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Degradation of Clavulanic Acid in Aqueous Alkaline Solution: Isolation and Structural Investigation of Degradation Products

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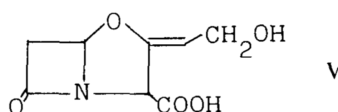
The degradation of potassium clavulanate in a weakly alkaline aqueous solution has been investigated. Potassium clavulanate was degraded in 0.1 M Na_2HPO_4 solution at various temperatures. Four degradation products were isolated and their structures were elucidated as 2,5-bis-(2-hydroxyethyl)pyrazine(I), 3-methyl-2,5-bis-(2-hydroxyethyl)pyrazine(II), 3-(2-carboxyethyl)-2,5-bis-(2-hydroxyethyl)pyrazine (III), and 3-ethyl-2,5-bis-(2-hydroxyethyl)pyrazine (IV) by mass spectroscopy and nuclear magnetic resonance spectroscopy. High-performance liquid chromatographic analysis of the reaction solution indicated that the reaction at 60 °C yielded all four pyrazine derivatives, whereas II was not formed at 35 °C and III was not formed at 100 °C. A reaction mechanism is proposed which involves 4-amino-3-oxobutanol (IX) as a key intermediate.

Keywords——clavulanic acid; clavulanic acid alkaline degradation; clavulanic acid degradation mechanism; pyrazine derivative NMR spectra; pyrazine derivative mass spectra

Introduction

Although clavulanic acid, (*Z*)-(2*R*,5*R*)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-azabicyclo[3.2.0]heptane-2-carboxylic acid (V), is a potent inhibitor of β-lactamases from



V

Gram-positive and Gram-negative bacteria and also from various clinical isolates,[1-4] it has only weak antibacterial activity itself. However, its use in combination with certain penicillins (amoxicillin and ticarcillin) is effective for clinical use.[5,6]

It is well known that penicillins are degraded in aqueous alkaline solution to yield the corresponding penicilloic acids, and that the latter compounds epimerize at the 5C position in aqueous solution.[7-10] However, the structures of the degradation products and the degradation mechanism of clavulanic acid in aqueous solution have not been well studied. Cherry and Newall reported that the degradation of lithium clavulanate yields formylacetic acid and 4-amino-3-oxobutanol.[11] In our previous work,[12] we investigated the stability of potassium clavulanate in aqueous solutions over a pH range of 3.2 to 10.1 at an ionic strength of 0.5 at 35 °C, and observed the formation of degradation products having absorption maxima (λ_{max}) around 280 nm in the weakly alkaline region. A preliminary high-performance liquid chromatographic (HPLC) analysis showed one major and three minor peaks of degradation products of potassium clavulanate in a weakly alkaline aqueous

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solution. The major peak substance was isolated and identified as 3-ethyl-2,5-bis-(2-hydroxyethyl)pyrazine (IV).[13] The present paper deals with the isolation and structural investigation of the other degradation products, and with the temperature dependence of the degradation reaction. The reaction mechanism is also discussed.

Experimental

Reagents and Materials——Potassium clavulanate was supplied by Beecham Yakuhin Co., Ltd. (Tokyo, Japan). Tetra-*n*-butylammonium bromide (TBAB), disodium hydrogen phosphate, and other chemicals of analytical reagent grade were purchased from Nakarai Chemicals Co. (Kyoto, Japan). Deionized and distilled water and distilled methanol were used to prepare the sample solutions and the eluents for HPLC and column chromatography.

Isolation of Degradation Products——(i) Potassium clavulanate (125 mg) was dissolved in 50 ml of 0.1 M Na_2HPO_4 solution, and kept at 50 °C for 6 h. The solvent was removed by evaporation under reduced pressure. The residue was dissolved in 2 ml of H_2O, and subjected to preparative column chromatography (LiChroprep RP-8, 310 × 25 mm i.d., E. Merck, Darmstadt, West Germany). The column was eluted successively with 280 ml of H_2O/MeOH = 80/1 (v/v) (1), 180 ml of H_2O/MeOH = 10/1 (v/v) (2), and methanol (3). The fractions between 80 and 100 ml of eluate 1, between 140 and 180 ml of eluate 2, and between 120 and 200 ml of eluate 3 were collected (fractions 1, 2, and 3, respectively).

