The Interaction of Chlorinated 6-Spiroepoxypenicillins with *Bacillus cereus* β-Lactamase I: Irreversible Inhibition and Turnover

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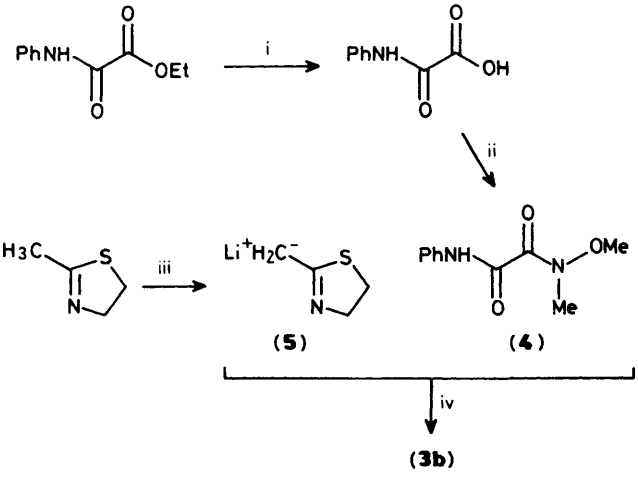
The chlorinated 6-spiroepoxypenicillin anilides (**1a**) and (**1b**) are irreversible inhibitors of β-lactamase I from *Bacillus cereus*, but they are also turned over by this enzyme to yield the same hydrolysis product, whose structure has been revealed to be an unusual 6-substituted-1,4-dihydrothiazine-3-carboxylate, α-ketoamide; a possible pathway for the turnover and inhibitory processes associated with these interactions is presented.

Recently, we have reported that the novel chlorinated 6-spiroexpoxypenicillins (**1**)[1] are potent *in vitro* inhibitors of a range of clinically important β-lactamases.[2] In view of the current interest in β-lactam-derived, mechanism-based inhibitors for these enzymes,[3] it was considered important to establish the nature of the molecular processes associated with their inhibitory activity. To this end we investigated the inhibition of the readily available and well characterised, class A[4] β-lactamase I from *Bacillus cereus*[5] by the anilides (**1a**) and (**1b**).

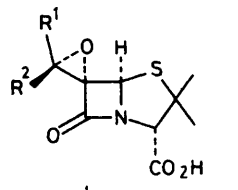
Kinetic analysis, after incubation of ion-exchange purified enzyme with (**1a**), or (**1b**) using nitrocefin as reporter substrate, revealed that the compounds are both turned over as substrates [$k_{cat.}/K_m$; (**1a**) 748 mol^{-1} dm^3 s^{-1}; (**1b**) 3 824 mol^{-1} dm^3 s^{-1}] prior to the irreversible inactivation event. In the case of (**1a**), the ratio of turnover to inactivation is 2 080 : 1; for (**1b**), this ratio is 10 800 : 1.†

In addition, incubation at 30 °C of a solution (pH 7.0) containing (**1a**), (**1b**), or a 1 : 1 mixture of (**1a**) and (**1b**) (0.16 mM), and β-lactamase I (4.0 μM) afforded a single turnover product (TP) (u.v.; $\lambda_{max.}$ 350 nm), which was separated from enzyme and buffer salts by hydrophobic interaction chromatography (h.i.c.) on Diaion HP-20 resin and elution with MeCN. The turnover product, as its sodium salt (TP–Na), was then purified to homogeneity by semi-preparative reverse-phase h.p.l.c.‡ After desalting again by h.i.c. and lyophilisation from water, TP–Na was isolated as a yellow amorphous powder.§ Overnight treatment of TP–Na (≤ 1 mg) at 0—5 °C with excess MeI in acetone (0.5 ml) led to the isolation of a methyl ester, TP–Me.§

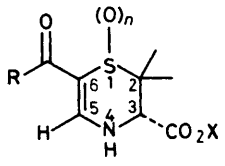
I.r. spectroscopic analysis of TP–Na indicated disappearance of the characteristic β-lactam carbonyl absorbance, and



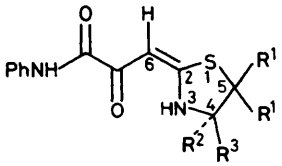
Scheme 1. *Reagents and conditions:* i, (a) KOH (1 equiv.), MeOH, (b) conc. HCl, Et_2O, 79%; ii, MeONHMe·HCl, $POCl_3$, CH_2Cl_2, pyridine (3 equiv.), 72%; iii, Bu^nLi, tetrahydrofuran (THF), −78 °C, 1.5 h; iv, (a) (**5**) (2 equiv.), and (**4**), THF, −78 to 0 °C, 1 h, (b) ≤ −30 °C, NH_4Cl (aq.), 76%.



