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Degradation of β-Lactamase Inhibitor, (2*S*,3*R*,5*S*)-3-Methyl-7-oxo-3-(1*H*-1,2,3-triazol-1-yl-methyl)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic Acid 4,4-Dioxide (YTR-830H), in Aqueous Solutions and Alkaline Methanol Solution: Pathway and Structural Elucidation of Products

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(2*S*,3*R*,5*S*)-3-Methyl-7-oxo-3-(1*H*-1,2,3-triazol-1-yl-methyl)-4-thia-1-azabicyclo[3.2.0]-heptane-2-carboxylic acid 4,4-dioxide (YTR-830H) is a new β-lactamase inhibitor. The degradation of this β-lactamase inhibitor in several buffer solutions, NaOH aqueous solution and NaOH-saturated methanol solution was investigated. In the initial step of degradation in aqueous solutions, YTR-830H was degraded to 2-amino-3-methyl-3-sulfino-4-(1*H*-1,2,3-triazol-1-yl)-butyric acid (YTR-830H-II) and formylacetic acid (YTR-830H-III) through (*E*)-5-methyl-5-sulfino-6-(1*H*-1,2,3-triazol-1-yl)-3-aza-1-heptene-1,4-dicarboxylic acid (YTR-830H-I) as an intermediate. The YTR-830H-II in all solutions then proceeded to an unidentified product, YTR-830H-IIa. In acidic solution it was converted to 1,2,3-triazole and several unidentified products (YTR-830H-IIa, -IIb, -IIc and -IId). Degradation at various pHs showed different patterns and rates. Following degradation in NaOH-saturated methanol solution, 1-methyl hydrogen (*E*)- and (*Z*)-5-methyl-5-sulfino-6-(1*H*-1,2,3-triazol-1-yl)-3-aza-1-heptene-1,4-dicarboxylic acid (YTR-830H-Ia and -Ib) were detected.

Keywords——(2*S*,3*R*,5*S*)-3-methyl-7-oxo-3-(1*H*-1,2,3-triazol-1-yl-methyl)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid 4,4-dioxide (YTR-830H); β-lactamase inhibitor; degradation pattern; structural elucidation; (*E*)-5-methyl-5-sulfino-6-(1*H*-1,2,3-triazol-1-yl)-3-aza-1-heptene-1,4-dicarboxylic acid (YTR-830H-I); 1-methyl hydrogen (*E*)-5-methyl-5-sulfino-6-(1*H*-1,2,3-triazol-1-yl)-3-aza-1-heptene-1,4-dicarboxylic acid (YTR-830H-Ia); 1-methyl hydrogen (*Z*)-5-methyl-5-sulfino-6-(1*H*-1,2,3-triazol-1-yl)-3-aza-1-heptene-1,4-dicarboxylic acid (YTR-830H-Ib); 2-amino-3-methyl-3-sulfino-4-(1*H*-1,2,3-triazol-1-yl)-butyric acid (YTR-830H-II); formylacetic acid (YTR-830H-III); 1,2,3-triazole

YTR-830H, (2*S*,3*R*,5*S*)-3-methyl-7-oxo-3-(1*H*-1,2,3-triazol-1-yl-methyl)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid 4,4-dioxide, is a new β-lactamase inhibitor, developed through with the cooperation of R.G.Micetich and Taiho Pharm. Co. (Tokushima, Japan).[1)]

This β-lactamase inhibitor, which is now undergoing studies as a combination therapy with piperacillin (PIPC), has been shown to extend the *in vitro* spectrum of β-lactam antibiotics to a number of resistant organisms. The enzymological and bacteriological activities of this β-lactamase inhibitor are distinct from those of clavulanic acid and sulbactam. It has also been demonstrated to have relatively little intrinsic biological activity.[2)]

The degradation of YTR-830H in several buffer, aqueous NaOH and NaOH-saturated methanol solutions was investigated, with monitoring by high-performance liquid chromatography (HPLC), ultraviolet (UV) absorption spectroscopy and ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectroscopy. Following identification of the structures of the degradation

products, the degradation pathways of YTR-830H were deduced.

