Decomposition of p-Nitrobenzyl 7-[D-(−)-α-Aminophenylacetamido]-3-chloro-3-cephem-4-carboxylate (1f).[6] A C_6H_6 solution (3 L) of **1f** (2.6 g, 5.1 mmol) was stirred under reflux for 100 h. The C_6H_6 solution was allowed to cool and the C_6H_6 removed in vacuo. The mixture was chromatographed over silica gel for dry-column chromatography. Amorphous piperazine-2,5-dione **3** was eluted with 1:1 ethyl acetate–cyclohexane: 0.6 g (38%); mp 176 °C dec; field desorption M^+ at 466; λ_{max} 265, 371 nm (ε 17900, 16600); IR (KBr) 1721 (ester), 1663 (C=C), 1640 (amide), 1340, 1510 cm^{-1} ($-NO_2$); $[\alpha]^{25}_D$ +139.7° (Me_2SO); proton double-resonance data for **3a**, 6.12 ppm (d of t) [collapse of 3.58 ppm, (d) to a s, sharpening of 11.69 ppm (br s)], 3.58 ppm (d) [collapse of 6.12 ppm (d of t) to br s], 8.85 ppm (d) [collapse of 4.98 ppm (d) to s]. Protons at 9.25 (s), 8.58 (d), and 11.69 ppm (br s) exchanged upon D_2O wash; an unsatisfactory analysis was obtained for C, H, and N, but **3** contained no Cl.

Reaction of p-Nitrobenzyl 7-Amino-3-chloro-3-cephem-4-carboxylate (1e)[6] with Isobutyl Alcohol. Chauvette and Pennington prepared **1e** by the PCl_5 treatment of *p*-nitrobenzyl 7-(thiophene-2-acetamido)-3-chloro-3-cephem-4-carboxylate, followed by cleavage of the imino chloride with isobutyl alcohol, which precipitates the crystalline HCl salt of **1e**.[6] If the filtrate from this procedure is allowed to stand, **4** crystallizes as the hydrochloride in yields up to 25%.

Dissolution of the HCl salt **4** in pyridine followed by precipitation with H_2O gave **4** as yellow-orange crystals. Recrystallization from ethanol gave **4**: mp 114 °C dec; field desorption M^+ 407; λ_{max} 263, 365 nm (ε 11000, 8000); IR (KBr) 1721 (ester), 1669 (C=C), 1350, 1521 cm^{-1} ($-NO_2$); proton double-resonance data for **4a**, 6.0 ppm (d of t) [collapse of 3.5 ppm (d) to s, sharpening of 10.5 ppm (br s)], 3.5 ppm (d) [collapse of 6.0 ppm (d) to s], 2.0 ppm (m) [collapse of 0.9 ppm (d) to s, collapse of 4.0 ppm (d) to s]. Protons at 10.5 (br s) and 2.8 ppm (v br) exchange upon D_2O wash. Anal. Calcd for $C_{18}H_{21}N_3O_6S$: C, 53.07; H, 5.16; N, 10.32; S, 7.86. Found: C, 52.75; H, 4.94; N, 10.16; S, 7.56.

Acknowledgment. The authors thank R. R. Chauvette for samples of cephalosporins, Dr. D. Dorman for suggestions in interpreting the ^{13}C NMR spectra, and Dr. L. D. Hatfield for bringing to our attention that **1e** decomposes in isobutyl alcohol.