(ii) Potassium clavulanate (125 mg) was degraded in 50 ml of 0.1 M Na_2HPO_4 solution at 100 °C for 6 h. The solvent was removed by evaporation under reduced pressure. The residue was extracted twice with 20 ml of methanol. The methanol solution was concentrated to a small volume, and subjected to preparative column chromatography as described above. The column was eluted successively with 90 ml of H_2O/MeOH = 80/1 (v/v), 225 ml of H_2O/MeOH = 10/1 (v/v), and H_2O/MeOH = 5/1 (v/v). The fraction between 200 and 250 ml of the last eluate was collected (fraction 4).

Product A: After removal of the solvent of fraction 2 by evaporation, the residue was dissolved in a small volume of methanol, and subjected to preparative HPLC for further purification. The stationary phase used was Develosil ODS-10 (Nomura Chemicals Co., Seto, Japan) packed in 25 cm × 10 mm i.d. stainless steel tubing. The eluent used was H_2O/MeOH = 5/1 (v/v). The fraction between 36 and 52 ml of the eluate was collected. Removal of the solvent by evaporation gave a reddish-yellow solid (product A). Ultraviolet (UV) $\lambda_{max}^{methanol}$: 206, 279, 296 (shoulder (sh)) nm. Fluorescence spectrum: λ_{ex}, 278 nm; λ_{em}, 339 nm.

Product B: Fraction 4 was treated in the same manner as described for the isolation of product A except that the eluent used was H_2O/MeOH = 4/1 (v/v). The fraction between 43 and 48 ml of the eluate was collected. Removal of the solvent by evaporation gave the product as white needles (product B). UV $\lambda_{max}^{methanol}$: 209, 278, 298 (sh) nm. Fluorescence spectrum: λ_{ex}, 279 nm; λ_{em}, 340 nm.

Product C: Fraction 1 was treated in the same manner as described for the isolation of product A except that the eluent used was H_2O. The fraction between 20 and 30 ml of the eluate was collected. The removal of the solvent by evaporation gave a reddish-yellow solid (product C). UV $\lambda_{max}^{methanol}$: 209, 279, 298 (sh) nm. Fluorescence spectrum: λ_{ex}, 280 nm, λ_{em}, 341 nm.

Product D: Fraction 3 was treated in the same manner as described for the isolation of product A except that the eluent used was H_2O/MeOH = 3/1 (v/v). The fraction between 43 and 48 ml of the eluate was collected. Removal of the solvent gave a reddish-yellow oily product (product D). UV $\lambda_{max}^{methanol}$: 209, 279, 298 (sh) nm. Fluorescence spectrum: λ_{ex}, 280 nm; λ_{em}, 340 nm.

Measurements——(i) UV Spectra: UV spectra were measured on a model 228 spectrophotometer (Hitachi Co., Ltd., Tokyo) using methanol as a reference solution. The UV spectra of HPLC peaks were obtained by stopping the flow of eluent and using a model 228 spectrophotometer equipped with a flow cell (Hitachi Co., Ltd.).

(ii) HPLC: A liquid chromatograph (TRIROTAR-V, Japan Spectroscopic Co., Ltd., Tokyo) equipped with a variable-wavelength detector (UVIDEC-100-V, Japan Scpectroscopic Co., Ltd.) or the model 228 spectrophotometer and a spectrofluorophotometer (RF-540, Shimadzu Co., Kyoto) was used in series with a stationary phase of Zorbax ODS (Du Pont Instrument Systems, Wilmington, Delaware, U.S.A.) packed in 150 × 4.6 mm i.d. stainless steel tubing. The eluent used was 5 mM TBAB + 5 mM Na_2HPO_4 + 5 mM NaH_2PO_4 solution/methanol = 5/1 (v/v) at a flow rate of 0.8 ml/min. The UV detection wavelength was 280 nm. The excitation and emission wavelengths for fluorometric detection were 280 and 340 nm, respectively. All operations were carried out at ambient temperature.

(iii) ^1H- and ^{13}C-Nuclear Magnetic Resonance (NMR) Spectra: ^1H- and ^{13}C-NMR spectra were measured on a JNM-FX 200 spectrometer (JEOL, Tokyo). The samples were dissolved in $CDCl_3$ or CD_3OD. The chemical shifts are given in parts per million (ppm) from internal tetramethylsilane and are reported in δ values.

