with NaCl, acidified to pH 2 with phosphoric acid, and exhaustively extracted with ethyl acetate and then with ethyl acetate–n-butanol (3 : 2). Concentration of the dried extracts afforded a crude product (0.13 g), the major component of which corresponded [t.l.c. (EtOAc–MeOH, 1 : 1, R_F 0.19) and ^1H n.m.r.] to the diacid sulphoxide (4).

Autoxidation Reactions of the Thiazine Monomethyl Ester (2b).—(a) *Autoxidation at* pH 4. The thiazine (2b) (0.5 g, 2.2 mmol) was dissolved in a pH 4 buffer (sodium acetate–acetic acid; 100 ml) and stirred at room temperature for 4 h under exposure to air. The solution was neutralised with sodium hydrogencarbonate and extracted with dichloromethane. From the dried extract was obtained a foam; trituration with ether afforded crystals of optically inactive methyl 3,4-*dihydro*-3-*hydroxy*-2,2-*dimethyl*-2H-1,4-*thiazine*-6-*carboxylate* (8) (0.20 g, 44%), m.p. 88—89 °C, $\nu_{max.}$ (KBr) 3 420, 3 300, 1 640, and 1 590 cm^{-1}, $\lambda_{max.}$ (EtOH) 303 nm (ε 10 500), δ 1.19 (3 H, s, CH_3), 1.33 (3 H, s, CH_3), 2.56br (1 H, s, OH), 3.72 (3 H, s, OCH_3), 4.29 (1 H, d, *J* 4.5 Hz, H-3), 5.8br (1 H, s, NH), and 7.47 (1 H, d, *J* 7 Hz, H-5) (addition of D_2O caused the disappearance of the NH and OH signals and collapse of the doublets to singlets) (Found: C, 47.4; H, 6.45; N, 6.8%; M^+ 203.061 0. $C_8H_{13}NO_3S$ requires C, 47.3; H, 6.45; N, 6.9%; M^+ 203.061 6.) The aqueous phase from the autoxidation was saturated with NaCl and extracted exhaustively with ethyl acetate. Concentration of the dried extracts afforded a foam (68 mg, 14%) containing a mixture of the alcohol sulphoxides (9) [R_F 0.55 and 0.25 (EtOAc–acetone, 1 : 3)] in which the less polar sulphoxide predominated (*ca.* 2 : 1). The aqueous phase was cooled to 0 °C, acidified to pH 2 with 2M-HCl, and extracted with ethyl acetate. The dried extract was concentrated to afford a foam (32 mg), the major component corresponding (t.l.c., i.r., and n.m.r.) to the thiazine sulphoxide (3b).

(b) *Autoxidation at* pH 2.8 (1% *acetic acid*). The thiazine (2b) (0.23 g, 1 mmol) was suspended in 1% acetic acid (40 ml) with vigorous stirring at room temperature for 10 h in air. The resulting, almost clear, solution was neutralised with sodium hydrogencarbonate and extracted with dichloromethane. Work-up, as described in (a), afforded the thiazine alcohol (8) (90 mg, 44%), m.p. 88—89 °C (from ether), and the alcohol sulphoxides (9) (12 mg, 6%).

(c) *Autoxidation in water*. The thiazine (2b) (0.15 g, 0.65 mmol) was suspended in water (25 ml) and shaken vigorously for 24 h in air. The reaction mixture was extracted with chloroform, washed with brine, dried, and concentrated to yield a foam (87 mg); preparative t.l.c. (chloroform–EtAc, 3 : 2) afforded the pure alcohol (54 mg, 36%), m.p. 88—89 °C.

(d) *Control*. When the thiazine (2b) (0.5 g) was suspended in 1% acetic acid (75 ml; previously refluxed under argon for 25 h) and vigorously stirred at room temperature, under argon for 28 h no change occurred. Under similar conditions in the presence of air, all the thiazine was consumed within this time.

Periodate Oxidation of the Alcohol (8).—The alcohol (0.40 g, 2.0 mmol) in tetrahydrofuran (10 ml) was oxidised with sodium periodate (0.47 g, 2.2 mmol) in water (10 ml) at room temperature for 2 h. The reaction mixture was diluted with water, concentrated *in vacuo*, and treated with barium acetate solution until no further precipitate formed. The mixture was filtered and the solution stirred with Amberlite 1RA-120 (H^+) to remove sodium ions before re-filtering and freeze drying, to give, as a foam (0.39 g), a mixture of the 3,4-*dihydro*-3-*hydroxy*-6-*methoxycarbonyl*-2,2-*dimethyl*-2H-1,4-*thiazine* 1-*oxides* (9). Preparative t.l.c. (EtOAc–acetone, 1 : 3) gave the less polar sulphoxide alcohol (R_F 0.55) (78 mg, 20%), m.p. 136—137 °C (from acetone–

toluene, $\nu_{max.}$ (KBr) 3 200br, 1 675, and 1 590 cm^{-1}, $\lambda_{max.}$ (EtOH) 275 nm (ε 15 200), δ [$(CD_3)_2SO$] 0.91, (3 H, s, CH_3), 1.68 (3 H, s, CH_3), 3.72 (3 H, s, OCH_3), 4.84 (1 H, d, *J* 9 Hz, H-3), 6.72 (1 H, d, *J* Hz, NH), 7.80 (1 H, d, *J* 7 Hz, H-5), 9.40 (1 H, br s, OH) (exchange with D_2O caused disappearance of the OH and NH signals and collapse of the doublets to singlets) (Found: C, 43.8; H, 6.0; N, 6.3%; M^+ 219.056 5, $C_8H_{13}NO_4S$ requires C, 43.8; H, 6.0; N, 6.4%; M^+ 219.056 5).

