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Short Communication

Formation of fluorescent pyrazine derivatives via a novel degradation pathway of the carbacephalosporin loracarbef

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Introduction

Recently an unknown degradation product of loracarbef (structure 1) was observed at low levels (~0.1%) in samples of the bulk drug substance that had been stored at 30°C for 36 months. Identification of this unknown was desired because of the potential for this unknown to form in the product during the shelf-life of the drug [1].

Isolation of such a minor impurity from limited quantities of sample is difficult and, therefore, more severe conditions were evaluated for generating higher levels of this degradation product. LC analysis with photodiode array detection indicated that this degradation product was present at higher levels in a sample of loracarbef stressed at 85°C for 8.5 months. Therefore, the degradation product (structure 4) and two other closely eluting degradation products (structures 5 and 6) were isolated concurrently from the 85°C-stressed sample by preparative LC. Visualization of all three of these products under long wavelength UV indicated that they were fluorescent. MS and NMR spectroscopic characterization of these three products indicated the structures (structures 4–6) were derivatives of the highly fluorescent 2-hydroxy-3-phenylpyrazine (structure 3).

It is well established that β-lactam antibiotics containing the phenyl glycine side chain will degrade under certain conditions to 2-hydroxy-3-phenyl pyrazine (structure 3) [2–4]. Degradation studies of cefaclor (structure 2) [5] and other phenyl glycine-containing β-lactams [4, 6] led to a proposed pathway for the formation of this pyrazine derivative (Scheme 1). The pathway to these 2-hydroxy-3-phenyl pyrazine derivatives involves hydrolysis of carbon-6 to reveal the "masked aldehyde", and subsequent cyclization and aromatization leading to the pyrazine structure.

In the case of the loracarbef, the sulphur at position 5 is replaced with a methylene, effectively blocking the possibility of hydrolysis leading to an aldehyde at position 6. Thus it was predicted that carbacepahlosporins such as loracarbef would not degrade to 2-hydroxy-3-phenyl pyrazine derivatives. In agreement with this prediction, no pyrazine derivatives were detected in a previous study of the aqueous degradation of loracarbef [7]. The discovery that substituted pyrazines were formed during solid-state degradation of loracarbef indicates the existence of a novel degradation pathway to pyrazines, distinct from the established cephalosporin degradation pathway. This report describes the isolation, characterization and proposed mechanism of formation for

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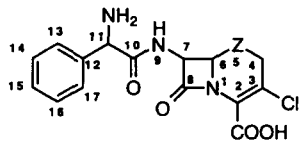

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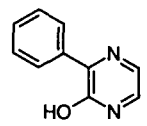

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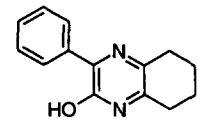

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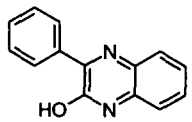

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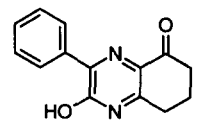

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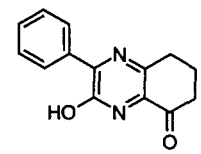

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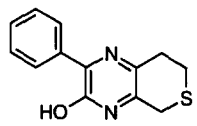

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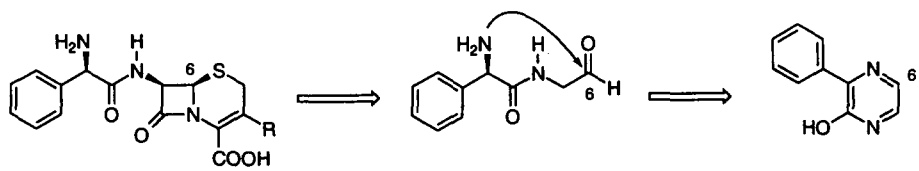
Structures



Scheme 1 *General Pathway to 2-Hydroxy-3-phenylpyrazine Derivatives via Phenylglycyl-Cephalosporin Degradation*

these substituted pyrazine degradation products.

