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Alkaline Degradation of Sulbactam

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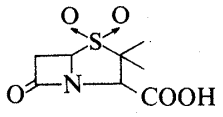
Alkaline degradation of sodium sulbactam in methanol and in an aqueous solution has been investigated. The degradation in alkaline methanol produced methyl 5-carboxy-6-methyl-6-sulfino-4-aza-2-heptenoate (II) which showed an ultraviolet (UV) absorption maximum at 279 nm. The UV absorption almost disappeared when the solution was acidified with methanolic HCl solution, and reappeared on subsequent realkalization, suggesting interconversion between II and its protonated form. The degradation in aqueous alkaline solutions yielded 5-carboxy-6-methyl-6-sulfino-4-aza-2-heptenoic acid (IV), which showed λ_{max} at 267 nm. IV was further degraded to 2-amino-3-methyl-3-sulfinobutanoic acid and formylacetic acid. The UV absorption of II and IV almost disappeared in aqueous acidic conditions, and reappeared on subsequent realkalization. These changes could be due to the hydrolysis of enamines II and IV to yield methyl formylacetate and formylacetic acid, respectively, in acidic conditions, and to the generation of the corresponding enolate ions on realkalization.

Keywords——sulbactam; sulbactam alkaline degradation; sulbactam alkaline degradation mechanism; sulbactam degradation product; sulbactam degradation product NMR spectra; sulbactam degradation product mass spectra; clavulanate

Introduction

Sulbactam, (2*S*,5*R*)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid 4,4-dioxide (I), is a potent β-lactamase inhibitor.[1]



I

In a previous paper,[2] we described the alkaline degradation of sodium sulbactam in methanol and in aqueous solutions, and reported the development of a high-performance liquid chromatographic (HPLC) method in which the alkaline degradation was employed as a postcolumn reaction for detection and quantitation of sulbactam. This paper deals with the structural investigation of the alkaline degradation products of sodium sulbactam in methanol and in aqueous solutions, and proposes a degradation mechanism.

Experimental

Reagents and Materials——Sodium sulbactam and potassium clavulanate were supplied by Pfizer-Taito Co., Ltd. (Tokyo, Japan) and Beecham Yakuhin Co., Ltd. (Tokyo, Japan), respectively. Tetra-*n*-butylammonium bromide (TBAB) and other chemicals of reagent grade were obtained from Nakarai Chemicals Co. (Kyoto, Japan), and were used without further purification. NaOD, D_2O, and CD_3OD used for the measurements of proton and carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR) spectra were purchased from CEA (Saclay, France).

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Deionized and distilled water and distilled methanol were used to prepare the eluents for HPLC and column chromatography.

Isolation of Alkaline Degradation Products——i) Methyl 5-Carboxy-6-methyl-6-sulfino-4-aza-2-heptenoate (II): Sodium sulbactam (10 mg) was dissolved in 2 ml of methanol, and 0.2 ml of NaOH-saturated methanol was added to the solution. After standing at room temperature for 30 min, the reaction solution was subjected to preparative column chromatography (LiChroprep Si-60, 310 × 25 mm i.d., E. Merck, Darmstadt, West Germany). The column was eluted successively with 120 ml of methanol–chloroform (1 : 1 (v/v)) and methanol–chloroform (2 : 1 (v/v)). The fraction between 140 and 200 ml of the second eluate was collected. Evaporation of the solvent under reduced pressure gave a yellowish-white solid. Fast atom bombardment (FAB) mass spectrum (MS),[3] *m/z* 266 $(M+H)^+$. ^{1}H-NMR (CD_3OD) δ: 1.04 (s, CH_3-*cis*), 1.08 (s, CH_3-*trans*), 1.14 (s, CH_3-*cis* and *trans*), 3.57 (s, OCH_3-*trans*), 3.59 (s, OCH_3-*cis*), 3.82 (CH-*trans*), 3.88 (s, CH-*cis*), 4.32 (d, CH–CO-*cis*, J=8.1 Hz), 4.73 (d, CH–CO-*trans*, J=13.2 Hz), 6.75 (d, =CH–NH-*cis*, J=8.1 Hz), 7.52 (d, =CH–NH-*trans*, J=13.2 Hz). UV λ_{max} 279 nm (methanol).