Experimental

Materials and Reagents——β-Lactamase inhibitor YTR-830H was supplied by our Synthetic Laboratory [IR (KBr): 1795, 1700 cm^{-1}. FAB-MS: m/z 301 $[M+1]^+$. ^{1}H-NMR (DMSO-d_6) δ ppm: 1.34 (s, 11-CH_3), 3.31, 3.72 (dd, J=1.5, 16.5 Hz, 4.5, 16.5 Hz, 6-CH_2), 4.79 (s, 3-CH), 4.90, 5.26 (d, J=15.5 Hz, 2-CH_2), 5.20 (m, 5-CH), 7.79 (d, J=1 Hz, triazole 4-CH=), 8.09 (d, J=1 Hz, triazole 5-CH=)]. 2-Amino-3-sulfino-3-(1*H*-1,2,3-triazol-1-ylmethyl)butyric acid (YTR-830H-II), which had been identified as a degradation product of YTR-830H, was prepared by hydrolysis of YTR-830H with sodium hydroxide solution and subsequent purification using cation exchange resin column (H^+-type) chromatography. Acetonitrile was of liquid chromatographic reagent grade. The NaOD, D_2O and CD_3OD used for the measurement of ^{1}H-NMR spectra were obtained from Merck (Darmstadt, G.F.R). The ion-pair chromatographic reagent, PIC-A® (low UV), containing tetra-*n*-butylammonium salt, was obtained from Waters Assoc. (Milford, MA, U.S.A.). Other chemicals used were all purchased from Wako (Osaka, Japan).

CHP-20P resin (150—300 μm particle size) and silica gel (Kieselgel 60, 70—230 mesh) were obtained from Mitsubishi Chemical Industry (Tokyo, Japan) and Merck, respectively. The PIC-A aqueous solution, used as a mobile phase, was prepared to 5 mM concentration by adding the contents of one vial (*ca.* 20 ml) of commercial PIC reagent to 1.0 l of distilled water.

The buffer solutions used were prepared as follows: Britton–Robinson buffer solution (pH 3.0) was prepared by adding 18.5 ml of 0.2 N NaOH to 1.0 l of ion-exchanged water containing 3.92 g of H_3PO_4, 2.40 g of CH_3COOH and 2.47 g of H_3BO_3. Phosphate buffer solution (pH 7.0) was prepared by mixing 40 ml of 1/15 M KH_2PO_4 and 60 ml of 1/15 M Na_2HPO_4. Clark–Lubs buffer solution (pH 9.0) was prepared by adding 21.3 ml of 0.2 N NaOH and 128.7 ml of ion-exchanged water to 50 ml of a mixture of 0.2 M boric acid and 0.2 N KCl.

Instruments——The IR spectra were measured on a Hitachi 260-50 infrared (IR) spectrophotometer (Tokyo, Japan) by the KBr tablet method. The UV spectra were recorded with a Shimadzu UV-265FW spectrophotometer (Kyoto, Japan). Measurement of ^{1}H-NMR spectra was carried out using a JEOL FX-100 NMR spectrometer coupled with an FAFT 70 data system and equipped with a Fourier transformed (Tokyo, Japan), using either tetramethylsilane or 3-(trimethylsilyl)propionic acid-d_4 as an internal standard. A JEOL DX-303 mass spectrometer linked to a JMA-DA5000 data system was used for the measurement of electron impact and fast atom bombardment mass spectra (EI- and FAB-MS). FAB-MS were measured using water or dimethylsulfoxide as a solvent and a mixture of glycerol and thioglycerol (1 : 1, v/v) as a matrix on the target. EI-MS were obtained under the following conditions: ionization energy, 70 eV; ionization current, 300 μA; and acceleration voltage, 3.0 kV. The high-performance liquid chromatographic system consisted of two LC-6A pumps, an SPD-6A variable-wavelength UV detector, a CTO-6A column oven, a CRB-6A reaction oven, a SIL-6A automatic injector and a C-R3A Chromatopac data system (Shimadzu, Kyoto, Japan). A third LC-6A pump was used for post-column reagent addition.

HPLC——The separation of the degradation products of YTR-830H was carried out under the following conditions.

i) HPLC Condition 1: Column, Develosil ODS-5 (Nomura Kagaku, Nagoya, Japan) 150 × 4.6 mm i.d.; column temperature, room temperature; mobile phase, acetonitrile/PIC-A (low UV) aqueous solution = 10 : 90 (v/v); flow rate, 1.5 ml/min; and detector, UV 220 nm (0.08 a.u.f.s.).

ii) HPLC Condition 2: Column, Develosil ODS-5, 150 × 4.6 mm i.d.; column temperature, room temperature; mobile phase, acetonitrile/PIC-A (low UV) aqueous solution = 1 : 99 (v/v); flow rate, 1.5 ml/min; reaction coil, 60 cm × 0.2 mm i.d.; reaction reagent, 0.5 N NaOH; flow rate of reagent, 0.5 ml/min; reaction temperature, room temperature; and detector, UV 265 nm (0.32 a.u.f.s.) and UV 220 nm (0.16 a.u.f.s.)

iii) HPLC Condition 3: Column, Develosil ODS-5, 150 × 4.6 mm i.d.; column temperature, room temperature; mobile phase, acetonitrile/PIC-A (low UV) aqueous solution = 15 : 85 (v/v); flow rate, 1.5 ml/min; and detector, UV 276 nm (0.08 a.u.f.s.)