References and Notes

(1) D. J. Tipper and J. L. Strominger, *J. Biol. Chem.*, **243**, 3169 (1968).
(2) J. M. Indelicato, T. T. Norvilas, R. R. Pfeiffer, W. J. Wheeler, and W. L. Wilham, *J. Med. Chem.*, **17**, 523 (1974).
(3) J. M. T. Hamilton-Miller, E. Richards, and E. P. Abraham, *Biochem J.*, **116**, 385 (1970).
(4) J. M. Indelicato and W. L. Wilham, *J. Med. Chem.*, **17**, 528 (1974).
(5) R. R. Chauvette and P. A. Pennington, *J. Am. Chem. Soc.*, **96**, 4986 (1974).
(6) R. R. Chauvette and P. A. Pennington, *J. Med. Chem.*, **18**, 403 (1975).
(7) J. F. Quay, W. R. Brown, M. R. Clarkson, and H. R. Sullivan, Abstracts, 16th Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, Ill., Oct 1976.
(8) H. R. Black, K. S. Israel, G. L. Brier, and J. D. Wolny, Abstracts, 16th Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, Ill., Oct 1976.
(9) D. A. Preston, Abstracts, 16th Interscience Conference on Antimicrobial Agents and Chemotherapy, Chicago, Ill., Oct 1976.
(10) H. Bundgaard, *Arch. Pharm. Chemi, Sci. Ed.*, **3**, 94 (1975).
(11) D. B. Boyd, R. B. Hermann, D. E. Presti, and M. M. Marsh, *J. Med. Chem.*, **18**, 408 (1975).
(12) H. Bundgaard, *Arch. Pharm. Chemi, Sci. Ed.*, **4**, 25 (1976).
(13) T. Yamana, A. Tsuji, K. Kanayama, and O. Nakano, *J. Antibiot.*, **27**, 1000 (1974).
(14) A. Dinner, *J. Med. Chem.*, following paper in this issue.
(15) J. M. Indelicato, T. T. Norvilas, and W. J. Wheeler, *J. Chem. Soc., Chem. Commun.*, 1162 (1972).
(16) A. I. Cohen, P. T. Funke, and M. S. Pour, *J. Pharm. Sci.*, **62**, 1559 (1973).

Cephalosporin Degradations[1]

Alan Dinner

Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46206. *Received October 26, 1976*

The acidic aqueous degradation of the 7α-aminophenylglycinamido-containing cephalosporin cephalexin (**1a**) has been examined. Two major degradation products have been isolated and characterized: 3-formyl-3,6-dihydro-6-phenyl-2,5(1*H*,4*H*)-pyrazinedione (**5**) and 3-hydroxy-4-methyl-2(5*H*)-thiophenone (**6**). By carrying out the reaction in ^{18}O-enriched H_2O, the intramolecular nature of the cephalexin degradation has been demonstrated.

The chemical reactivity of β-lactam-containing antibiotics is linked to antimicrobial activity and bacterial resistance.[2] This has evoked considerable interest in the chemical degradation of cephalosporin antibiotics.[3,4] Three reports have recently appeared which detail the alkaline hydrolysis of the clinically useful antibiotics, cephalexin [7-(D-2-amino-2-phenylacetamido)-3-methyl-3-cephem-4-carboxylic acid; cephalexin monohydrate, KEFLEX, Lilly] (**1a**) and cephradine (**2**). In 1973, Cohen[5] reported that the degradation of **2** in Na_2CO_3 at 5 °C affords the diketopiperazine **3a**; in 1974, Yamana[6] speculated that diketopiperazine **4** forms from the hydrolysis of cephalexin at pH 8, and in 1976, Bundgaard[7] actually isolated such a compound from the alkaline hydrolysis of cephalexin.[8]

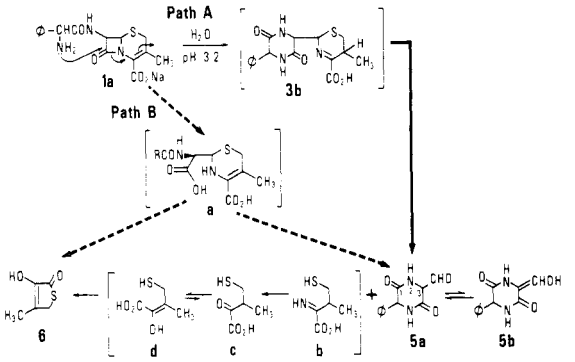
Since cephalexin possesses oral antibiotic activity, an acidic rather than a basic degradation study should better mimic any chemical reaction that might occur in the stomach. Hence, we wish to report the identification of two major products from the acidic degradation of **1a** and to propose a route to their formation. We also report herein preliminary toxicological data on the cephalexin degradation products.

