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COMMUNICATIONS

The degradation of temocillin, a 6α-methoxypenicillin, and identification of the major degradation products

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Temocillin, 6β-(*R,S*-2′-carboxy-2′-thien-3-ylacetamido)-6α-methoxypenicillanic acid, shows good stability in mild aqueous acid or base; in stronger acid the methoxypenillic acid is formed whereas alkaline or enzyme hydrolysis results in the formation of the methoxypenicilloic acid and the C-5‡ epimer.

Several 7α-methoxycephalosporins (cephamycins) have been reported for their useful antibacterial properties, but temocillin, 6β-(*R,S*-2′-carboxy-2′-thien-3-ylacetamido)-6α-methoxypenicillanic acid (1), is the only 6α-methoxypenicillin that has been developed (Slocombe et al 1981). Compared with most traditional penicillins, temocillin shows remarkably high stability to a very wide range of bacterial β-lactamases (Edmondson et al 1981) and to mild aqueous acid and base; aqueous solutions of temocillin in pH 5, 7 and 9 buffers at a concentration of 100 μg ml^{-1} showed <5% degradation over 48 h at ~20 °C when examined by reverse phase HPLC.

Acid degradation (Scheme I)

HPLC analysis of solutions of temocillin below pH 4 showed slow degradation, $t_{\frac{1}{2}}$ ~ 24 h at pH 4, $t_{\frac{1}{2}}$ ~ 9 h at pH 3, and the formation of a less polar material. Isolation by preparative HPLC gave a small amount of material identified as 7-carboxy-2,2,dimethyl-7-methoxy-5-(thien-3-ylmethyl)-7,8-dihydroimidazo-[5,1-*b*]thiazolidin-3-ylcarboxylic acid (41) from its NMR, IR and mass spectra. The same penillic acid is more readily formed, $t_{\frac{1}{2}}$ ~ 6 h at pH 3, from 6α-methoxy-6β-(2′-thien-3-ylacetamido)penicillanic acid (2). The observation that a small amount of (2) is always present in the solutions of temocillin at pH <4 suggests that decarboxylation to give (2) is the first step of the acid degradation. In stronger acid (0·1 M HCl), temocillin degrades to give several products as detected by HPLC (Bird et al 1984). No attempt has been made to identify these other products.

Although a penillic acid such as (4b) is an acid degradation product of classical penicillins, it is usually one of several products including penamaldic and penicilloic acids, and in some cases penicillenic acids, which ultimately lead to penilloic acids (Kessler et al 1983).

The 6α-methoxy substituent blocks the formation of the penicillenic acid (6) and as a consequence the penillic acid (4a) is formed instead of the penamaldic acid. By analogy with studies on benzylpenicillin (Bundgaard 1971), the oxazolone (3) is the presumed intermediate. Unlike benzylpenillic acid, (4a) does not degrade further in aqueous acid. Even heating (4a) in methanol does not give the penilloic acid (see below).

After the diacid (1) (10·6 g) had been stored as a foam at 20 °C for 1 month, HPLC analysis showed a 2:3 mixture of (4a):(1). Crystallization from methanol afforded (4a) as a white solid, 1·76 g, 19%, mp 212 °C (d), IR spectrum, ν_{max} (KBr) 3420 (broad), 1725, 1675, 1583, 1450, 1374, 1215 and 772 cm^{-1}. NMR spectrum, δ_H (90 MHz, DMSO-d_6) 1·41 (6H, s, 2 × CH_3), 3·33 (3H, s, OCH_3), 3·75 (2H, s, CH_2) , 4·47 (1H, s, 3-H), 5·72 (1H, s, 8-H), 6·95–7·55 (3H, m, thienyl protons), 8·5–10·5 (2H, br, 2 × CO_2H). Mass spectrum (bis-trimethylsilyl ester), found *m/z* 514·1453 (M^+, calculated for $C_{21}H_{34}N_2O_5S_2Si_2$ 514·1440). The mother liquors were evaporated to dryness, triturated with ethyl acetate to remove temocillin, then redissolved in hot methanol. Reverse phase HPLC showed a new peak, more retained than (4a), which became the major component after the methanol solution had been heated under reflux for 4 h. The product was adjusted to pH 7 and purified on 'Sephadex G25 fine' eluting with water. Acidification to pH 2 and crystallization from water then provided 7-carboxy-2,2-dimethyl-5-(thien-3-ylmethyl)imidazo[5,1-*b*]thiazolidin-3-ylcarboxylic acid (5), 0·76 g, mp 185–187 °C (d). IR spectrum, ν_{max} (KBr) 3410 (broad), 1705, 1600, 1545, 1375, 1200 and 775 cm^{-1}. NMR spectrum, δ_H (90 MHz, DMSO-d_6) 1·53 (3H, s, CH_3), 1·59 (3H, s, CH_3), 3·93 (2H, s, CH_2), 4·67 (1H, s, 3-H), 6·85–7·55 (3H, m, thienyl protons). Mass spectrum (bis-trimethylsilyl ester), *m/z* 482 (M^+, $C_{20}H_{30}N_2O_4S_2Si_2$).