ii) 2-Amino-3-methyl-3-sulfinobutanoic Acid (VIII): Sodium sulbactam (50 mg) was dissolved in 10 ml of 0.15 M borate buffer (pH 8.8), and kept in a water-bath at 65 °C for 90 min. Then the solvent was removed by evaporation. The residue was dried *in vacuo*, and extracted twice with 10 ml of methanol. The methanolic solution was concentrated to about 2 ml, and subjected to preparative column chromatography (LiChroprep Si-60). The column was eluted with methanol–chloroform (3 : 1 (v/v)), and the fraction between 140 and 200 ml of the eluate was collected. After evaporation of the solvent, the residue was dissolved in 2 ml of H_2O, and subjected to reversed-phase column chromatography (LiChroprep RP-8, 310 × 25 mm i.d., E. Merck). The column was eluted with H_2O and the fraction between 50 and 70 ml of the eluate was collected. Evaporation of the solvent gave a yellowish-white solid. FAB-MS,[3] *m/z* 182 $(M+H)^+$. ^{1}H-NMR (0.5 N NaOD–D_2O) δ: 0.94 (3H, s, CH_3), 1.03 (3H, s, CH_3), 3.42 (1H, s, CH). ^{13}C-NMR (0.5 N NaOD–D_2O) δ: 14.3 (q, CH_3), 16.9 (q, CH_3), 60.2 (s, 3C), 60.9 (d, 2C), 180.3 (s, C=O). UV λ_{max} 229 nm (methanol).

Measurements——i) UV Spectra: UV spectra were measured on a model 228 spectrophotometer (Hitachi Co., Ltd., Tokyo). The UV spectra of HPLC peaks were obtained by the stopped-flow method using the model 228 spectrophotometer equipped with a flow-through cell.

Alkaline Degradation in Methanol: A 1 ml aliquot of methanolic solution of sodium sulbactam (54 μg/ml) was added to 3 ml of 10-fold diluted NaOH-saturated methanol. The UV spectra between 320 and 220 nm were measured at 0 and 120 min after mixing (Fig. 1).

Alkaline Degradation in Aqueous Solutions: A 2 ml aliquot of aqueous sodium sulbactam solution (15 μg/ml) was mixed with 2 ml of 0.5 N NaOH solution (final pH 13.2). The UV spectra of the solution between 290 and 240 nm were recorded at reaction times of 0 (immediately after addition of NaOH solution), 5, 10, 20, 40, and 80 min (Fig. 3). A 2 ml aliquot of aqueous sodium sulbactam solution (15 μg/ml) was added to 2 ml of 0.5 N NaOH aqueous solution (final pH 13.2). The UV spectrum of the solution was measured 10 min after addition (Fig. 4A). A 2 ml aliquot of the alkaline solution was neutralized with 1 ml of 0.5 N HCl solution (final pH 7.0). The UV spectrum of the neutralized solution was measured (Fig. 4B). A 3 ml aliquot of the neutral solution was realkalized with 1 ml of 0.5 N NaOH solution (final pH 13.1). The UV spectrum of the solution was measured (Fig. 4C).

ii) HPLC: A liquid chromatography (TRI ROTAR-V, Japan Spectroscopic Co., Ltd., Tokyo) equipped with a variable wavelength detector (UVIDEC-100-V, Japan Spectroscopic Co., Ltd. or a model 228 spectrophotometer equipped with a flow-through cell, Hitachi Co., Ltd.) was used in a reversed-phase mode. The reagent solution for the postcolumn reaction was delivered with a double-plunger pump (NP-DX-2, Nihon Seimitsu Kagaku Co., Ltd., Tokyo). A 0.5 mm i.d. × 1 m piece of Teflon tubing was used as the reactor. All operations were carried out at ambient temperature. Detailed conditions are given in the legends to Figs. 2 and 5.

iii) NMR Spectra: ^{1}H- and ^{13}C-NMR spectra were obtained on a JNM-FX 200 spectrometer (JEOL, Tokyo) at probe temperatures of about 30 and 60 °C. The chemical shifts are given in ppm from internal tetramethylsilane, sodium 3-(trimethylsilyl)propionate-d_4, or dioxane.

Degradation Products of Sodium Sulbactam in NaOD–D_2O: Sodium sulbactam (10 mg) was dissolved in 0.5 N NaOD–D_2O solution. The ^{1}H-NMR spectra were obtained at reaction times of about 10, 20, 40, 60, 80, 100, 120, 140, and 240 min. Sodium sulbactam (20 mg) was dissolved in 0.5 N NaOD–D_2O solution. The solution was allowed to stand at room temperature for 4 h, then the ^{13}C-NMR spectrum was obtained.