Experimental Section

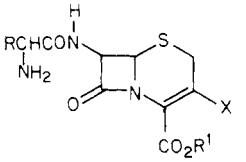
General Procedures. Melting points were determined with a Mel-Temp apparatus and are uncorrected. Infrared spectra were determined on a Beckman IR-12 spectrometer, NMR spectra were recorded on a Varian T-60 spectrometer, and mass spectra were recorded on a Hitachi RMU-6D spectrometer at 70 eV. Elemental analyses obtained are within ±0.3% of the theoretical values.

Cephalexin Degradation. A solution of 1.0 g of cephalexin in 100 mL of deionized water (resulting pH 3.3) was warmed to 75 °C. Periodic examination of the solution by TLC [5:2:1:1, EtOAc–CH_3COCH_3–HOAc–H_2O; R_f (cephalexin) = 0.14] revealed that most of the starting material had degraded within 90 min and two major degradation products (R_f = 0.78, 0.91) were formed. The aqueous solution was then cooled and extracted with $CHCl_3$. The less polar product (R_f = 0.91) was isolated from the $CHCl_3$ extract (200 mg), purified via sublimation (100 °C, 100 μ), and identified on the basis of its spectral data as the known[9] **3-hydroxy-4-methyl-2(5*H*)-thiophenone (6)**: IR (KBr) 3400–3200 (OH), 1690 (C=O), 1640 cm^{-1} (C=C); ^1H NMR ($CDCl_3$) δ 2.1 (3

Scheme I

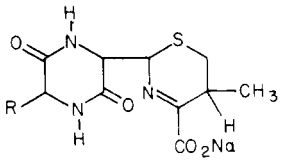


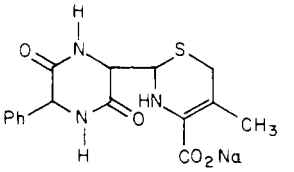
H, t, J = 1 Hz), 3.8 (2 H, q, J = 1 Hz), 6.0 (1 H, br s, exchangeable); mass spectrum M^+ at m/e 130.0090, calcd for $C_5H_6O_2S$, m/e 130.0088; mp 127–128 °C.



1a, R = C_6H_5; R^1 = H; X = CH_3
b, R = C_6H_5; R^1 = Na; X = Cl
c, R = C_6H_5; R^1 = Na; X = CH_2OAc

2, R =  ; R^1 = Na; X = CH_3





4

3a, R = 

b, R = C_6H_5

The more polar product was isolated from the aqueous solution by lyophilization (425 mg) and purified by silica gel column chromatography (elution solvent 5% MeOH in $CHCl_3$), sublimation (175 °C, 50 μ), and finally recrystallization (1:1, MeOH–CH_2Cl_2) to give white crystals, mp 205–206 °C. The spectral data are consistent with structure **5**, **3-formyl-3,6-dihydro-6-phenyl-2,5(1*H*,4*H*)-pyrazinedione (5)**:[10] UV (EtOH) 214 (5250), 257 nm (3050); IR (KBr) 3300–2800 (OH, NH, CH), 1710–1630 cm^{-1} (aldehyde, amide, C=C); ^1H NMR (Me_2SO-d_6) δ 4.0 (m, C*H*CHO), 5.0 (m, PhCH and -C=C*H*OH), 7.4 (s, aromatic protons), 8.0–9.4 (m, NH, exchangeable), 10.0 (m, CHO), 12.2 (v br s, OH, exchangeable); mass spectrum M^+ at m/e 218.0691, calcd for $C_{11}H_{10}N_2O_3$ 218.0696. Anal. ($C_{11}H_{10}N_2O_3$) C, H, N.