Degradation in Buffer Solutions——YTR-830H was dissolved in Britton–Robinson buffer (pH 3.0), phosphate buffer (pH 7.0) and Clark–Lubs buffer (pH 9.0) solutions to a concentration of 500 μg/ml. Each solution, after standing at 35 °C for 3, 12, 15, 20, 36, 42, 60, 66, 84, 107 and 160 h, was directly injected into the chromatograph. Then the degradation products were analyzed under HPLC conditions 1 and 2.

Degradation in Aqueous Alkaline Solution——To 2 ml of aqueous YTR-830H (100 μg/ml) solution, 2 ml of 1 N NaOH solution and 4 ml of distilled water were added (final pH 12). The solution was mixed well and the UV spectra were recorded at reaction times of 1, 5, 15, 30, 60 and 120 min. The UV spectral changes of YTR-830H in alkaline, neutral and realkalinized solutions were obtained using the following preparations. A 2 ml aliquot of aqueous YTR-830H solution (100 μg/ml) was diluted with 4 ml of 0.5 N NaOH solution. Then a 2 ml aliquot of the alkaline solution was neutralized with 1 ml of 0.5 N HCl solution. Finally, a 3 ml aliquot of the neutral solution was realkalinized with 1 ml of 0.5 N NaOH solution.

YTR-830H (15 mg) was dissolved in 0.3 ml of 0.5 N NaOD–D_2O and ^{1}H-NMR spectra were measured after

allowing the solution to stand at room temperature for 10, 60 and 120 min, and 16 h.

The reaction in aqueous NaOH solution was also analyzed, both with and withour post-column alkalization HPLC (HPLC condition 2), to monitor the products.

Degradation in NaOH-Saturated Methanol——A 1 ml aliquot of methanol solution containing 100 μg/ml of YTR-830H was diluted with 0.3 ml of NaOH-saturated methanol and 2.7 ml of methanol, mixed and then allowed to stand at room temperature for 1, 30 and 60 min. The UV spectra and chromatograms, under the HPLC condition 3, were recorded for each preparation.

For the ^{1}H-NMR spectral investigations, 15 mg of YTR-830H was dissolved in 0.25 ml of CD_3OD and 10 μl of 40% NaOD/D_2O and the resulting solution was left standing at room temperature for 10, 20, 30, 60 and 90 min, after which the spectra were measured.

Isolation of a Mixture of YTR-830H-Ia and YTR-830H-Ib——YTR-830H (20 mg) was dissolved in 2 ml of methanol and 0.2 ml of NaOH-saturated methanol. After standing at room temperature for 30 min, the reaction solution was applied to a silica gel column (250 mm × 20 mm i.d., packed using chloroform), which was washed with 50 ml of a mixture of methanol and chloroform (1 : 1, v/v) and then eluted with a mixture of methanol and chloroform (2 : 1, v/v). The collected fraction between 50 and 100 ml of the second eluate was evaporated to dryness under nitrogen gas *in vacuo*. It yielded 10 mg of a yellow-white powder consisting of YTR-830H-Ia and YTR-830H-Ib.

Isolation of YTR-830H-II——An aqueous solution containing 0.5 g of YTR-830H was diluted with 3 ml of 1N NaOH and reacted at room temperature for 1 h. The resultant solution was next applied to a cation-exchange resin column (Diaion WK-10S, H^+-type, 10 cm × 2 cm i.d.) and eluted with distilled water. The combined elution products were lyophilized to yield 100 mg of YTR-830H-II as a yellow-white powder.

Results and Discussion

Degradation in Buffer Solutions

The time-dependent degradation of YTR-830H in buffer solutions at pH 3.0, 7.0 and 9.0, and in distilled water was investigated at 35 °C with monitoring by HPLC (condition 1). The chromatograms for each solution are shown in Fig. 1. The time-dependent changes of the chromatograms of YTR-830H and its degradation products, prepared by plotting the relative peak area of each compound, are illustrated in Figs. 2—5.

The degradation of YTR-830H in buffer solution at pH 3.0 and in distilled water proceeded very slowly, as shown by the fact that even after the reaction had continued for 160 h, only *ca.* 20% of the initial amount of YTR-830H was degraded (Figs. 2 and 5). YTR-830H-II (peak b in Figs. 1A and 1D) and other degradation products (1,2,3-triazole, YTR-830H-IIa, -IIb, -IIc and -IId, peaks c, d, e, f and g in Figs. 1A and 1D) were detected. These products were not observed in buffer solutions at pH 7.0 and 9.0 (Figs. 1B and 1C). The product YTR-830H-II is stable for more than 24 h under the reaction conditions, showing