(**1**) **a**: R^1 = PhNHCO–, R^2 = Cl
b: R^1 = Cl, R^2 = PhNHCO–



(**2**) **a**: R = PhNHCO–, *n* = 0, X = Na
b: R = MeO–, *n* = 0, X = H
c: R = MeO–, *n* = 1, X = K
d: R = N_3–, *n* = 0, X = Me
e: R = PhNHCO–, *n* = 0, X = Me
f: R = PhNH–, *n* = 0, X = Me



(**3**) **a**: R^1 = Me, R^2 = CO_2Na, R^3 = H
b: R^1 = R^2 = R^3 = H

† The significance of the five-fold difference between the turnover numbers for (**1a**) and (**1b**), which differ only in their stereochemistry at one centre, and β-lactamase I will be reported in future publications, however both values compare favourably with the turnover number of 20 000 reported for the established β-lactamase inhibitor, sulbactam and this enzyme,[6] but not with the essentially stoicheiometric inhibition by 6β-bromopenicillanic acid.[7]

‡ C_{18}-Octadecylsilane (ODS), isocratic elution with 30% v/v MeCN in 0.1 M NaH_2PO_4 (pH 4).

§ *Selected physical data* for TP–Na: i.r. (KBr), 3450 sh, 3350, 3060, 2970, 2930, 1665, 1620, 1605, 1560, 1500 cm^{-1}; ^1H n.m.r. (D_2O, 400 MHz) δ 1.27 (s, 3H), 1.49 (s, 3H), 3.98 (s, 1H), 7.28 (m, 1H), 7.44 (m, 2H), 7.51 (m, 2H), 8.51 (s, 1H); f.a.b.-m.s. (thioglycerol), found 343 (MH^+), 321 (MH^+ of free acid); $C_{15}H_{15}N_2O_4SNa$ requires 343 (MH^+).

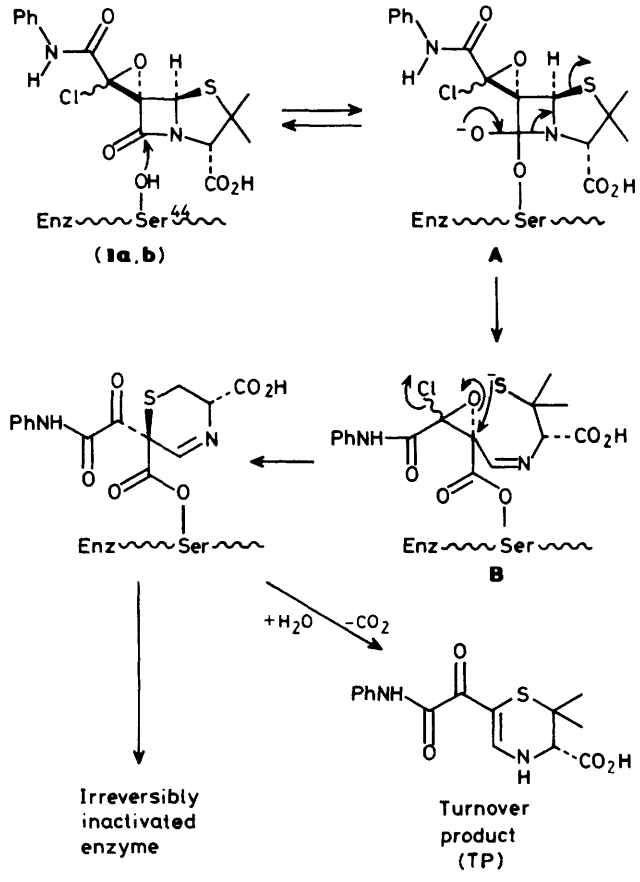
For TP–Me: ^1H n.m.r. ($CDCl_3$, 400 MHz), δ 1.24 (s, 3H), 1.61 (s, 3H), 3.88 (s, 3H), 4.16 (d, 1H, *J* 1.8 Hz), 6.14 (br., m, 1H), 7.16 (m, 1H), 7.36 (m, 2H), 7.62 (m, 2H), 9.29 (br., s, 1H), 9.46 (d, 1H, *J* 6.9 Hz). *N.B.* On irradiation of the signal at δ 6.14, the signals at δ 4.16 and 9.46 collapsed to singlets. Electron ionization (e.i.)-m.s., found M^+ 334.0970; $C_{16}H_{18}N_2O_4S$ requires 334.0985.

For (**3b**): m.p. 184—185 °C; u.v., $\lambda_{max.}$ (MeOH) 343 nm (ε 23 740 m^2 mol^{-1}); i.r. (KBr), 3310, 1670, 1600, 1590, 1510, 1475, 1440 cm^{-1}; ^1H n.m.r. ($CDCl_3$, 90 MHz), δ 3.30 (t, 2H, *J* 7.5 Hz), 3.98 (t, 2H, *J* 7.5 Hz), 6.42 (s, 1H, exchangeable with D_2O), 7.00—7.75 (m, 5H), 9.33 (br., s, 1H, slowly exchangeable with D_2O). *N.B.* Enamine N–H could not be detected. E.i.-m.s., found M^+ 248.0618; $C_{12}H_{12}N_2O_2S$ requires 248.0620.

Table 1. ^{13}C N.m.r. data and assignments for (**2c**), TP–Na, and (**3b**).