(iv) Mass Spectra: Electron-impact (EI) mass spectra were recorded on a JMS-O1SG mass spectrometer (JEOL) and an M-80A mass spectrometer (Hitachi Co., Ltd.). Field-desorption (FD) mass spectra were measured with an M-80A mass spectrometer equipped with an FD ion source.

(v) Fluorescence Spectra: Fluorescence spectra were recorded on a RF-540 spectrofluorophotometer

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(Shimadzu Co.).

Results and Discussion

HPLC of Degradation Products

Figure 1 shows chromatograms of potassium clavulanate degraded in 0.1 M Na_2HPO_4 solution (pH 9.2) at various temperatures (35—100 °C) for 6 h. When the reaction was carried out at 35 °C, one major and several minor peaks due to degradation products were observed on a chromatogram (Fig. 1a). At this stage of the reaction, about 50% of potassium clavulanate remained unchanged (based on HPLC detection at 230 nm), but it was not detected here because potassium clavulanate itself has no UV absorption at the detection wavelength (280 nm). The UV spectrum of the major peak (peak 5) scanned by the stopped-flow method showed λ_{max} at 278 nm, and fluorometric detection gave no response. The intensity of peak 5 decreased with time and finally disappeared after 24 h. This peak was also observed initially in the cases of the reactions at 50 and 60 °C, but not at 100 °C. These results suggest that the product in peak 5 is a labile intermediate which is difficult to isolate by preparative chromatography. Among the minor peaks in Fig. 1a, peak 3 was more intense than peaks 1 and 4. HPLC of the degradation products (Fig. 1b) obtained by reaction at 50 °C for 6 h (at this stage, potassium clavulanate was completely degraded) revealed three major peaks (peaks 1, 3, and 4), but no peak corresponding to peak 5 in Fig. 1a appeared. The retention time of peak 4 (12.6 min) coincide with that of 3-ethyl-2,5-bis(2-hydroxyethyl)pyrazine (IV).[13] The retention time of peak 3 was markedly shortened when the cationic ion-pair reagent (TBAB) was not used as a component of the mobile phase. This suggests that this product has an anionic site in the molecule. Figure 1c shows the HPLC separation of the products formed by degradation at 60 °C for 6 h, indicating a new peak (peak 2) with a retention time between those of peaks 1 and 3. The relative intensity of peak 3

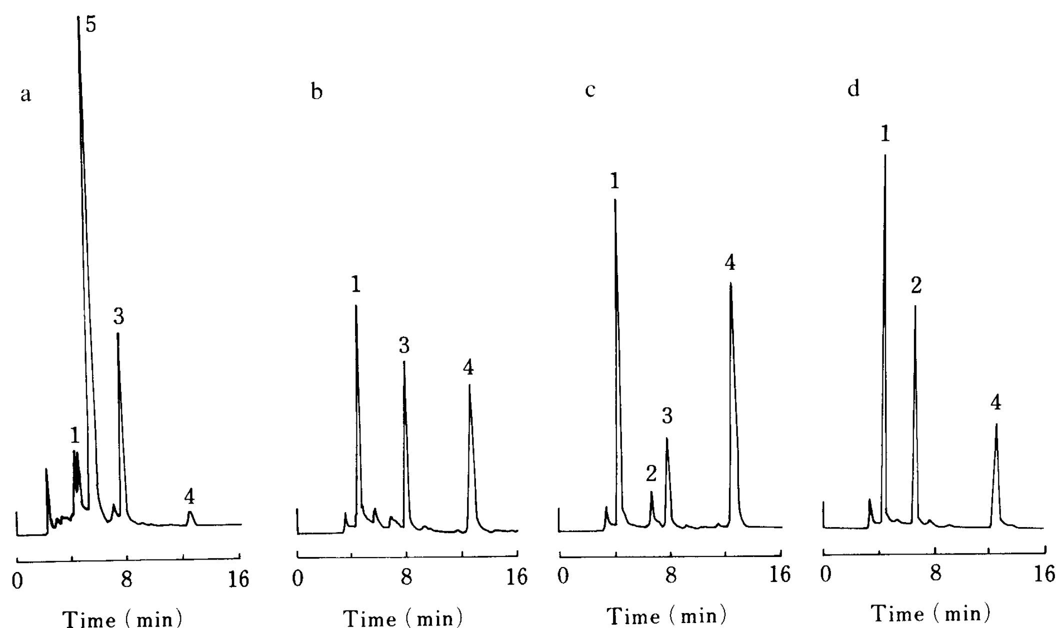

Fig. 1. Chromatogram of Potassium Clavulanate Degraded in 0.1 M Na_2HPO_4 Solution at Various Temperatures