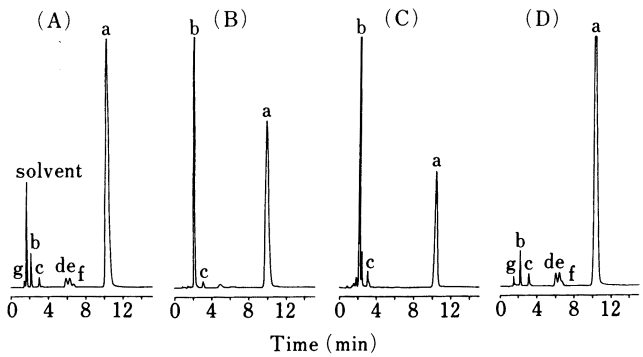

Fig. 1. Chromatograms of YTR-830H after Degradation Under Various Conditions

(A) In Britton–Robinson buffer (pH 3.0) at 35 °C for 84 h, (B) in phosphate buffer (pH 7.0) at 35 °C for 160 h, (C) in Clark–Lubs buffer (pH 9.0) at 35 °C for 36 h and (D) in distilled water at 35 °C for 84 h.

a, YTR-830H; b, YTR-830H-II; c, YTR-830H-IIa; d, YTR-830H-IIb; e, YTR-830H-IIc; f, YTR-830H-IId; g, 1,2,3-triazole. HPLC condition 1 was used.

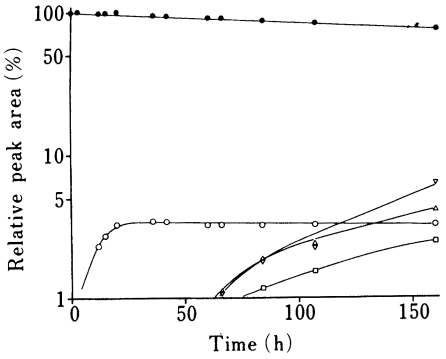

Fig. 2. Changes of YTR-830H and Its Degradation Products in Britton–Robinson Buffer (pH 3.0) at 35 °C

●, YTR-830H; ○, YTR-830H-II; □, YTR-830H-IIa; △, YTR-830H-IIb; ▽, YTR-830H-IIc. HPLC condition 1 was used.

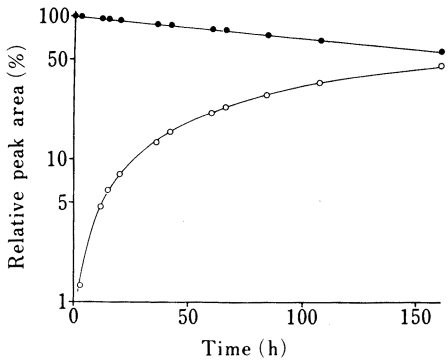

Fig. 3. Changes of YTR-830H and Its Degradation Products in Phosphate Buffer (pH 7.0) at 35 °C

●, YTR-830H; ○, YTR-830H-II. HPLC condition 1 was used.

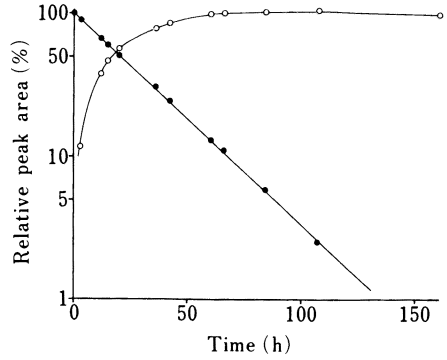

Fig. 4. Changes of YTR-830H and Its Degradation Products in Clark–Lubs Buffer (pH 9.0) at 35 °C

●, YTR-830H; ○, YTR-830H-II. HPLC condition 1 was used.

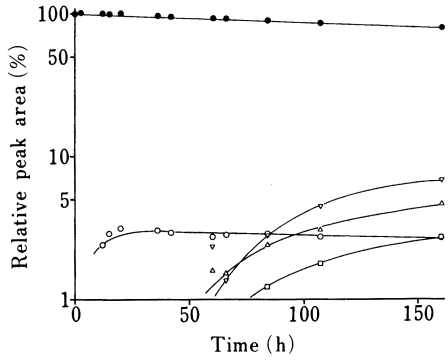

Fig. 5. Changes of YTR-830H and Its Degradation Products in Distilled Water at 35 °C

●, YTR-830H; ○, YTR-830H-II; □, YTR-830H-IIa; △, YTR-830H-IIb; ▽, YTR-830H-IIc. HPLC condition 1 was used.

only *ca.* 2—3% degradation. The amount of this product present decreases gradually on prolonged exposure to the reaction solutions, while the other products increase in amount over time. It was also found from the results obtained for the degradation of YTR-830H-II in buffer solution at pH 3.0 and in distilled water that 1,2,3-triazole, YTR-830H-IIa, -IIb, -IIc and -IId were all produced from YTR-830H-II. Figure 6 shows the chromatograms of YTR-830H-II under HPLC condition 1, after degradation at 35 °C for 4 h in buffer solution (pH 3.0) or in distilled water. These products could not be isolated by the usual methods of column chromatography or derivatization. Attempts to isolate these products are in progress.