Degradation Products of Sodium Sulbactam in NaOD–CD_3OD: Sodium sulbactam (10 mg) was dissolved in 0.5 N NaOD–CD_3OD solution. The ^{1}H-NMR spectrum was obtained after 10 min standing at room temperature.

iv) Mass Spectra: MS were obtained with a JMS-DX 300 (JEOL) mass spectrometer equipped with a FAB gun. The samples were dissolved in methanol and then mixed with glycerol on the target.

Results and Discussion

Alkaline Degradation in Methanol

Figure 1 shows the UV spectra of an alkaline methanol solution of sodium sulbactam

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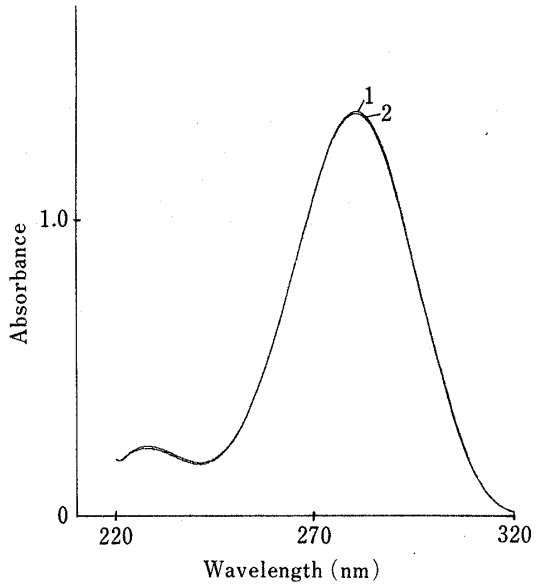


Fig. 1. UV Spectra of Sodium Sulbactam Degraded in Alkaline Methanol

Concentration: 13.5 μg/ml. Reaction time: 1, 0 min; 2, 120 min. Sample preparation: see Experimental.

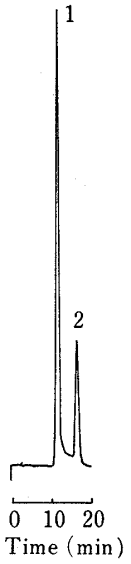
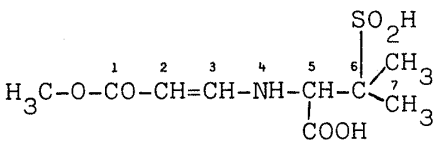


Fig. 2. Chromatogram of Sodium Sulbactam after Degradation in Alkaline Methanol

Concentration: 13.5 μg/ml. Reaction time: 30 min. HPLC conditions: column, Develosil ODS-10 (25 cm × 4.6 mm i.d.); eluent, 5 mM TBAB + 1 mM Na_2HPO_4 + 1 mM NaH_2PO_4–methanol (2 : 1 (v/v)); flow rate, 1.0 ml/min; detection, 280 nm; sensitivity, 0.016 aufs; injection volume, 5 μl. Peaks 1 and 2 are assigned, respectively, to *trans* and *cis* isomers of methyl 5-carboxy-6-methyl-6-sulfino-4-aza-2-heptenoate (II). Assignments: see the text.

kept standing at room temperature for 0 and 120 min. The spectra indicate the rapid degradation of sodium sulbactam (sodium sulbactam itself has no UV absorption above 200 nm) to yield a product(s) having λ_{max} 280 nm, and this product(s) is stable for at least 2 h. This UV absorption almost disappeared when the solution was acidified with methanolic HCl solution, and reappeared on subsequent realkalization with NaOH-saturated methanol. Figure 2 shows a chromatogram of the alkaline degradation products. Two peaks (1 and 2) with an area ratio of 3 : 1 were observed by detection at 280 nm. The UV spectra of peaks 1 and 2 measured by the stopped-flow method showed λ_{max} at 278 and 285 nm, respectively. When the eluates containing the peak 1 and 2 compounds were fractionated and re-chromatographed under the same conditions, peaks 1 and 2 were again obtained from each fraction. This suggests that relatively rapid interconversion occurs between these compounds. These results are in accordance with the fact that methyl 5-carboxy-6-methyl-6-sulfino-4-aza-2-heptenoate (II), which was isolated from the degradation solution (see Experimental), has λ_{max} at 279 nm (methanol), and gave two peaks with an area ratio of 3 : 1 with retention times