(2c)		TP–Na		(3b)	
δ[a,b]	Assignment[d]	δ[a,b]	Assignment[e]	δ[a,c]	Assignment[e]
18.10	>C(CH_3)$_2$	24.84	>C(CH_3)$_2$	—	
23.70		28.21		—	
53.13	C-2	39.11	4°	29.41	–CH_2–(C-5)
54.82	–OCH_3	—		—	
57.68	C-3	66.62	>CH–	50.03	–CH_2–(C-4)
—		—		85.71	=CH–(C-6)
96.48	C-6	100.41	4°	—	
—		122.96	Aromatic C–H	119.56	Aromatic C–H
—		127.03		124.43	
—		130.11		129.40	
—		136.69	Aromatic 4°	137.40	Aromatic 4°
150.52	C-5	150.85	=CH–	—	
171.35	2 × C=O	165.76	3 × 4°	161.24	C-2 and 2 × C=O
175.78		174.79		174.37	
—		181.51		177.50	

[a] Chemical shift relative to Me_4Si. [b] Solvent D_2O. [c] Solvent $CDCl_3$. [d] See ref. 13. [e] From distortionless enhancement by polarization techniques (DEPT) spectrum.



Scheme 2. Hypothetical pathway for turnover and irreversible inhibition, along with possible active site rearrangements, following the interaction of (**1a, b**) with β-lactamase I.

fast atom bombardment mass spectrometry (f.a.b.-m.s.) revealed a molecular ion corresponding to overall loss of Cl, C, and O from (**1**). These data were compatible with two possible structures for the turnover product: (**2a**), a 6-substituted-1,4-dihydrothiazine, or (**3a**), a 2-acylidene-1,3-thiazolidine derivative.

6-Substituted-(2*H*)-3,4-dihydro-2,2-dimethyl-1,4-thiazine-3-carboxylates [*e.g.* (**2b—d**)] are well-documented and spectroscopically well-characterised rearrangement products from both the enzymatic[8] and chemical hydrolysis of penams with leaving groups at C-6,[9] but an extensive search of the chemical literature for models of the alternative 2-acylidene-5,5-dimethyl-1,3-thiazolidine-4-carboxylate structure (**3**) failed to unearth entirely suitable analogues for spectroscopic comparison.[10] Synthesis of the simplified analogue (**3b**) could, however, be envisaged by utilisation of the lithiated 2-methyl-2-thiazoline chemistry developed by Meyers[11] combined with Weinreb's dimethylhydroxamate-derived ketone synthesis.[12] Indeed, reaction of (**4**), derived from ethyl oxanilate, with two equivalents of the lithium salt (**5**) readily yielded the desired 2-acylidene-1,3-thiazolidine analogue (**3b**) (Scheme 1).§

Despite similarities between the u.v. spectra of (**3b**) and TP–Na, a comparison of the ^{13}C-n.m.r. spectra of TP–Na (**3b**), and the known compound (**2c**)[13] (Table 1), particularly with respect to the chemical shifts of the C-5 carbon of (**2c**) (δ 150.52), the C-6 carbon of (**3b**) (δ 85.71), and the analogous carbon in TP–Na (δ 150.85), suggested that TP–Na possessed the 6-substituted-1,4-dihydrothiazine structure (**2a**). Furthermore, ^1H-n.m.r. analysis of TP–Me indicated a close similarity, particularly in the coupling pattern, to another 6-substituted-1,4-dihydrothiazine (**2d**).[9a] This provided strong evidence for the 1,4-dihydrothiazine α-ketoamide structures (**2a**) and (**2e**) for TP-Na and TP–Me, respectively.

Further support for the dihydrothiazine structure¶ was provided by studies directed towards mimicking chemically the turnover of (**1**) by the enzyme; reaction of the methyl ester of (**1b**) with sodium methoxide yielded (**2f**), whose structure was proven by independent synthesis. ‖

¶ In a preliminary report of this work,[2] we tentatively assigned the structure (**3a**) to the turnover product. On the basis of the data now reported in this communication we suggest that this assignment was incorrect, and we now favour (**2a**) as the structure for TP–Na.

‖ By treatment of 6α-chloropenicillanic acid with excess redistilled aniline at 80—90 °C followed by methylation with CH_2N_2 (R. E. Shute, unpublished results). The mechanism for the additional loss of C=O from the methyl ester of (**1b**) on treatment with NaOMe is currently under investigation.

From the above observations, coupled with the knowledge that β-lactamase I is a serine proteinase-related enzyme,[4,8] it is possible to propose a series of chemical transformations which are likely to occur following interaction of the chlorinated 6-spiroepoxypenicillin inhibitors (**1**) with the active site amino acid residue, serine-44 (Scheme 2). Attention should be drawn to the essential *cis* imine geometry in the intermediate **B**. The exact mechanism by which **B** is formed is not, as yet, clearly understood, but one possibility is that it arises from the tetrahedral intermediate **A** by a concerted β-lactam ring opening and elimination of thiolate (*cf.* ref. 3).

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