YTR-830H in buffer solution at pH 7.0 was much more stable than that at pH 9.0 (Figs. 1B, 1C, 3 and 4), but its degradation pattern was quite similar. The respective degradation percentages for YTR-830H after 20 h in buffer solution at pH 7.0 and 9.0 were *ca.* 5% and 50% as shown in Figs. 3 and 4. The major product (YTR-830H-II, peak b in Figs. 1B and 1C) and the minor product (YTR-830H-IIa, peak c in Figs. 1B and 1C) were only detected by chromatography after the reaction had proceeded for 160 h.

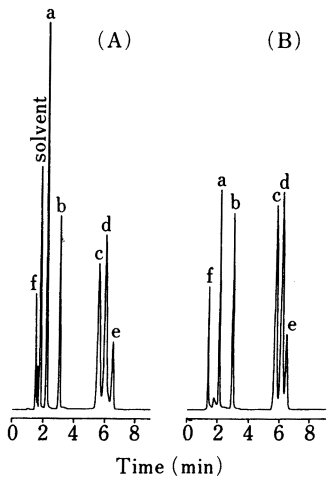

Fig. 6. Chromatograms of YTR-830H-II after Degradation under Various Conditions

(A) In Britton–Robinson buffer (pH 3.0) and (B) in distilled water at 35 °C for 4 h.
a, YTR-830H-II; b, YTR-830H-IIa; c, YTR-830H-IIb; d, YTR-830H-IIc; e, YTR-830H-IId; f, 1,2,3-triazole. HPLC condition 1 was used.

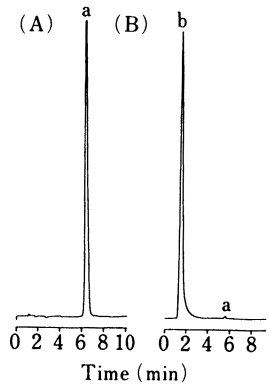

Fig. 7. Chromatograms of YTR-830H after Degradation in Aqueous Alkaline Solution Followed by Neutralization

Detection (A) at UV 220 nm and (B) at UV 265 nm, using the post-column alkalization technique.
a, YTR-830H-II; b, YTR-830H-III. HPLC condition 2 was used.

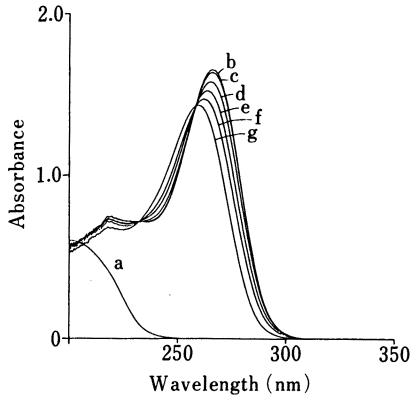

Fig. 8. UV Absorption Spectra of YTR-830H

a, in aqueous solution and b—g, after degradation in aqueous alkaline solution.
Reaction time: b, 1 min; c, 5 min; d, 15 min; e, 30 min; f, 60 min; g, 120 min.

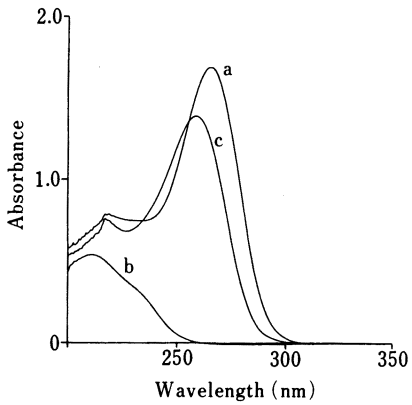

Fig. 9. UV Absorption Spectra of YTR-830H

a, alkaline; b, neutral; c, realkalinized aqueous solutions.

Furthermore, the product YTR-830H-III was found in the YTR-830H solutions at all pH ranges by the post-column alkaline degradation HPLC method (HPLC condition 2), as shown in Fig. 7B. This YTR-830H-III could not be detected under HPLC condition 1 (Fig. 7A), since this product was decarboxylated under acidic conditions, having no UV absorption, as mentioned later.

Degradation in Aqueous Alkaline Solution

The UV spectral changes of YTR-830H, degraded in aqueous alkaline solutions (pH 12.0) for various reaction time periods, are shown in Fig. 8.