II

coinciding with those of peaks 1 and 2 in Fig. 2. It is known that λ_{max} of the *trans* isomer of β-amino-α,β-unsaturated carbonyl compound is at shorter wavelength by about 10 nm than that of the *cis* isomer.[4] From these and the NMR results mentioned later, it is concluded that the degradation of sodium sulbactam in alkaline methanol yields *trans* and *cis* isomers of

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II, which is responsible for the appearance of peaks 1 and 2 in Fig. 2. The appearance and disappearance of UV absorption at 279 nm could result from protonation and deprotonation of the nitrogen atom at the 4-position.

It is interesting to compare the above results with those for clavulanic acid, which is also a potent β-lactamase inhibitor; Davies and Howarth[5] reported that the degradation of potassium clavulanate in methanol yielded methyl 8-hydroxy-6-oxo-4-aza-2-octenoate (III). III exists as a 3 : 2 mixture of *trans* and *cis* isomers in chloroform and assumes a *trans* form in dimethyl sulfoxide[5] and in acetone.[6]

$$H_3C\text{–}O\text{–}CO\text{–}CH=CH\text{–}NH\text{–}CH_2\text{–}CO\text{–}CH_2CH_2OH \qquad \text{III}$$

The ^{1}H-NMR spectrum of II measured in CD_3OD at about 60 °C showed the loss of the 2C proton signals (4.32 ppm for the *cis* isomer and 4.73 ppm for the *trans* one) and change of the 3C proton signals (6.75 ppm for the *cis* isomer and 7.52 ppm for the *trans* one) from doublet to singlet. When sodium sulbactam was degraded in NaOD–CD_3OD, the ^{1}H-NMR spectrum showed the same signals as above except that the methyl proton of the methoxy group was absent. These results may reflect the tautomerism between the enamine II and the imine.

Alkaline Degradation in Aqueous Solution

Figure 3 shows the UV spectra of sodium sulbactam degraded in aqueous alkaline solutions (pH 13.2) for various time periods. The λ_{max} of the reaction solution moved from 267 to 260 nm with a concomitant decrease in the intensity at λ_{max}, as the reaction proceeded. The maximum absorbance at 267 nm was attained at a reaction time of 5 min. Alkaline degradation for 10 min at pH 13.2 at room temperature (Fig. 4A) followed by neutralization with an aqueous HCl solution to pH 7.0 resulted in the disappearance of the UV absorption (Fig. 4B). When the neutral solution of Fig. 4B was realkalized with an aqueous NaOH solution to pH 13.1, UV absorbance reappeared at 258 nm (Fig. 4C). Kemal and Knowles[7] reported that the degradation of sodium sulbactam in 0.5 N NaOH solution may yield 5-carboxy-6-methyl-6-sulfino-4-aza-2-heptenoic acid (IV). Cherry and Newall[8] reported that

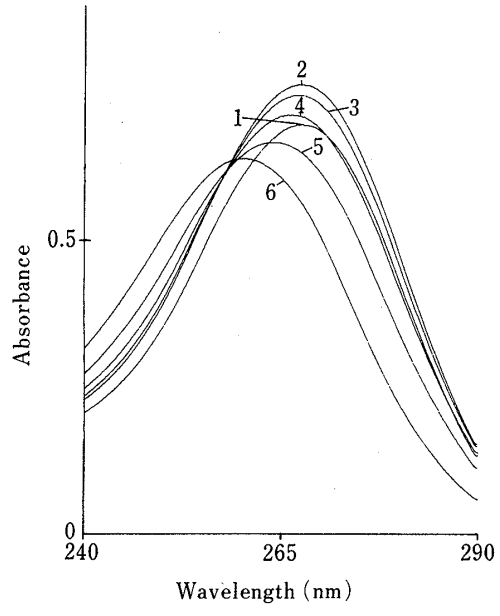

Fig. 3. UV Spectra of Sodium Sulbactam Degraded in Alkaline Aqueous Solutions

Reaction time: 1, 0 min; 2, 5 min; 3, 10 min; 4, 20 min; 5, 40 min; 6, 80 min. Sample preparation: see Experimental.