The more polar sulphoxide (R_F 0.25) was obtained as colourless crystals (125 mg, 29%), m.p. 194—195 °C, $\nu_{max.}$ (KBr) 3 150, 1 695, and 1 585 cm^{-1}, $\lambda_{max.}$ (EtOH) 274 nm (ε 13 200), δ [$(CD_3)_2SO$] 0.78 (3 H, s, CH_3) 1.27 (3 H, s, CH_3), 3.66 (3 H, s, OCH_3) 4.81br (1 H, s, H-3), 6.97 (1 H, d, *J* 6 Hz, NH), 7.62 (1 H, d, *J* 6 Hz, H-5), and 8.72 (1 H, s, OH) (exchange with D_2O caused disappearance of the OH and NH signals and collapse of the doublets to a singlet) (Found: C, 43.9; H, 6.1; N, 6.15%; M^+ 219.056 5. $C_8H_{13}NO_4S$ requires C, 43.8; H, 6.0; N, 6.4; M^+ 219.056 5).

Oxidation of the sulphide (8) (0.2 g, 1 mmol) in aqueous acetone was also effected with hydrogen peroxide (3.5 mmol) at room temperature over 22 h. The reaction mixture was concentrated *in vacuo*, saturated with NaCl, and extracted with ethyl acetate to give a mixture of the sulphoxides (9) (0.15 g, 68%) in which the less polar component predominated by a factor of 3 : 1 (^1H n.m.r.).

Autoxidation of the Thiazine Diester (6b).—(a) *With free-radical initiator.* A solution of the thiazine diester (50 mg, 0.2 mmol) in acetonitrile (3 ml) and 1% acetic acid (3 ml) was warmed to 45 °C whilst air was bubbled through it. Portions of azobisisobutyronitrile (32 mg) were added during the course of the reaction. After 25 days no starting material remained. Work-up, by preparative t.l.c. (ethyl acetate–acetone, 3 : 1) afforded three products. The least polar material (R_F 0.79) corresponded to the hydroxythiazine (12) and was isolated as an oil (14 mg, 26%) which crystallised on addition of ether, m.p. 116—118 °C (lit.,[18] 116—118 °C), $\nu_{max.}$ (KBr) 3 360, 3 340, 1 735, 1 680, and 1 600 cm^{-1}, $\lambda_{max.}$ (EtOH) 306 nm (ε 10 000), δ 1.18 (3 H, s, CH_3), 1.45 (3 H, s, CH_3), 3.38br (1 H, s, OH), 3.81 (3 H, s, OCH_3), 3.96 (3 H, s, OCH_3), 6.28br (1 H, s, NH), and 7.66 (1 H, d, *J* 8 Hz, H-5) (the signals at δ 3.38 and 6.28 were exchanged with D_2O) (Found: C, 46.0; H, 5.8; N, 5.6; Calc. for $C_{10}H_{15}NO_5S$: C, 46.0; H, 5.8; N, 5.35%).

The second component (R_F 0.53), a crystalline solid (9 mg, 16%), was 3,4-*dihydro*-3-*hydroxy*-3,6-*bismethoxycarbonyl*-2,2-*dimethyl*-2H-1,4-*thiazine* 1-*oxide* (13), m.p. 158—160 °C $\nu_{max.}$ (KBr) 3 360, 3 180, 1 745, 1 645 and 1 590 cm^{-1}, $\lambda_{max.}$ (EtOH) 276 nm (ε 12 000) δ 1.02 (3 H, s, CH_3), 1.98 (3 H, s, CH_3), 3.90 (3 H, s, OCH_3), 3.96 (3 H, s, OCH_3), 7.50br (1 H, s, NH), 7.93 (1 H, d, *J* Hz, H-5), and 8.34br (1 H, s, OH) (Found: C, 43.4; H, 5.5; N, 5.2%; M^+ 277.060 4. $C_{10}H_{15}NO_6S$ requires C, 43.3; H, 5.2; N, 5.05%; M^+ 277.062 0.)

The most polar product (R_F 0.29) was the diester sulphoxide (5), crystalline solid (14 mg, 26%).