Although YTR-830H alone in aqueous solution has no UV absorption above 200 nm, a

maximum absorption (λ_{max}) at 265 nm (spectrum b in Fig. 8) was observed following the addition of NaOH solution. Subsequently this λ_{max} moved from 265 to 258 nm (spectrum g in Fig. 8), with a concomitant decrease in the intensity at λ_{max}, during reaction at room temperature for 120 min. The UV absorption (λ_{max} at 265 nm) observed in the alkaline solution almost disappeared following neutralization with an aqueous HCl solution to pH 7.0 (spectrum b in Fig. 9). It reappeared (λ_{max} at 258 nm) on realkalization with NaOH solution to pH 12.0 (spectrum c in Fig. 9).

The information obtained from the above change of the UV spectral change of YTR-830H in aqueous alkaline solution could not be obtained by the HPLC method. Following neutralization with aqueous HCl solution, two peaks (YTR-830H-II and -III) on the chromatograms, the same as shown in Fig. 7, were observed. This result suggests that the degradation products are more stable in aqueous alkaline solution, but are unstable under neutral or acidic conditions.

Since these alkaline degradation products could not be isolated using the above methods,

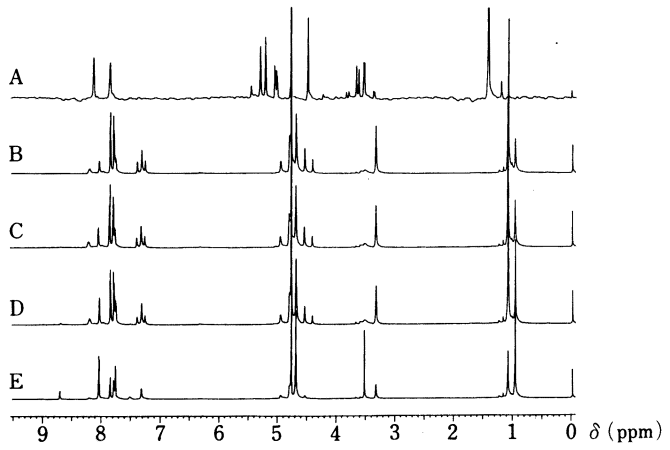

Fig. 10. ^{1}H-NMR Spectra of YTR-830H

A, in D_2O solution; B—E, after degradation in NaOD–D_2O solution. Reaction time: B, 10 min; C, 1 h; D, 2 h; E, 16 h.

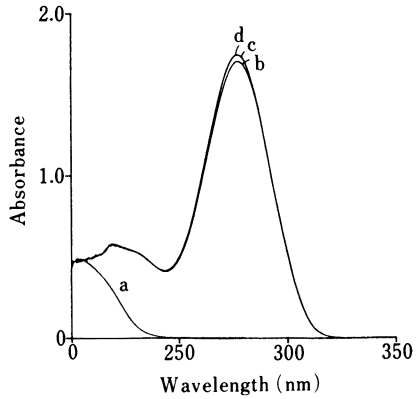

Fig. 11. UV Absorption Spectra of YTR-830H

a, in methanol; b—d, after degradation in NaOH-saturated methanol.
Reaction time: b, 1 min; c, 30 min; d, 60 min.

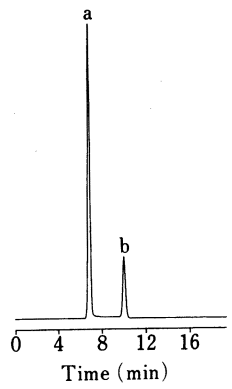

Fig. 12. Chromatograms of YTR-830H after Degradation in NaOH-Saturated Methanol

a, YTR-830H-Ia; b, YTR-830H-Ib. HPLC condition 3 was used.

the ^{1}H-NMR spectral method was then used for the investigation of the alkaline degradation of YTR-830H. The ^{1}H-NMR spectral changes of YTR-830H measured in 0.5 N NaOD–D_2O solution are given in Fig. 10. The signals of intact YTR-830H were not observed in NaOD–D_2O solution, but the signals of degradation products could be observed in the spectra. From the spectral changes during time-dependent degradation, three products (YTR-830H-I, -II and -III) could be observed.

Degradation in Alkali-Saturated Methanol Solution

The UV spectra of YTR-830H, degraded in alkali-saturated methanol solution at room temperature for 1, 30 and 60 min periods, are shown in Fig. 11.

Although YTR-830H in methanol solution has no UV absorption above 200 nm, the addition of NaOH-saturated methanol solution to this solution resulted in a UV absorption of λ_{max} at 277 nm, and this λ_{max} did not change for at least 60 min. This UV absorption was also seen to disappear following neutralization with methanolic HCl solution, and to reappear on subsequent realkalization with NaOH-saturated methanol solution.

The chromatogram performed under HPLC condition 3, of the degradation products of YTR-830H in NaOH-saturated methanol solution, is shown in Fig. 12. Two products (YTR-830H-Ia and -Ib, peaks a and b in Fig. 12), having retention times (t_R) of 6.5 and 10 min were observed. These products were obtained as a mixture by silica gel column chromatography, but the respective products could not be isolated.