Experimental

Liquid chromatography

Analytical. The analytical scale reversed-phase (RP)–HPLC system utilized a photodiode array detector with UV detection from 200 to 400 nm at 4-nm resolution (Waters 991, Milford, MA, USA). The LC was run in a gradient mode from 0 to 100% solvent B in 30 min and held at 100% solvent B for 10 min. Solvent A was an aqueous solution containing 6.9 g l^{-1} $NH_4H_2PO_4$ with the pH adjusted to 2.5 using H_3PO_4. Solvent B was a mixture of acetonitrile and solvent A (60:40, v/v). The flow rate was 1.0 ml min^{-1}. A C-18 reversed-phase column was used (4.6 × 250 mm, 5 μM, YMC, Wilmington, NC, USA).

Preparative. Degradation products 4–6 were isolated by semi-preparative LC from a sample

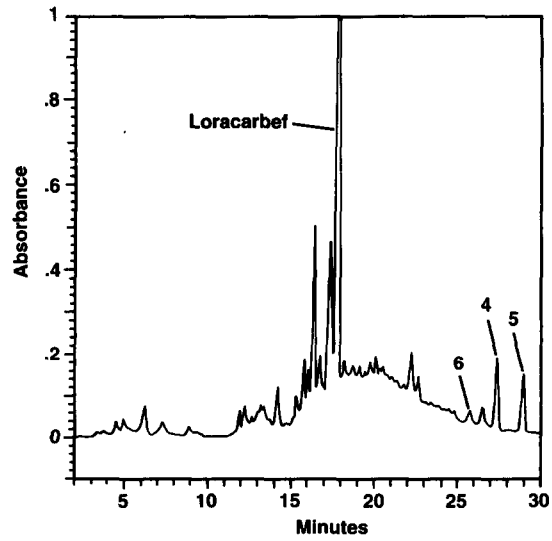


Figure 1
HPLC chromatogram (UV, 220 nm) of a sample of loracarbef stressed at 85°C for 8.5 months. The peaks corresponding to compounds 4, 5, and 6 are indicated on the chromatogram.

of loracarbef that was stressed at 85°C for 8 months (Fig. 1). The stressed loracarbef sample was dissolved in 0.1% acetic acid in water at a level of 5 mg ml^{-1} and was kept at 0°C during the course of preparative LC. The system (Rainin HPXL LC system, and Dynamax FC-1 fraction collector, Rainin Instrument Co., Woburn, MA, USA) used a semi-preparative C-18 reversed-phase column (20 × 250 mm, 5 μm, YMC). The LC was run in a gradient mode from 0 to 100% solvent B in 45 min. Solvent A was an aqueous solution of 0.1% acetic acid, and solvent B consisted of a mixture of acetonitrile, water and acetic acid (60:40:0.1, v/v/v). The flow rate was 20 ml min^{-1}. A total of 102 preparative injections (30 ml/injection) were made over the course of several days, and the fractions containing 4–6 were collected in containers cooled to 0°C to minimize post-column degradation. The solvent was removed using lyophilization.

NMR

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX-500 spectrometer. Spectra were recorded in DMSO-d_6 with a trace of trifluoroacetic acid and referenced to internal tetramethylsilane on the basis of the chemical shifts of residual solvent peaks.

Mass spectrometry

Fast-atom bombardment (FAB)-MS and FAB-MS/MS data were obtained using a VG ZAB-2SE (BE) two sector mass spectrometer. Samples were dissolved in "Magic Bullet" (a 5:1 solution of dithiothreitol–dithioerythritol in methanol) and bombarded with Cs ions having a net energy of 12 KeV in the ZAB-2SE. For MS/MS experiments, the ions leaving the ion source were collisionally activated with helium in the first field-free region and the products were separated using a constant B/E linked-scan.