Degradation of 3b. A mixture of 100 mg of **3b**[7] in 10 mL of deionized water was warmed to 75 °C (solution pH ≃3.0). After 90 min the solution was worked up as above to afford **5** and **6** as shown by IR and TLC comparison with previously obtained material.

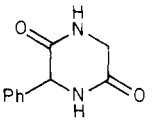
Hydrolysis in $H_2{}^{18}O$. For either **1a** or **3b**, 60 mg of sample was placed in 3 mL of 10 atom % oxygen-18 enriched water (Bio-Rad Laboratories) and the mixture was allowed to react as above. The amount of ^{18}O incorporation in resulting **5** was determined by measuring the P + 2/P intensity ratio in the mass spectrum and subtracting from it the P + 2/P ratio in unenriched **5**. Similarly, the amount of ^{18}O incorporation in **5** not contained in the 3-formyl portion of the molecule was obtained by measuring the 192/190 intensity ratio and subtracting from it the 192/190 intensity ratio in unenriched **5**.

Results and Discussion

The ^1H NMR assignments of **5** indicate that this material exists in Me_2SO-d_6 as an equilibrium mixture of 50% **5a** and 50% **5b**. Consistent with this is the observation that **5** gives a positive $FeCl_3$ test, analogous to 2-formylcyclohexanone,[11] whereas model compound **7** does not give a positive response. The chemical shifts of the benzal proton (δ 4.9) and aromatic protons (δ 7.4) in 3-phenyl-2,5-piperazinedione (**7**)[12] are also in accord with the observed δ values in **5** (vide supra). The mass spectrum of **5**, after an initial loss of m/e 28, exhibits the same major fragments as those observed in **7** (m/e 147, 118, 104).

A possible route which accounts for the formation of **5** and **6** is shown in Scheme I. Intramolecular attack of the phenylglycineamino function in **1a** on the lactam carbonyl affords diketopiperazine **3b**. At the pH (3.3) of the reaction medium, the thioaminal carbon of **3b** undergoes hydrolysis which results in the formation of **5** and imine b. This imine intermediate rapidly hydrolyzes to ketone c (reminiscent of the structure proposed by Abraham[13] to account for the cephalosporin c degradation products), which is then transformed into thiolactone **6** from enol form d.

The intact diketopiperazine **3b** is not isolated during this reaction process. However, if prepared by an alternative synthesis[7] and then hydrolyzed at pH 3.3, it rapidly degrades to **5** and **6**.



7

The intramolecular nature of the cephalexin degradation is further demonstrated by carrying out the reaction in ^{18}O-enriched H_2O. If the reaction follows path A of Scheme I, then the piperazinedione **5** should have labeled ^{18}O only in the 3-formyl oxygen. Alternatively, if the β-lactam in cephalexin is hydrolytically cleaved via path B in Scheme I to give a cephalosporate such as a (which could subsequently be transformed to **5** and **6**), then not only would the formyl oxygen in **5** be labeled but also the oxygen at C-4 would be enriched in ^{18}O. We have observed that **5** formed from cephalexin contains no more ^{18}O than **5** prepared from independently synthesized[7] **3b** under identical conditions. Furthermore, analyses of the mass spectra of these piperazines reveal that after an initial loss of m/e 28 (side chain C=O), as expected, neither remaining piperazine fragment (m/e 190) contains any unnatural ^{18}O.

The acute oral toxicity of a mixture of **5** and **6** has been obtained in mice. Groups of ten fasted female cox ICR mice, weighing 16–18 g, were given a single oral dose (1000–5000 mg/kg) of sample prepared as an aqueous suspension containing 15% sample and 10% acacia. The LD_0 for the mixture of **5** and **6** is greater than 5000 mg/kg. These results are similar to the toxicity found for cephalexin (LD_0 > 5000 mg/kg).