Structure of YTR-830H-I

YTR-830H-I, signals of which were observed in the ^{1}H-NMR spectra of YTR-830H, measured in NaOD–D_2O (Fig. 10), could not be isolated since this compound is very unstable in aqueous solution, except in NaOH-alkaline solution. Therefore, structural elucidation was first attempted on YTR-830H-Ia and YTR-830H-Ib produced in NaOH-saturated methanol solution, which were thought to have related structures.

The mixture of YTR-830H-Ia and -Ib gave a quasi-molecular ion peak at m/z 333 $(M+1)^+$ (Found, 333.0790; Calcd, 333.0868 for $C_{11}H_{17}N_4O_6S$) in its FAB-MS. In the ^{1}H-NMR spectrum (D_2O), the signals of CH_3 at δ 1.09 and δ 1.03, CH at δ 3.46 and δ 3.61, OCH_3 at δ 3.65 and δ 3.70, =CHCO at δ 4.53 (d, coupling constant (J) = 14 Hz) and δ 4.50 (d, J = 9 Hz), CH_2 at δ 4.73 (AB-q), =CHNH at δ 7.63 (d, J = 14 Hz) and δ 6.69 (d, J = 9 Hz), =CH at the C-4′ position of the triazole moiety at δ 7.80 (d, J = 1 Hz), =CH at the C-5′ position of the triazole moiety at δ 7.91 (d, J = 1 Hz) and 8.00 (*d*, J = 1 Hz) (the former values, where two values are given, are due to YTR-830H-Ia) were assigned.

On the basis of these ^{1}H-NMR spectral findings, it was assumed that YTR-830H-Ia and -Ib are *cis–trans* isomers with a signal intensity ratio of 1 : 2. The peaks of t_R at 6.5 (YTR-830H-Ia) and 10.0 min (YTR-830H-Ib) observed in Fig. 12, showed λ_{max} at 279 and 291 nm, respectively, by the photo-diode array detection method. The difference between these λ_{max} absorptions corresponded to that of the *cis–trans* isomers of a β-amino-α,β-unsaturated carbonyl compound reported by Ostercamp.[3] The λ_{max} of the *trans* isomer was at a shorter wavelength by about 10 nm, compared to that of the *cis* isomer.

From these results, the degradation products of YTR-830H-Ia and -Ib in NaOH-saturated methanol solution were identified as 1-methyl hydrogen (*E*)- and (*Z*)-5-methyl-5-sulfino-6-(1*H*-1,2,3-triazol-1-yl)-3-aza-1-heptene-1,4-dicarboxylic acid. The chemical structures of YTR-830H-Ia and -Ib in NaOH-saturated methanol solution are similar to the degradation products of sodium sulbactam.[4] Haginaka *et al.*[4] reported that the degradation products of sodium sulbactam in NaOH-saturated methanol solution were *trans* and *cis* isomers of methyl 5-carboxy-6-methyl-6-sulfino-4-aza-2-heptenoate, which were observed as two peaks on an HPLC chromatogram with a 1 : 3 ratio of *cis–trans* isomers.

In addition, the ^{1}H-NMR spectrum of a mixture of YTR-830H-Ia and -Ib measured in

D_2O after standing at room temperature for a longer time-period, or that in NaOD–CD_3OD, showed the disappearance of the signal of the CH proton at the 2 position (δ 4.50 for the *cis* or *Z* isomer and δ 4.53 for the *trans* or *E* isomer) and the appearance of one at the 3 position (δ 6.69 for the *cis* or *Z* isomer and δ 7.63 for the *trans* or *E* isomer). These results suggested enamine–imine tautomerism, as reported for the degradation products of sodium sulbactam.[4] This tautomerism was too fast to be observed.

Next, on the basis of the above structural elucidations of YTR-830H-Ia and -Ib, the identification of YTR-830H-I produced in aqueous NaOH solution was investigated using ^{1}H-NMR spectral data measured in NaOD–D_2O, since it could not be isolated because of its instability.

The ^{1}H-NMR spectra of YTR-830H-I measured in NaOD–D_2O showed a pattern complicated by the partially deuterium-exchanged protons due to enamine–imine tautomerism, but after the solution was left standing for 16 h the spectrum became simplified.

Among the signals observed in the initial step of the degradation, the signals at δ 1.10 of CH_3, δ 3.35 of CH, δ 4.50 (d, J = 14 Hz) of =CHCO, δ 4.77 (AB-q) of CH_2, δ 7.36 (d, J = 14 Hz) of =CHNH, δ 7.81 (d, J = 1 Hz) of =CH at the C-4′ position of the triazole moiety and δ 7.87 (d, J = 1 Hz) of =CH at the C-5′ position of the triazole moiety were assigned as signals of YTR-830H-I. These chemical shifts of YTR-830H-I largely corresponded to those of YTR-830H-Ia and -Ib except that the methyl proton of the methoxy group was absent.