Potassium clavulanate (1 mg/ml) was degraded at reaction temperatures of 35 °C (a), 50 °C (b), 60 °C (c), and 100 °C (d) for 6 h. Injection volume: 5 μl. Sensitivity: 0.08 aufs. Other HPLC conditions are given in Experimental.

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with respect to peaks 1 and 4 was smaller than those obtained in the reactions at 35 and 50 °C. The UV spectra of all these peaks measured by the stopped-flow method exhibited λ_{max}'s around 280 nm with shoulders at about 300 nm. Fluorometric detection indicated that all these substances were weakly fluorescent. After reaction at 100 °C, the intensities of peaks 1 and 2 were increased with concomitant disappearance of peak 3, while peak 4 became smaller than in Fig. 1c. As mentioned below we isolated the four products and determined their structures in order to elucidate the reaction mechanism. The isolated products A, B, C, and D

TABLE I. ^{1}H Chemical Shifts and Spin–Spin Coupling Constants of Degradation Products of Potassium Clavulanate and Their Assignments

Assignment	Chemical shift[a]			
	Product A[b]	Product B[b]	Product C[c]	Product D[b]
3C-H	8.40 (s)[e]			
6C-H	8.40 (s)	8.21 (s)	8.22 (s)	8.21 (s)
7C-H[d]	3.04 (t)	2.99 (t)	2.94 (t)	3.02 (t)
8C-H[d]	4.05 (t)	4.05 (t)	3.91 (t)	4.06 (t)
9C-H[d]	3.04 (t)	2.99 (t)	3.09 (t)	3.05 (t)
10C-H[d]	4.05 (t)	4.10 (t)	3.92 (t)	4.08 (t)
11C-H		2.53 (s)	3.15 (m)	2.86 (q)
12C-H			2.61 (m)	1.30 (t)
OH	−1.6 (br)	−3.6 (br)		−3.9 (br)
Coupling constant[f]				
$J_{7C\text{-}H,8C\text{-}H}$	5.8	5.4	6.4	4.8
$J_{9C\text{-}H,10C\text{-}H}$	5.8	5.4	6.7	4.8
$J_{11C\text{-}H,12C\text{-}H}$				7.1

a) In ppm. *b*) In $CDCl_3$. *c*) In CD_3OD.
d) Tentative assignment except for product A.
e) The abbreviations used are as follows: s, singlet; d, doublet; t, triplet; m, multiplet; q, quartet; br, broad.
f) In Hz.

TABLE II. ^{13}C Chemical Shifts and Assignments of Degradation Products of Potassium Clavulanate

Assignment	Chemical shift[a]			
	Product A[b]	Product B[b]	Product C[c]	Product D[b]
2C[d]	153.4 (s)[e]	151.6 (s)	151.6 (s)	151.4 (s)
3C[d]	143.6 (d)	151.9 (s)	156.7 (s)	157.9 (s)
5C[d]	153.4 (s)	152.4 (s)	153.2 (s)	153.2 (s)
6C	143.6 (d)	140.5 (d)	141.9 (d)	142.3 (d)
7C[d]	36.4 (t)	35.3 (t)	37.4 (t)	37.5 (t)
8C[d]	61.4 (t)	60.4 (t)	62.0 (t)	62.0 (t)
9C[d]	36.4 (t)	36.2 (t)	38.7 (t)	38.8 (t)
10C[d]	61.4 (t)	61.6 (t)	62.0 (t)	62.2 (t)
11C		21.6 (q)	32.0 (t)	28.4 (t)
12C			37.7 (t)	13.3 (q)
13C			181.4 (s)	

a) In ppm. *b*) In $CDCl_3$. *c*) In CD_3OD.
d) Tentative assignment except for product A.
e) Multiplicities were determined by off-resonance decoupling (s, singlet; d, doublet; t, triplet; q, quartet).