Sullivan has demonstrated[14] in mice and rats that following the oral administration of a single dose of cephalexin-^{14}C, 99% of the radioactivity is recovered from 24-h urine and feces samples. His examination of these samples by TLC revealed that the only radioactive entity present had an R_f value corresponding to unchanged **1a**. The R_f values for **5**[15] in the three systems utilized by Sullivan[14] do not correspond to those of **1a**. Hence, the products of the acidic degradation of cephalexin do not appear to be produced in the mouse or rat.

Acknowledgment. I thank Dr. W. D. Broddle and Dr. G. K. Hanasono of Eli Lilly and Co. for arranging and performing the biological tests. I also thank Dr. J. In-

delicato, Dr. L. Hatfield, and Dr. R. Schirmer of these laboratories for helpful conversations.

References and Notes

(1) Reported in part at the 172nd National Meeting of the American Chemical Society, San Francisco, Calif., Sept 1976, MEDI 93.
(2) J. Strominger and D. Tipper, *Am. J. Med.*, **39**, 708 (1965).
(3) (a) J. Indelicato, T. Norvilas, and W. Wheeler, *J. Chem. Soc., Chem. Commun.*, 1162 (1972); (b) J. Hamilton-Miller, G. Newton, and E. Abraham, *Biochem. J.*, **116**, 371 (1970).
(4) E. Roets, A. Vlietinck, G. Janssen, and H. Vanderhaeghe, *J. Chem. Soc., Chem. Commun.*, 484 (1973).
(5) A. Cohen, P. Funke, and M. Puar, *J. Pharm. Sci.*, **62**, 1559 (1973).
(6) T. Yamana, A. Tsuji, K. Kanayama, and O. Nakano, *J. Antibiot.*, **27**, 1000 (1974).
(7) H. Bundgaard, *Arch. Pharm. Chemi, Sci. Ed.*, **4**, 25 (1976).
(8) The $\Delta^{3,4}$ location for the double bond in the diketopiperazine reported by Bundgaard[7] (**4**) is incorrect. It is apparent from an examination of the NMR spectrum of this compound that the doublet at δ 1.2 with J = 7 Hz is indicative of a methyl function at C_3 adjacent to one hydrogen. One would expect the methyl at C_3 in a $\Delta^{3,4}$ compound to have $\delta \simeq 2.0$ and $J \simeq 0$ Hz as in cephalexin. The correct structure for the diketopiperazine is **3b**, analogous to the one proposed by Cohen[5] for the cephradine degradation.
(9) D. Green, A. Long, P. May, and A. Turner, *J. Chem. Soc.*, 766 (1964).
(10) We have isolated **5** in low yield (<5%) from the acidic aqueous degradations of cephaloglycin (**1b**) and cefaclor (**1c**). The plethora of products formed from both compounds under these conditions indicates that a structure analogous to **3b** is probably not a principal intermediate in the degradations of these compounds.
(11) W. Johnson and H. Posvic, *J. Am. Chem. Soc.*, **69**, 1361 (1947).
(12) K. Kopple and M. Ohnishi, *J. Am. Chem. Soc.*, **91**, 962 (1969).
(13) E. Abraham and G. Newton, *Biochem. J.*, **79**, 377 (1961).
(14) H. Sullivan, R. Billings, and R. McMahon, *J. Antibiot.*, **22**, 195 (1969).
(15) The radiolabel in the tagged cephalexin comes from 2-phenylglycine-^{14}C. Hence, only **5** and not **6** would be expected to possess a radiolabel if the metabolism mimicked the acidic degradation.