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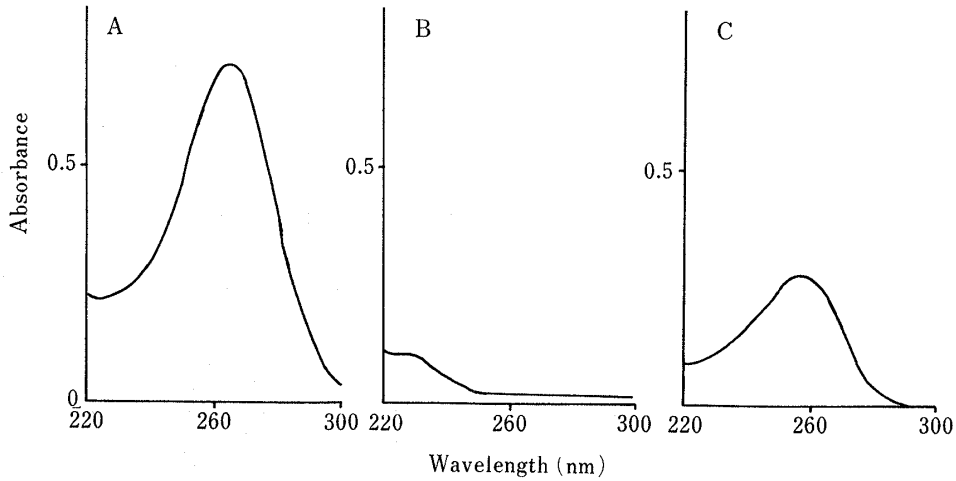
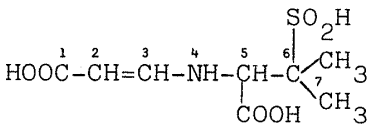


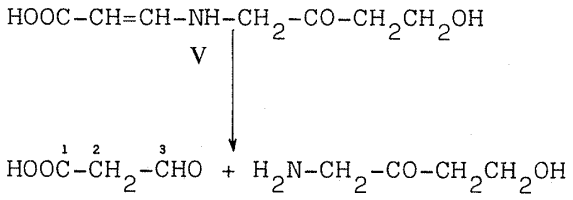
Fig. 4. pH Dependent Spectral Change of Sodium Sulbactam after Degradation in Alkaline Aqueous Solutions

pH of the solution: A, 13.2; B, 7.0; C, 13.1. Sample preparation: see Experimental.



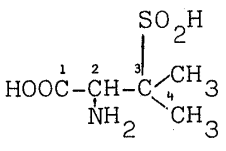
IV

the degradation of lithium clavulanate in alkaline aqueous solutions yields 8-hydroxy-6-oxo-4-aza-2-octenoic acid (V), which is very labile, being readily hydrolyzed to formylacetic acid (VI) and 1-amino-4-hydroxybutan-2-one (VII), and that the dianioic form of VI has a UV absorption maximum at 258 nm. They also found that acidification of a solution of the dianion resulted in the disappearance of the maximum at 258 nm because of the formation of VI, and this UV absorption was regenerated by realkalization due to the formation of the dianion.[8] Thus, the UV spectral changes shown in Fig. 4 can be reasonably explained by considering that sodium sulbactam is degraded in aqueous alkaline solutions to yield VI (which shows λ_{max} at 258 nm in alkaline solutions) and 2-amino-3-methyl-3-sulfinobutanoic acid (VIII) *via* IV (which shows λ_{max} at 267 nm). These considerations were also supported by the HPLC results. Figure 5A shows a chromatogram of sodium sulbactam degraded in 0.5 N NaOH solution followed



V

VI VII



VIII

by neutralization. Figure 5B shows a chromatogram of sodium sulbactam treated in the same manner as in Fig. 5A except that NaOH solution was delivered as a postcolumn reagent. Peak 1 with a retention time corresponding to that of VIII is observed on both chromatograms. When the eluate was alkalized with the NaOH solution, a new peak 2 with a retention time of 8.3 min appeared (Fig. 5B). The UV absorption spectrum of the peak 2 compound in an alkaline solution showed λ_{max} at 258 nm. The alkaline degradation of potassium clavulanate followed by neutralization also yielded the peak 2 compound, which was detected only by