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TABLE III. EI Mass Spectra of Degradation Products of Potassium Clavulanate[a)]

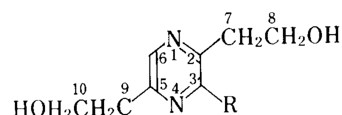
	Mass number[b)]		
	Product A	Product B	Product D
M^+	168 (39.2)[c)]	182 (39.5)	196 (100)
M^+-17	151 (63.2)	165 (47.9)	179 (62.8)
M^+-30	138 (68.9)	152 (48.9)	166 (69.1)
M^+-43	125 (40.6)	139 (20.7)	153 (12.6)
M^+-61	107 (100)	121 (100)	135 (82.2)

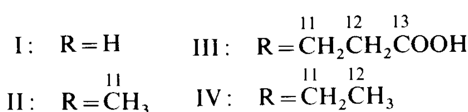
a) The EI mass spectrum of product C was not obtained.
b) In *m/z*. *c)* Relative intensity (%) to the base peak.

showed retention times corresponding to those of peaks 1, 2, 3, and 4, respectively. Thus, it follows that the degradation of potassium clavulanate in 0.1 M Na_2HPO_4 solution yields one labile intermediate and four relatively stable products.

Structures of Degradation Products

The observed chemical shifts, spin–spin coupling constants, and assignments of ^{1}H-NMR spectra of the isolated degradation products are summarized in Table I, and the ^{13}C-NMR chemical shifts and assignments are listed in Table II. The mass spectral data are given in Table III. The ^{1}H-NMR spectrum of product A gave signals due to CH_2 protons (3.04 and 4.05 ppm, coupling constant 5.8 Hz), aromatic protons (8.40 ppm), and OH protons (−1.6 ppm). The ^{13}C-NMR spectrum showed signals due to CH_2 carbons (36.4 and 61.4 ppm), olefinic CH (143.6 ppm), and quaternary carbons (153.4 ppm). These results indicate that product A is a symmetrically disubstituted pyrazine derivative, I. This was also





supported by the EI mass spectrum, which showed a molecular ion peak at *m/z* 168 and fragment ion peaks at *m/z* 151 (M^+-OH), 138 (M^+-CH_2O), 125 (M^+-CH_2CHO), and 107 ($M^+-CH_2O-CH_2OH$). Thus, product A was concluded to be 2,5-bis(2-hydroxyethyl)-pyrazine (I).

The ^{1}H-NMR spectrum of product B showed that this molecule has two CH_2CH_2OH groups, one aromatic proton, and one methyl group. The ^{13}C-NMR spectrum revealed the signals of one methyl carbon, four CH_2 carbons, one CH carbon, and three quaternary carbons. These results suggest that one aromatic proton on the pyrazine skeleton of I is replaced by a methyl group. The EI mass spectrum showed a molecular ion peak at *m/z* 182 and fragment ion peaks at *m/z* 165, 152, 139, and 121. These mass numbers are larger by 14 mass units than those of I. Thus, product B was concluded to be 3-methyl-2,5-bis(2-hydroxyethyl)pyrazine (II).

The ^{1}H- and ^{13}C-NMR spectra of product C are different from those of products A, B, and D; two multiplet proton signals were observed at 2.61 and 3.15 ppm, while a carbonyl

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carbon signal appeared at 181.4 ppm. It has been reported that ^{1}H-NMR signal of the CH_2CH_2 fragment in 3-arylpropanoic acid is of the AA′-BB′-type with the low-field half broadened by long-range coupling to the aromatic protons.[14] Therefore, the two multiplet proton signals can be assigned to CH_2CH_2 of a carboxyethyl group attached to the pyrazine skeleton. The presence of a carboxyl group in this molecule was suggested by the ^{13}C-NMR spectrum and the HPLC behavior as mentioned above. These results lead to the structure III for product C. The FD mass spectrum exhibited peaks at *m/z* 263, 285 (base peak), and 154, which are assignable to $(M+H)^+$, $(M+Na)^+$, and $(M+2Na)^{2+}$, respectively. Thus, product C is concluded to be the sodium salt of 3-(2-carboxyethyl)-2,5-bis(2-hydroxyethyl)pyrazine (III).

As reported previously,[13] product D was identified as IV.