Results and Discussion

Elucidation of structures

As recorded in Table 1, compounds 4–6 showed spectroscopic properties that were consistent with the 2-hydroxy-3-phenylpyrazine substructure: (1) the compounds had UV maxima near 360 nm (see Fig. 2), (2) the aromatic region of the proton NMR spectra is consistent with a phenyl group attached to a

Table 1
Spectroscopic assignments for compounds 4–6

Spectroscopic properties		4	5	6*
UV (λ_{max})		367 nm	306, 361 nm	362 nm
Mol form		$C_{14}H_{14}N_2O$	$C_{14}H_{10}N_2O$	$C_{14}H_{12}N_2O_2$
MH^+	found	227.1171	223.0871	241.0968
	calc.	227.1184	223.0872	241.0977
MS/MS fragments	MH-18 (H_2O)	209 (100)	205 (100)	223 (65)
	MH-28(CO)	198 (35)	194 (65)	213 (100)
	MH-56 (C_4H_8)	170 (60)	—	—
	Ph-CN	104 (32)	104 (12)	104 (35)
	Ph	77 (16)	77 (16)	77 (20)
NMR assignments†	2	135.40/-	132.19/-‡	127.13/-
	3	25.85/2.58	115.20/7.36§	192.88/-
	4	21.15/1.73	123.44/7.34‖	37.12/2.95
	5	22.44/1.76	130.36/7.55‖	21.54/2.10
	6	28.78/2.63	128.85/7.85§	29.31/2.63
	7	129.77/-	132.14‡	129.10/-
	10	147.52/-	154.25/-	141.46/-
	11	155.49/-	154.70/-	154.98/-
	12	136.39/-	135.76/-	135.70/-
	13, 17	128.45/8.29	129.31/8.33	129.35/8.37
	14, 16	127.90/7.40	127.92/7.52	128.30/7.49
	15	129.09/7.42	130.25/7.52	131.14/7.49
	Exch. H	12.27	12.59	11.48

*NMR assignments tentative, based on comparison to compounds 4 and 5.
†Numbering of carbons for NMR is based on the proposed origin from the parent loracarbef molecule (see Scheme 2).
‡Assignment may be interchanged.
§Assignment may be interchanged.
‖Assignment may be interchanged.

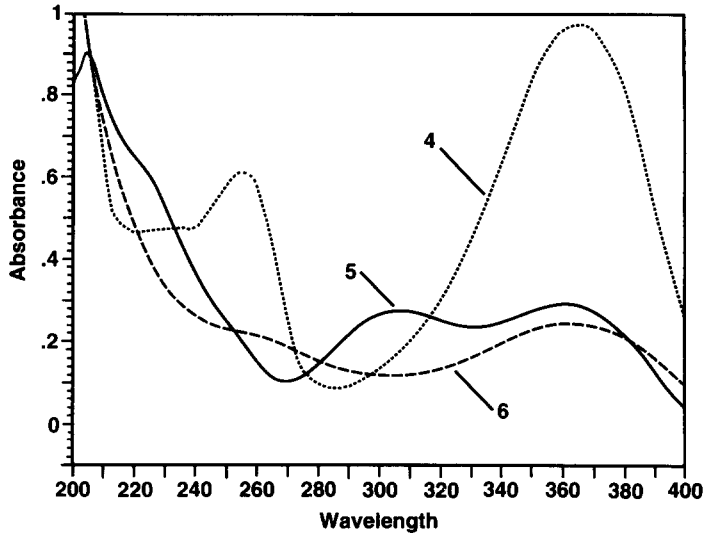


Figure 2
Overlaid UV spectra of degradation products 4, 5 and 6. The UV spectra were obtained on the photodiode array-UV detector as the compounds eluted from the column.

pyrazine ring [Baertschi *et al.*, manuscript in preparation]; and (3) there were diagnostic fragment ions for phenyl (*m/z* 77) and benzonitrile (*m/z* 104) in the MS/MS spectra of the protonated molecular ion. The proton NMR spectra of these compounds included resonances below δ12, characteristic of the exchangeable proton of these ring systems.