3-Hydroxyisoxazole-5-hydroxamic Acid

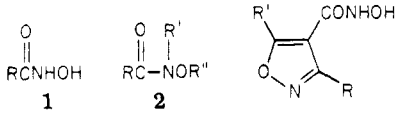
John W. Hines, Jr., and Charles H. Stammer*

Chemistry Department, University of Georgia, Athens, Georgia 30602. *Received December 13, 1976*

The synthesis of the title compound, 3-hydroxyisoxazole-5-hydroxamic acid (**4b**), by two procedures is described. The first, involving the treatment of dimethyl acetylenedicarboxylate with hydroxylamine, had previously been reported to give the 3-hydroxyisoxazole-5-carboxylic acid (**4a**). In the second, treatment of chlorofumaroyl dichloride with hydroxylamine also gave the intermediate chlorofumarodihydroxamic acid (**6**). Compound **6** was found to have some activity against P388 lymphocytic leukemia.

Since hydroxamic acids **1** have many different kinds of biological activities[1] we were interested in combining this functionality with that of the 3-hydroxyisoxazole moiety[2] in order to investigate the antibacterial and anticancer activity of this structure.

Hydroxamic acids are well known as strong chelating agents of Cu(II) and Fe(III) ions. Various aromatic and heterocyclic hydroxamic acids show powerful activity against mycobacteria and fungi.[3,4] Alkylated hydroxamic acids **2** also show antibacterial and antifungal activities.[5] Hydroxamic acid derivatives of isoxazole **3** are also known[6]

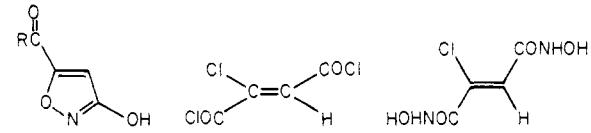


1 2 3

to show in vitro bacteriostatic activity. Of great interest also is the antileukemic activity of *N*-hydroxyurea[7] and the antimicrobial, fungicidal, and herbicidal activities of its derivatives.[8] Finally, simple aliphatic and aromatic hydroxamic acids are known to be potent inhibitors of ureas.[9]

In 1968, Nakamura[10] reported the synthesis of the acid **4a** and its methyl ester **4c** by the low-yield conversion of dimethyl acetylenedicarboxylate into **4a** with hydroxylamine in strongly basic solution. In our hands, this reaction yielded a very crude hydroxamic acid **4b** which could be converted into the crystalline methyl ester **4c** in acceptable yield. Saponification of **4c** gave the same acid (**4a**) reported by Nakamura in about 20% overall yield.

Several methods, including the treatment of β-keto esters with hydroxylamine[11] and the hydrolysis of 3-haloisoxazoles,[12] are available for the synthesis of 3-



4a, R = OH
b, R = NHOH
c, R = OCH_3

5 **6**

hydroxyisoxazoles, but none of these was appropriate to the synthesis of the desired hydroxamic acid, **4b**. An alternative procedure which yielded not only **4b** but also the intermediate dihydroxamic acid **6** proceeded through the diacid chloride **5**.[13] This compound was readily prepared from monopotassium acetylenedicarboxylate by treatment with HCl followed by thionyl chloride. When **5** was treated with 2 equiv of hydroxylamine at room temperature under basic conditions, only a red oil giving a positive $FeCl_3$ test for the hydroxamic acid function was obtained. However, when the reaction was carried out carefully at 0 °C under a nitrogen atmosphere, the solid dihydroxamic acid **6** was obtained in 67% yield. The ring closure of **6** to the isoxazole **4b** was carried out at room temperature in sodium hydroxide solution under a nitrogen atmosphere in 45% yield. This product (**4b**) had an infrared spectrum consistent[14] with the 3-hydroxyisoxazole structure. Methanolysis of the pure **4b** followed by saponification of the resulting ester gave the acid **4a** in approximately 60% yield. The infrared and NMR spectra of **4a** were consistent with the structure, while its melting point, 238 °C sublimes, differed appreciably from those given in the literature.[1b,11]

To our knowledge the mechanism of this kind of ring closure (**6** → **4b**) has not been investigated. It seems