Degradation Mechanism

A possible mechanism leading to the formations of I, II, III, and IV (Fig. 2) is as follows; the first step is initiated by attack of a hydroxy ion on the β-lactam ring of clavulanate. Since the ring-opened product thus formed is unstable as compared with penicilloic acids from penicillins, it immediately undergoes fission of the C–O bond on the oxapenam ring. The resulting product (VI) is then decarboxylated to yield 8-hydroxy-6-oxo-4-aza-2-octenoic acid (VII), and/or undergoes hydrolytic elimination of formylacetic acid to afford 2-amino-5-hydroxy-3-oxopentanoic acid (VIII). This step of reaction is followed by hydrolytic elimination of formylacetic acid from VII, and/or by decarboxylation of VIII, both yielding 4-

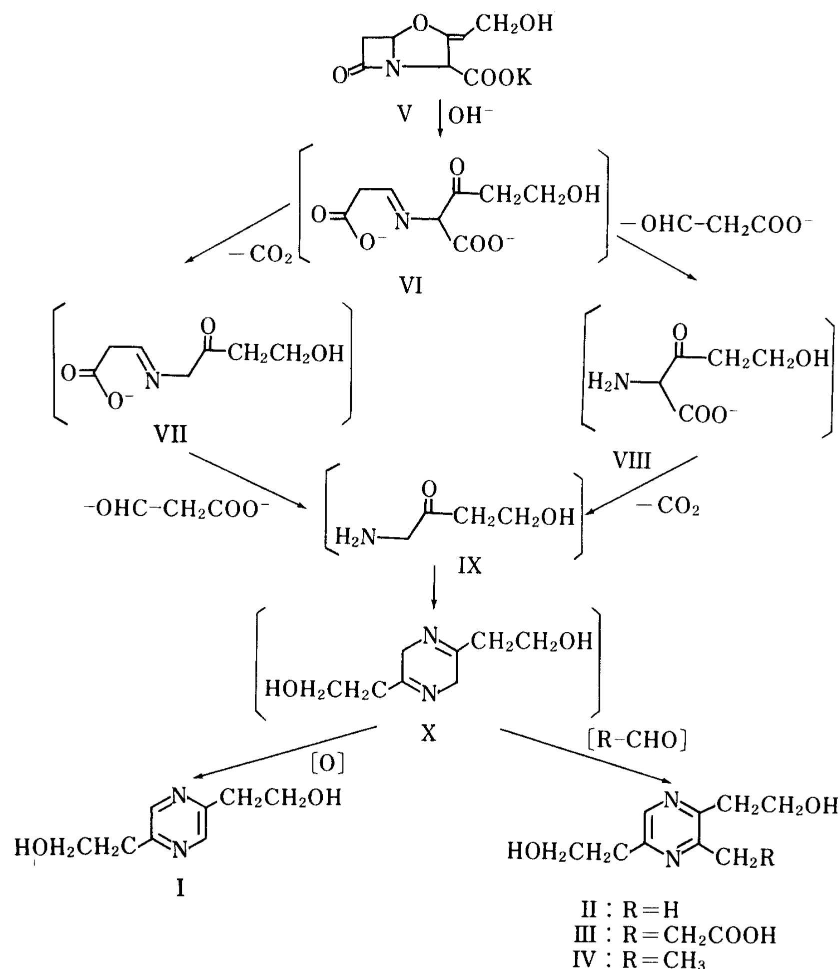

Fig. 2. Mechanism of Formation of Pyrazine Derivatives from Clavulanate

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amino-3-oxobutanol (IX) as a key intermediate. The three steps described above are consistent with the formation of IX, as suggested by Cherry and Newall.[11] The dimerization of IX yields a dihydropyrazine derivative (X), which seems to be rather unstable as compared with those substituted with alkoxycarbonyl and phenyl groups.[15] The spontaneous oxidation of X in the presence of air can yield I. The attacks of formaldehyde, formylacetic acid, and acetaldehyde, respectively, lead to II, III, and IV. Acetaldehyde can be supplied by decarboxylation of formylacetic acid, but the origin of formaldehyde is not clear. In order to investigate how these aldehydes participate in the reaction, a 1—10% (v/v) aqueous solution of formaldehyde or acetaldehyde was added to the reaction solution (100 °C). HPLC analysis indicated that no pyrazine derivatives were formed by addition of formaldehyde. This is possibly because Schiff's base formation between formaldehyde and the amino group of VIII or IX preceded the dimerization of IX. On the other hand, when acetaldehyde was used instead, IV was obtained as a major product. The role of formylacetic acid is obviously temperature-dependent, since the decarboxylation yielding acetaldehyde is favored at elevated temperature. As a result, the HPLC peak of III obtained by reaction at 35 °C (peak 3 in Fig. 1a) appeared to be larger than that of IV (peak 4), while they were similar at 50 °C (peaks 3 and 4 in Fig. 1b). Further elevation of the reaction temperature resulted in the reversal of their peak intensities at 60 °C (Fig. 1c), and finally in the disappearance of peak 3 at 100 °C.