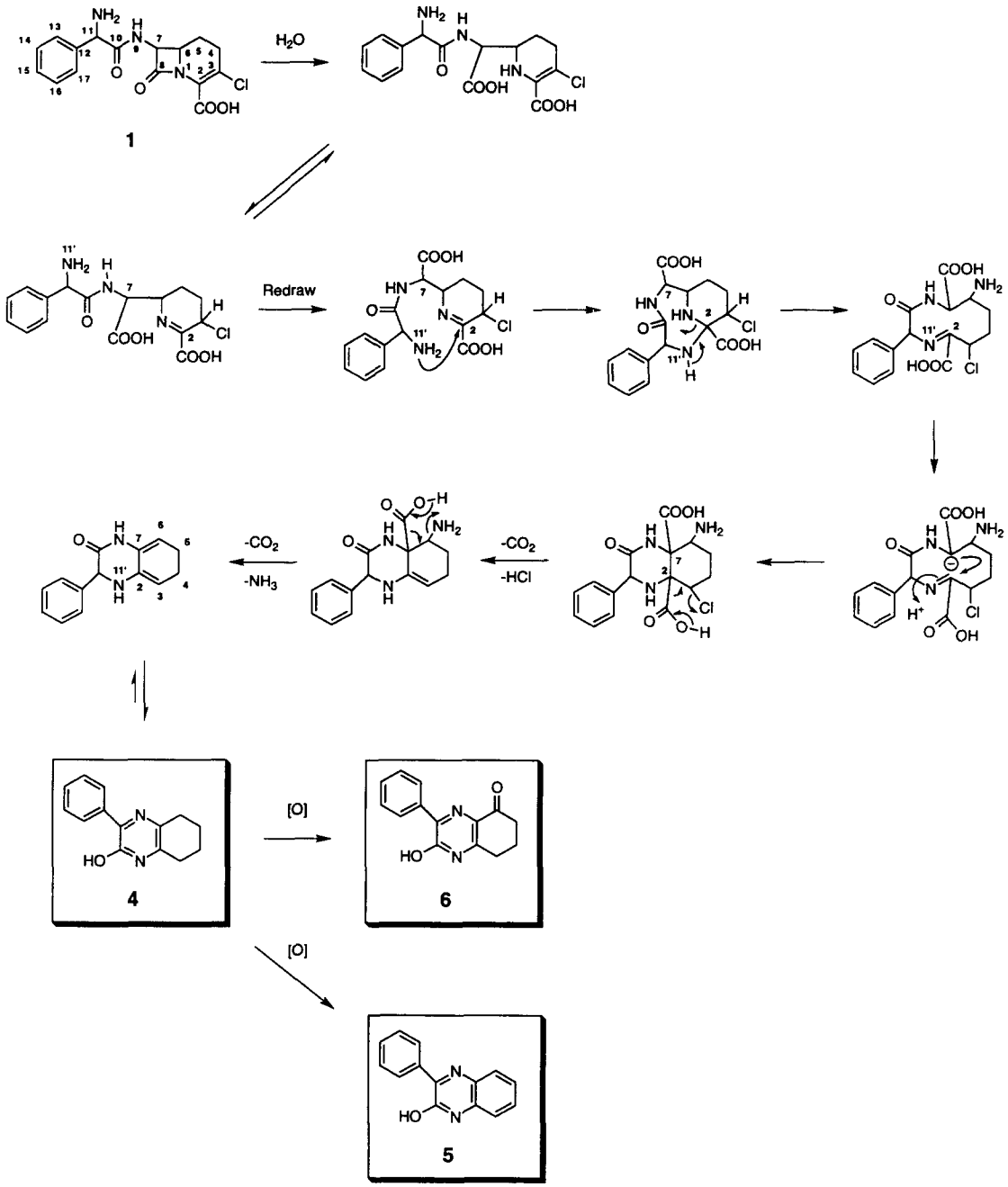
In the case of compound 4, the NMR data indicated, in addition to the 2-hydroxy-3-phenylpyrazine substructure, the presence of four methylene carbons. Given the molecular formula of compound 4 ($C_{14}H_{14}N_2O$, see Table 1), the presence of a 2-hydroxy-3-phenylpyrazine with the phenyl and exchangeable sites unsubstituted, and four additional methylenes, computer-aided structure analysis using GENOA [8] results in only one candidate structure for compound 4 (5,6,7,8-tetrahydro-2-hydroxy-3-phenylquinoxaline).