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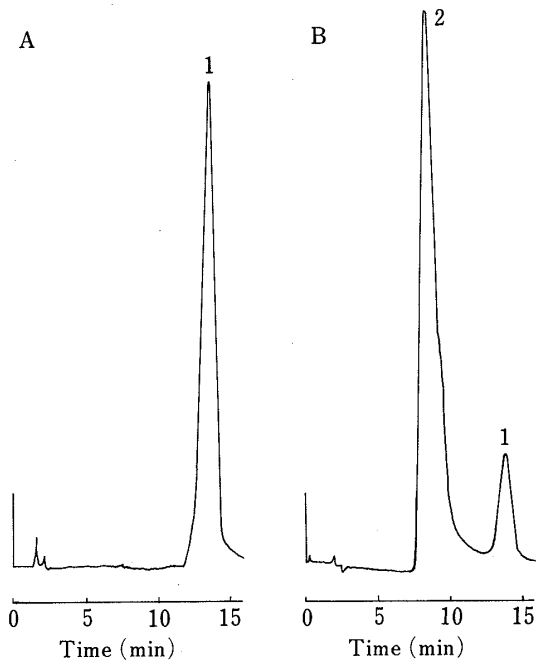

Fig. 5. Chromatogram of Sodium Sulbactam after Degradation in Alkaline Aqueous Solutions Followed by Neutralization

A 1 ml aliquot of aqueous solutions of sodium sulbactam (1 mg/ml) was mixed with 0.5 ml of 0.5 N NaOH solution. After standing at room temperature for 80 min, the reaction solution was neutralized with aqueous 0.5 N HCl solution to pH 7.0. HPLC conditions: column, Cosmosil $5C_{18}$ (15 cm × 4.6 mm i.d.); eluent, 5 mM TBAB + 1 mM Na_2HPO_4 + 1 mM NaH_2PO_4–methanol (20 : 1 (v/v)); flow rate, 1.0 ml/min; sensitivity, 0.04 aufs (A), 0.08 aufs (B); injection volume, 10 μl. In Fig. 5B, H_2O–MeOH (20 : 1 (v/v)) containing 0.5 N NaOH was delivered as a postcolumn reagent at a flow rate of 0.5 ml/min. Peak 1: 2-amino-3-methyl-3-sulfinobutanoic acid (VIII). Peak 2: formylacetic acid (VI). Assignments: see the text.

postcolumn alkalization. These results suggest that peak 2 could be due to VI, and that sodium sulbactam is degraded to yield VI and VIII.

In a previous paper,[2] we reported that the alkaline degradation of sodium sulbactam in methanol followed by degradation in aqueous alkaline solutions causes a hypsochromic shift of λ_{max} (279 nm) with a concomitant decrease in the intensity at λ_{max}, and that the alkaline degradation of sodium sulbactam in methanol followed by acidification with aqueous HCl solution to pH 0.7 results in the disappearance of the UV absorption, while realkalization of the solution results in a UV absorption maximum at 264 nm. The acidic solution was subjected to HPLC under the same conditions as in Fig. 5B. Two peaks were observed on a chromatogram; one has the same retention time as peak 1 in Fig. 5B, and the other was detected only by postcolumn alkalization (retention time, 6.2 min). The UV spectrum of the latter peak compound in alkaline solutions showed λ_{max} at 264 nm.

The alkaline degradation of potassium clavulanate in methanol followed by acidification also yielded a product with a retention time of 6.2 min. Cherry and Newall[8] reported that the alkaline degradation product of lithium clavulanate in methanol, III, is hydrolyzed in aqueous HCl solutions to yield VII and methyl formylacetate (IX), of which the latter has λ_{max} at

H_3C–O–CO–CH_2–CHO IX

264 nm in alkaline solutions, and that acidification of the solution of IX causes the disappearance of the UV absorption, while realkalization of the acidic solution regenerates the UV absorption. These results suggest that the methanolysis product of sodium sulbactam is hydrolyzed in aqueous HCl solutions to yield VIII and IX.

The NMR spectral results also support the view that the alkaline degradation of sodium sulbactam in aqueous solutions yields VI and VIII *via* IV. Figures 6, 7, and 8 show the ^{1}H-NMR spectral changes of sodium sulbactam degraded in 0.5 N NaOD–D_2O solution. Figure 9 shows the ^{13}C-NMR spectrum of sodium sulbactam degraded in 0.5 N NaOD–D_2O solution for 4—11 h. The ^{1}H-NMR signals of IV in 0.5 N NaOD–D_2O solution can be assigned as follows; 1.02 and 1.10 ppm to methyl protons, 3.66 ppm to the 5C proton, and 4.63 ppm (d, J = 13.4 Hz) and 7.27 ppm (d, J = 13.4 Hz) to the 2C and 3C protons, respectively. It is clear from the coupling constant that IV takes a predominantly *trans* configuration in NaOD–D_2O