It is known that aminopenicillins and aminocephalosporins are degraded under various conditions to yield highly fluorescent pyrazine derivatives.[16-19] The reactions are routinely used for assays of these antibiotics in pharmaceutical preparations and in body fluids. Though the mechanisms of these reactions are different from that mentioned above, it is interesting to note that pyrazine derivatives are commonly produced in the degradation of β-lactam antibiotics.

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References and Notes

1) P. A. Hunter, K. Coleman, J. Fisher, and D. Talor, *J. Antimicrob. Chemother.*, **6**, 455 (1980), and references cited therein.
2) D. Greenwood, F. O'Grady, and P. Baker, *J. Antimicrob. Chemother.*, **5**, 539 (1979).
3) M. Matsuura, H. Nakazawa, T. Hashimoto, and S. Mitsuhashi, *Antimicrob. Agents Chemother.*, **17**, 908 (1980).
4) M. H. Cynamon and G. S. Palmer, *Antimicrob. Agents Chemother.*, **23**, 935 (1983).
5) Abstracts of Papers, 29th Annual Meeting of the Japan Society of Chemotherapy, Nagasaki, June 1981, p. 171.
6) S. Bennett, R. Wise, D. Weston, and J. Dent, *Antimicrob. Agents Chemother.*, **23**, 831 (1983).
7) R. Busson, P. J. Claes, and H. Vanderhaeghe, *J. Org. Chem.*, **41**, 2556 (1976).
8) R. D. Carroll, S. Jung, and C. G. Sklavounos, *J. Heterocycl. Chem.*, **14**, 503 (1977).
9) J. P. Degelaen, S. L. Loukaes, J. Freeney, G. C. K. Roberts, and A. S. V. Burgen, *J. Chem. Soc., Perkins Trans. 2*, **1979**, 86.
10) A. E. Bird, E. A. Cutmore, K. R. Jennings, and A. C. Marshall, *J. Pharm. Pharmacol.*, **35**, 138 (1983).
11) P. C. Cherry and C. E. Newall, "Chemistry and Biology of β-Lactam Antibiotics," Vol. 2, ed. by R. B. Morin and M. Gorman, Academic Press, Inc., New York, 1982, p. 361.
12) J. Haginaka, T. Nakagawa, and T. Uno, *Chem. Pharm. Bull.*, **29**, 3334 (1981).
13) J. Haginaka, H. Yasuda, T. Uno, and T. Nakagawa, *Chem. Pharm. Bull.*, **31**, 1812 (1983).
14) S. F. Spassov and S. D. Simova, *J. Chem. Soc., Perkins Trans. 2*, **1978**, 1113.
15) G. W. H. Cheeseman and E. S. G. Werstiuk, "Advances in Heterocyclic Chemistry," Vol. 14, ed. by A. R. Katritzky and A. J. Boulton, Academic Press, Inc., New York, 1972, p. 99.
16) R. H. Barbhaiya, R. C. Brown, D. W. Payling, and P. Turner, *J. Pharm. Pharmacol.*, **30**, 224 (1978).
17) M. J. Lebelle, A. Villim, and W. L. Wilson, *J. Pharm. Pharmacol.*, **31**, 441 (1979).
18) T. Uno, M. Masada, Y. Kuroda, and T. Nakagawa, *Chem. Pharm. Bull.*, **29**, 1344 (1981).
19) J. M. Indelicato, D. E. Dorman, and J. L. Engel, *J. Pharm. Pharmacol.*, **33**, 119 (1981).

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