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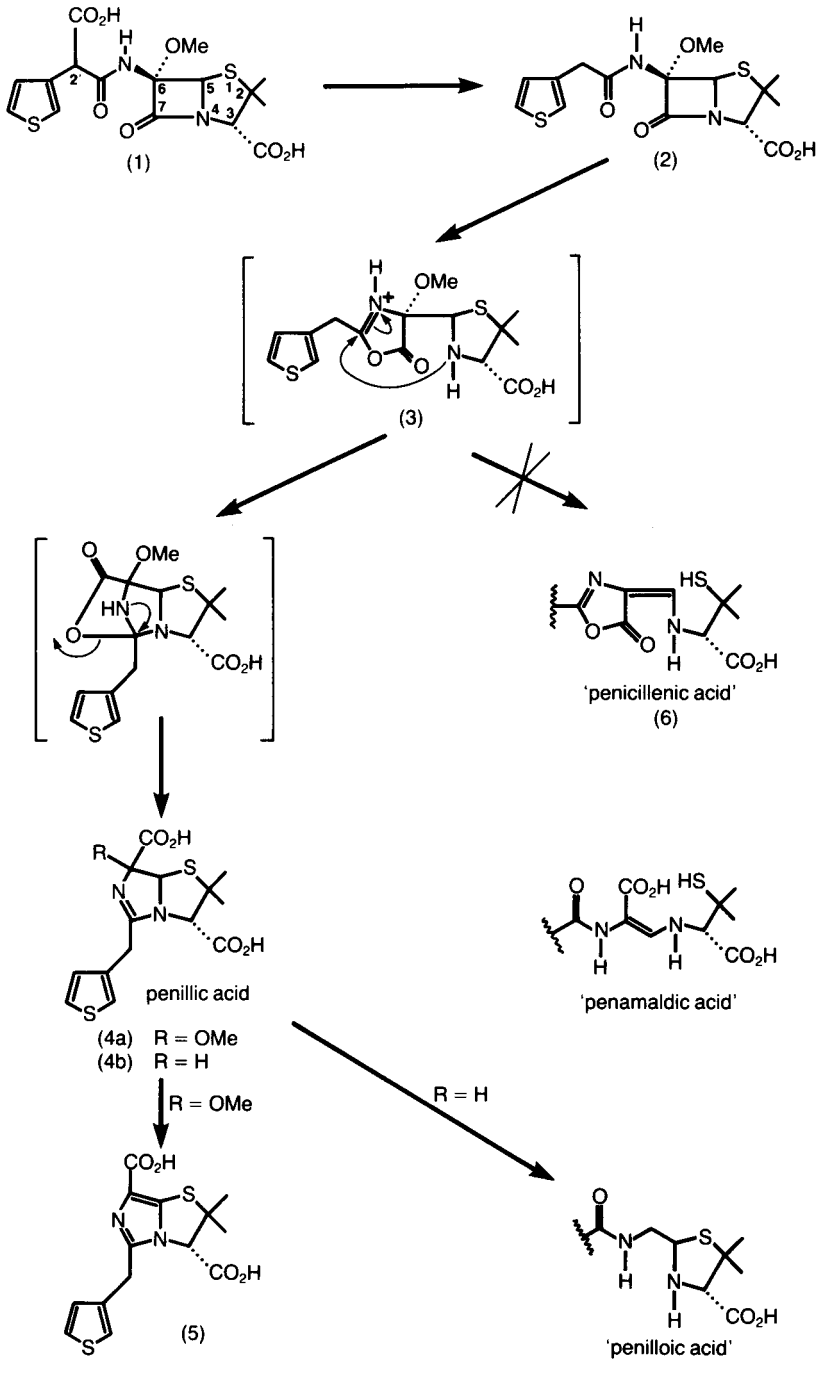
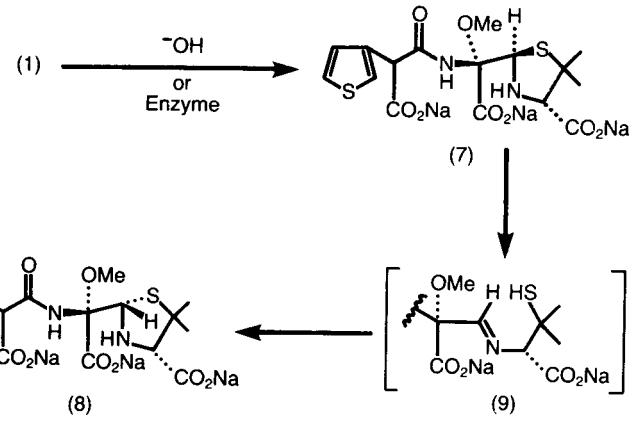