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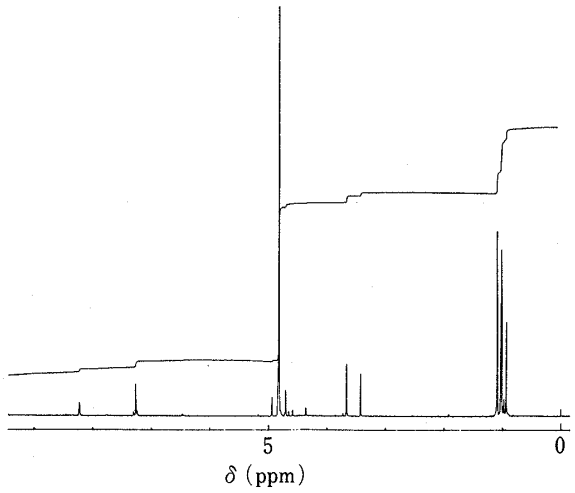

Fig. 6. ^{1}H-NMR Spectrum of Sodium Sulbactam Degraded in 0.5 N NaOD–D_2O Solution

Reaction time: 10 min. FT-NMR conditions: spectral width, 2000 Hz; acquisition time, 4.09 s; pulse flipping angle, 45°; number of data points, 16 K; number of recycles, 20.

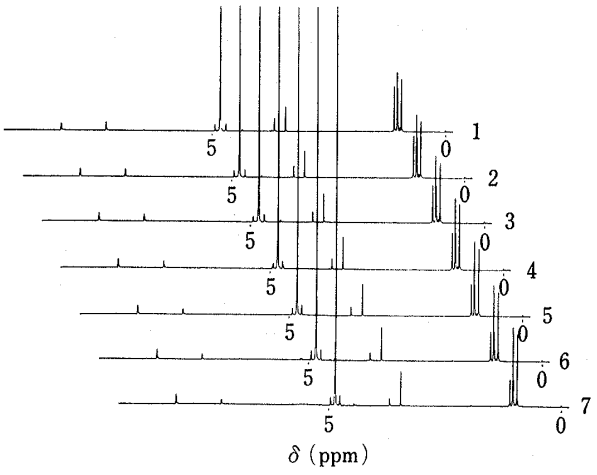

Fig. 7. ^{1}H-NMR Spectra of Sodium Sulbactam Degraded in 0.5 N NaOD–D_2O Solution

Reaction time: 1, 20 min; 2, 40 min; 3, 60 min; 4, 80 min; 5, 100 min; 6, 120 min; 7, 140 min. FT-NMR conditions: see the legend to Fig. 6.

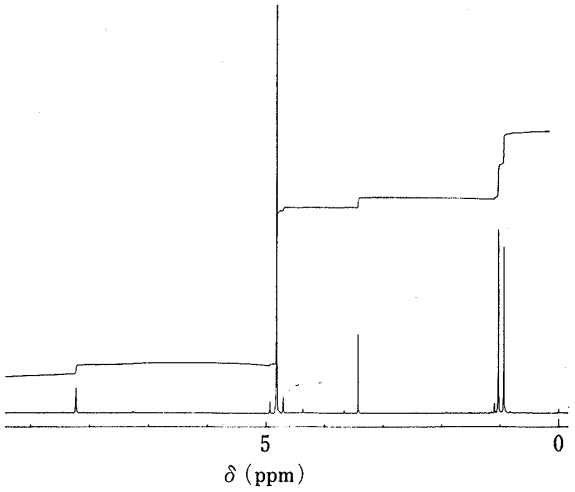

Fig. 8. ^{1}H-NMR Spectrum of Sodium Sulbactam Degraded in 0.5 N NaOD–D_2O Solution

Reaction time: 240 min. FT-NMR conditions: see the legend to Fig. 6.