(b) *Without free-radical initiator.* The thiazine (6b) (50 mg) in acetonitrile (3 ml) and 1% aqueous acetic acid (3 ml) was stirred at room temperature in air for 6 weeks. Work-up gave mainly starting material (25 mg) together with a mixture of three products shown (t.l.c.) to be the same as those isolated from the reaction using a free-radical initiator.

Stability of the Thiazine Sulphoxide (3b) *to Acid.*—(a) 1% *Acetic acid.* A solution of the sulphoxide (3b) in 1% acetic acid was stirred at room temperature. No change was detected (t.l.c. and u.v.) after 14 days.

(b) 0.1M-*Hydrochloric acid.* The sulphoxide (3b) was recovered unchanged after treatment with 0.1M-HCl at room temperature for 22 days.

Stability of the Alcohol (17) *to Acid.*—A solution of the sodium salt of the acid (17) (8 mg), prepared [18] by treatment of (12) with 0.1M-sodium hydroxide for 1 h at room temperature, was acidified to pH 3.5 with glacial acetic acid and stirred in air for 72 h. None of the alcohol (8) was detected after this time and, after freeze-drying, the ^1H n.m.r. spectrum of the residue corresponded to that of the hydroxy-acid (17).

Oxidation of the Dihydrothiazine (2b) *with Hydrogen Peroxide.*—The thiazine (2b) (0.23 g, 1 mmol) was partially dissolved in acetic acid (25 ml) and treated with hydrogen peroxide (1 mmol) at room temperature for 5 h. The solution was neutralised to pH 7 ($NaHCO_3$), saturated with NaCl, and extracted with ethyl acetate to give, as a foam (75 mg, 20%), the sulphoxides (9), in which the less polar sulphoxide predominated (3 : 1).

Methyl 3,4-*Dihydro*-3-*methoxy*-2,2-*dimethyl*-2H-1,4-*thiazine*-6-*carboxylate* (18).—The thiazine (2b) (0.5 g, 2.2 mmol) in methanol (20 ml) containing glacial acetic acid (0.2 ml) was stirred at room temperature for 12 days. The solution was diluted with ether (100 ml) and shaken with pH 7.5 buffer (100 ml). The organic layer was washed with more buffer, dried, and concentrated to give the thiazine (18) (0.13 g, 27%), m.p. 122—123 °C (from CH_2Cl_2–toluene) (lit.,[18] 128 °C), $\nu_{max.}$ (KBr) 3 315, 1 655, and 1 600 cm^{-1}, $\lambda_{max.}$ (EtOH) 305 nm (ε 8 150), δ 1.20 (3 H, s, CH_3), 1.38 (3 H, s, CH_3), 3.47 (3 H, s, OCH_3), 3.77 (3 H, s, OCH_3), 4.08 (1 H, d, *J* 4 Hz, H-3), 5.74br (1 H, s, NH), and 7.60 (1 H, d, *J* 6 Hz, H-5) (Found: 49.7; H, 7.0; N, 6.25. Calc. for $C_9H_{15}NO_3S$: C, 49.75; H, 7.0; N, 6.45%).

Liberation of the Diacid (4) *from Modified* β-*Lactamase* I.—Labelled enzyme (23.6 mg, specific radioactivity 2.24 μCi μmol^{-1}) in 1% ammonium hydrogencarbonate (2.2 ml) was treated with 20 μl of 100 mM-sodium periodate for 30 min and then dialysed against 1% w/v ammonium hydrogencarbonate (5 ml), first for 3.5 h at 4 °C (to remove most of the excess of sodium periodate) and then for 48 h at 28 °C. The diffusate was examined by ion-pair reversed-phase (surfactant) h.p.l.c. on a Waters Corasil C18 column, elution being carried out with triethylamine acetate (0.1M-acetic acid brought to pH 5.1 with triethylamine) containing 5mM-cetyltrimethylammonium bromide. The peak (detected by absorption at 275 nm) contained the radioactive material, which was eluted at the same position as the diacid (4) and extracted with chloroform (to remove cetyltrimethylammonium bromide). The radioactive product, examined by paper electrophoresis at pH 4.5 and pH 6.5 (under the conditions described earlier [3]) and by paper chromatography in the solvent [19] described above, had the same mobility as the diacid (4).

Oxidations with DDQ.—The thiazine (2b) (46 mg) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) (45 mg, 1 equiv.) were stirred at room temperature in a sodium acetate–acetic acid buffer (pH 4.0) (10 ml) for 2.5 h. The solution was adjusted to pH 9 (dilute NaOH), extracted with CH_2Cl_2, dried, filtered, and evaporated to give the alcohol (8) (33 mg, 82%), m.p. 88—89 °C (from ether), identical (^1H n.m.r., t.l.c.) to the sample prepared above.

Oxidation of the diester (6b) (245 mg) with DDQ (227 mg, 1 equiv.) in wet acetone (10 ml) at room temperature over-

night, followed by normal work-up, afforded the hydroxy-ester (12) (247 mg, 95%), m.p. 116.5—117.5 °C (from ether), identical (i.r., ^{1}H n.m.r., t.l.c.) to the compound described above.

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