Scheme I. Acid degradation of temocillin (1).



Scheme II. Alkaline or enzyme hydrolysis of temocillin (1).

Alkaline and enzymic degradation (Scheme II)
Although temocillin is stable in pH 9 buffer for at least 48 h, a solution maintained at pH 12 for 1·5 h, neutralized to pH7 with Amberlite IR 120 (H^+) and freeze-dried gave material retaining 6% of temocillin, the rest being the penicilloic acids (7).

Most bacterial β-lactamases do not hydrolyse temocillin (Edmondson et al 1981). However, two strains of *Providencia alcalifaciens* induced with benzyl penicillin cause hydrolysis to the penicilloic acids (7).

The course of the chemical degradation was followed by NMR and HPLC (Tables 1 and 2). The 250 MHz ^1H NMR spectrum of temocillin in 0·1 M NaOD-D_2O at 10 mg ml^{-1} was recorded at time intervals up to 20 days and samples were removed for HPLC examination. Loss of temocillin as (7) was formed could be clearly seen, as could the appearance of another material, (8), the corresponding 5*S*‡-epimer, after ~24 h. As has been reported (Ghebre-Sellassi et al 1984) for benzylpenicillin the 5*S*‡-epimer predominates at equilibrium, also the ^1H spectrum of (8) shows no incorporation of deuterium at C-5‡.

An NMR experiment with (1) in 5 M NaOD-D_2O initially gave a similar result, but then further degradation could be seen. For the enzyme hydrolysis, temocillin was dissolved in cell free enzyme solution, prepared as described by Edmondson et al (1981), and the reaction followed by HPLC. A sample of enzyme solution was freeze dried then redissolved in D_2O containing temocillin for a parallel NMR experiment. The lower concentration or activity of enzyme resulted in a slower reaction but the loss of temocillin to give (7) and epimerization to (8) was in accord with the results of the HPLC experiment.

Table 1. Relative proportions of (7) and (8) from the hydrolysis of temocillin (1).

Reaction time	Alkaline hydrolysis (0·1 M NaOD) (1):(7):(8)	Enzyme hydrolysis (pH 7) (1):(7):(8)
1 h	1:30:0	2:1:0
3 h	0:20:1	1:2:0
24 h	0: 3:1	1:3:1
48 h	0: 5:4	0:3:2
20 days	0: 1:3	

Table 2. NMR spectra (δ) of temocillin (1) (D_2O), (7) and (8) (0·1 M NaOD-D_2O)

		2 × Me-2	OMe	H-3	H-2′	H-5	Thienyl
(1)	R C-2′ epimer	(a) 1·36, 1·40	3·53	4·23	4·70	5·48	7·1–7·45
	S C-2′ epimer	(b) 1·38, 1·43	3·43	4·25	4·63	5·50	
(7)	Major C-2′ epimer	(a) 1·17, 1·47	3·18	3·55	exch	5·43	7·1–7·45
	Minor C-2′ epimer	(b)	3·13			5·42	
(8)	Major C-2′ epimer	(a) 1·08, 1·52	3·18	3·32	exch	5·08	7·1–7·45
	Minor C-2′ epimer	(b) 1·11	3·10			5·11	