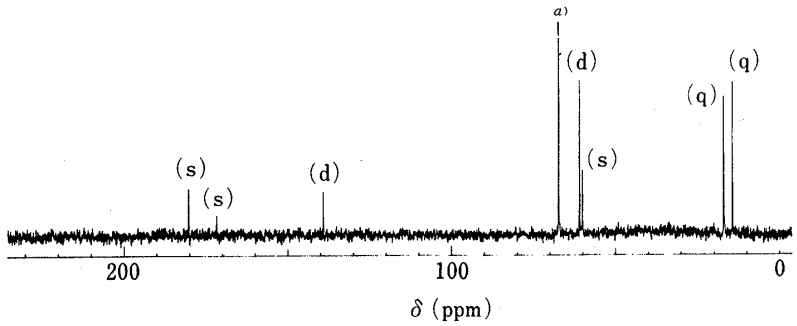

Fig. 9. ^{13}C-NMR Spectrum of Sodium Sulbactam Degraded in 0.5 N NaOD–D_2O Solution

Reaction time: 4 h. FT-NMR conditions: spectral width, 12004 Hz; acquisition time, 0.682 s; pulse flipping angle, 45°; number of data points, 16 K; number of recycles, 10000. Signal multiplicities obtained from the off-resonance decoupled spectrum are given on top of each line. *a*) indicates the solvent.

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solution. As shown in Fig. 6, the doublet for the 3C proton changed to a broad singlet upon deuteration of the 2C proton. The changes found in Fig. 7 reveal a decrease in the intensity of the signals assigned to IV and a concomitant increase in that of the signals assigned to other degradation product(s). The ^{1}H-NMR spectrum of the isolated product VIII in 0.5 N NaOD–D_2O solution showed signals at 0.94 and 1.03 ppm due to methyl protons, and 3.42 ppm due to the 2C proton. The ^{13}C-NMR signals of VIII in 0.5 N NaOD–D_2O solution are as follows: 14.3 and 16.9 ppm due to methyl carbons, 60.2 ppm due to the 3C carbon, 60.9 ppm due to the 2C carbon, and 180.3 ppm due to the carbonyl carbon. These results suggest that IV is further degraded to yield VIII. The ^{1}H-NMR signal at 8.22 ppm (Fig. 8) and the ^{13}C-NMR signals at 139.2 ppm and 171.1 ppm (Fig. 9) might be assigned to the dianion of VI whose 2C protons were deuterated. The ^{1}H-NMR spectrum of potassium clavulanate degraded in 0.5 N NaOD–D_2O solution showed a broad singlet signal at 8.22 ppm. These results suggest that the alkaline degradations of both sodium sulbactam and potassium clavulanate in aqueous solutions yield VI as a common product.

In a previous paper,[6] we suggested that the alkaline degradation of potassium clavulanate might yield V (λ_{max} 258 nm), and that the acid-base interconversions between V and its protonated form, and between III and its protonated form may be responsible for the reappearance and disappearance of the UV absorptions. As described above, it was found that the λ_{max} at 258 nm was due to the dianion of VI, and the disappearance and reappearance of the UV absorptions were due to the acid-base induced interconversions between VI and its dianion, and between IX and its anion.

Degradation Mechanism

A possible mechanism of the alkaline degradation of I in aqueous solutions and in methanol is illustrated in Chart 1. The first step of the reaction is initiated by the attack of

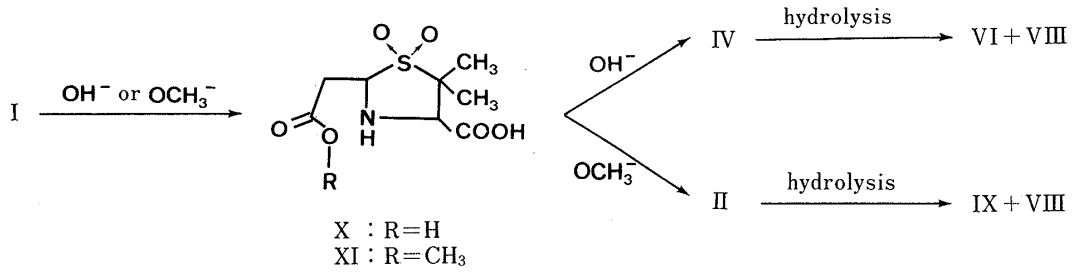

Chart 1. Proposed Degradation Mechanism of Sulbactam

hydroxide ion or methoxide ion on the β-lactam ring. The resulting products (X and XI) are short-lived intermediates, and the subsequent fission of C–S bonds of X and XI produces IV and II, respectively. IV is rather stable in the high pH region, but is very unstable under neutral and acidic conditions, as reported by Kemal and Knowles.[7] The hydrolysis of IV yields VI and VIII. II is rather stable at high pH, but is unstable under acidic conditions. The hydrolysis of II yields IX and VIII.

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