The structure of compound 5 contains two more sites of unsaturation than compound 4. Direct comparision of the spectra of these two compounds (see Table 1) suggests that in contrast to the presence of four *methylenes* in compound 4, compound 5 contains four sp^2-hybridized *methines*. When the molecular formula ($C_{14}H_{10}N_2O$), the 2-hydroxy-3-phenylpyrazine substructure with the phenyl and exchangeable sites unsubstituted, and four additional sp^2-hybridized methines, were input into the program GENOA, only structure 5 (2-hydroxy-3-phenylquinoxaline) was generated as a possible structure.

From the accurate mass of the MH^+ ion in the mass spectrum of compound 6, the molecular formula was deduced to be $C_{14}H_{12}N_2O_2$ (see Table 1). The ^{13}C NMR spectrum included a resonance typical of conjugated ketones (δ192.88). In addition, the proton NMR spectrum gave evidence for the presence of a chain of three methylene groups. This would be consistent with the replacement of two protons of a methylene group of structure 4 with an oxygen. There are two possible structures consistent with these data, structures 6 and 6a. Comparison of the NMR carbon–proton assignments of carbon 6 for compound 4 (carbon δ28.78, proton δ2.63) to the analogous carbon-proton pair in compound 6 shows a close correlation (carbon δ29.31, proton δ2.63). On this basis the keto group has been assigned to carbon 3, and the resulting structure is 7,8-dihydro-2-hydroxy-3-phenyl-5(6H)-quinoxalinone (compound 6).

Proposed mechanism of formation

The established phenylglycyl-cephalosporin degradation pathway to 2-hydroxy-3-phenyl pyrazine derivatives involves hydrolysis of carbon-6 to reveal the "masked aldehyde", and subsequent intramolecular cyclization and aromatization leading to the pyrazine structure (Scheme 1). In the case of loracarbef, the formation of the aldehyde intermediate is blocked because the sulphur is replaced by a methylene. Thus, the pathway to the pyrazine derivatives 4–6 must involve a different route.



Scheme 2 *Proposed Degradation Pathway to Substituted Pyrazine Derivatives*

The pathway to 4 involves: (1) hydrolysis of the β-lactam ring; (2) formation of new bonds between carbon 2 and nitrogen atom 11′ and between carbons 2 and 7; (3) eliminating the carboxyl groups, the chlorine atom, and nitrogen atom 1; and (4) tautomerizing the double bonds in the resulting molecule to yield the aromatic pyrazine group. The order of these steps cannot be determined with the present evidence, but a possible mechanism is proposed in Scheme 2. Compounds 5 and 6 can be viewed as resulting from the oxidation of compound 4. Alternative pathways for the formation of 4 from loracarbef may also be envisioned; however, the formation of the fused-bicyclic ring seems to be most straightforward as represented in Scheme 2. An analogous compound (structure 7) has been isolated from degradation studies of cefaclor (structure 2) [Baertschi *et al.*, manuscript in preparation], the sulpha-analogue of loracarbef. The presence and position of the

sulphur atom in 7 is consistent with the mechanism proposed in Scheme 2. This novel pathway to pyrazine derivatives from phenylglycyl cephalosporins is, therefore, not limited to carbacephalosporins, and may be present in other phenylglycyl cephalosporins. Further studies on the solid-state degradation of loracarbef (in progress) may help to further delineate this degradation pathway.

Acknowledgement — We thank Dwaine D. Slipher for his excellent work in isolating the degradation products described in the manuscript.

References

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[8] GENOA is a computer program designed to generate all candidate structures consistent with the molecular formula and a given set of substructural constraints; *cf.* R.E. Carhart *et al.*, *J. Org. Chem.* **46**, 1708–1718 (1981).