Ratio of side chain (C-2′) epimers, (1) 1·7–1·9:1, (7) 1·2–1·3:1, (8) 1·0–1·1:1

The presence of a 6-methoxy substituent and the lack of deuterium incorporation at C-5‡ of (8) provides strong evidence for the imine (9) being intermediate for the conversion of (7) to (8). Indeed Bird et al (1983) suggested that all penicilloic acids epimerize at C-5‡ between pH 4 and 12 by this mechanism. The formation of a small amount of (7) or the corresponding derivative of (2) in the acid decomposition of temocillin cannot be ruled out, but the observation that (7) in pH 2 or 3 acid buffer gives (8) but not (4a) suggests that (9) is not an intermediate in the formation of (4a).

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‡ Penicillin numbering.

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The pH-dependence of chloroquine uptake by phosphatidylcholine vesicles

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The pH-dependence of chloroquine uptake by phosphatidylcholine bilayered vesicles was investigated to examine the relative affinity of ionized and un-ionized chloroquine species to membrane bilayers. The results suggest that the neutral species and monocation are taken up by the bilayers but only the un-ionized species interacts significantly with the hydrocarbon interior.

The action of chloroquine as an antimalarial appears to depend on accumulation of the drug in the food vacuole of the parasite (Yayon & Ginsberg 1983). This requires passage of chloroquine across at least four membranes: the host erythrocyte membrane, the membrane of the parasitophosphorus vacuole, the external parasite membrane and the membrane of the food vacuole. Clearly, the action of chloroquine depends critically on its ability to cross membranes.

The drug (CQ) is a base with two sites of ionization (pK_a values of 10·1 and 8·1 (Irvin & Irvin 1947)). At physiological pH it is present largely as the dication (CQ^{2+}; about 83%), with some monocation (CQ^+; about 17%), while the neutral species (CQ°) is present only in very small quantities (less than 0·05%). Such observations led Homewood et al (1972) to propose that it is CQ^+ which passes through cell membranes at physiological pH.

Phospholipid bilayered vesicles have been widely used as model membranes. Calorimetric studies by Chawla et al (1979) on the interaction of chloroquine with phosphatidylcholine (PC) bilayers, at relatively high chloroquine concentrations (10^{-3} to 10^{-1} M), showed competitive binding between chloroquine and ionic lipids. Those workers also provided evidence, from proton NMR studies, that chloroquine interacts with the methylene groups of the hydrocarbon chain rather than with the methyl groups of the quaternary nitrogen of the choline group of PC. Lullman & Wehling (1979) studied the binding of a range of cationic drugs, including chloroquine, to various types of phospholipid vesicles, including PC, and demonstrated that chloroquine binding to PC was low when compared with the other drugs, though particularly high compared with phosphatidylserine, an anionic phospholipid. The evidence therefore suggests that both ionic and hydrophobic interactions are important.

Our study aimed to investigate these matters further by studying the pH dependence of chloroquine uptake by PC bilayered vesicles to determine the relative affinity of CQ°, CQ^+ and CQ^{2+} for membrane bilayers and provide information on the likely contribution of these species to passive diffusion across membranes.

PC was chosen because it is the most prevalent phospholipid in erythrocyte membranes (Rouser et al 1968) and its charge is pH-independent over the pH range used in the study (Bruni & Palatini 1982), which avoids the complication of possible ion binding being dependent on the variable charge of the phospholipid.

Materials and methods

Chloroquine as the diphosphate salt, was supplied by Sigma Chemical Co. (St. Louis, MO, USA). All glassware coming into contact with solutions of the drug was silanized using Aquasil silanizing liquid (Pierce, Rockford, IL, USA). The buffer used contained 20 mM Na_2HPO_4 and 125 mM NaCl and was adjusted to the required pH using 1 M HCl or NaOH.

PC vesicles were prepared by evaporating the solvent from a solution of PC (Type XI-E, Sigma Chemical Co. St Louis, MO, USA) in chloroform, weighing the dried PC (~100 mg), then adding 20 ml of buffer. The mixture was then sonicated for 3 min using a Heat-System Ultrasonic W-375 sonicator. The presence of multibilayered vesicles was verified by electron microscopy.

Binding of chloroquine to the PC vesicles was determined by equilibrium dialysis using glass dialysis cells and a cellophane semipermeable membrane (Type

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