As described in connection with the HPLC and ^{1}H-NMR spectral results, YTR-830H-I was further converted to YTR-830H-II and -III. Thus, the previously observed UV spectral changes of YTR-830H, where λ_{max} of the reaction solution alters from 265 to 258 nm under alkaline, neutral, or realkalization conditions (Fig. 9), can be reasonably explained by presuming that YTR-830H is degraded in aqueous alkaline solution to produce YTR-830H-II (no UV absorption) and -III (λ_{max} 258 nm in alkaline solution) through YTR-830H-I (λ_{max} 265 nm in alkaline solution).

Combining the above results with information on the coupling constant (J = 14 Hz) between the protons at the C-2 and C-3 positions and the chemical structures of YTR-830H-II and YTR-830H-III mentioned later, YTR-830H-I was identified as (*E*)-5-methyl-5-sulfino-6-(1*H*-1,2,3-triazol-1-yl)-3-aza-1-heptene-1,4-dicarboxylic acid (the *trans* isomer).

The methyl esters designated as YTR-830H-Ia and -Ib were assigned as a mixture of the *trans* and *cis* isomers (2 : 1 ratio), while YTR-830H-I was only observed as the *trans* isomer. The ^{1}H-NMR spectrum of YTR-830H-I, measured in NaOD–D_2O, showed that enamine–imine tautomerism was slower than that observed for YTR-830H-Ia and -Ib.

Haginaka *et al.*[4] and Kemal and Knowles[5] have reported that the degradation of sodium sulbactam in alkaline aqueous solution yields 5-carboxy-6-methyl-6-sulfino-4-aza-2-heptenoic acid, which is related in chemical structure to YTR-830H-I.

Chemical Structure of YTR-830H-II

The chemical structure of YTR-830H-II observed in buffer solutions and NaOH aqueous solution was investigated using a sample prepared from the NaOH degradation solution.

YTR-830H-II gave a quasi-molecular ion peak of m/z 249 $(M+1)^+$ (Found, 249.0652; Calcd, 249.0657 for $C_7H_{13}N_4O_4S$) and sodium adduct ion peaks of m/z 271 $(M+Na)^+$ and m/z 293 $(M+2Na-1)^+$ in its FAB-MS. In the ^{1}H-NMR spectrum (NaOD–D_2O), signals of CH_3 at δ 0.98, CH at δ 3.53, CH_2 at δ 4.71, and =CH at the C-4′ and C-5′ positions of the triazole moiety at δ 7.79 and δ 8.08 (each d, J = 1 Hz), were assigned. Furthermore, the signals of this YTR-830H-II were also observed in the ^{1}H-NMR spectra of YTR-830H, measured in NaOD–D_2O after the solution had been left standing for 16 h (Fig. 10). The IR spectrum of YTR-830H-II showed the characteristic absorption of a carboxy group ($\nu_{C=O}$) at 1702 cm^{-1} but not that of β-lactam ($\nu_{C=O}$). This product has no UV absorption.

Based on these spectral data, the t_R value on HPLC chromatography and comparison with an authentic sample, it was concluded that YTR-830H-II is 2-amino-3-methyl-3-sulfino-4-(1*H*-1,2,3-triazol-1-yl)-butyric acid. This YTR-830H-II has a similar chemical structure to 2-amino-3-methyl-3-sulfinobutanoic acid, which was reported as a degradation product of sodium sulbactam.[4] This compound was also identified as a metabolite of YTR-830H.[6]

Chemical Structure of YTR-830H-III

The degradation product YTR-830H-III could not be isolated, but the results obtained in the post-alkalization HPLC (Fig. 7B), the ^{1}H-NMR spectra measured in NaOD–D_2O (Fig. 10) and the UV spectral changes in aqueous alkaline solution (Figs. 8 and 9) were identical to those obtained with formylacetic acid, which is one of the degradation products of sodium sulbactam reported by Haginaka *et al.*[4] and Kemal and Knowles.[5]

In the ^{1}H-NMR spectra of YTR-830H measured in NaOD–D_2O (Fig. 10), the singlet signal at δ 8.23 was assigned as a =CH proton of YTR-830H-III. The signal at δ 8.23 was observed as a singlet, since the =CH proton at the C-3 position was substituted by deuterium. As shown in Fig. 7B, this YTR-830H-III could be detected only by the post-alkalization method, showing the λ_{max} at 258 nm. Results for UV spectra (Fig. 9) show that the alkaline degradation of YTR-830H in NaOH aqueous solution, followed by neutralization with HCl, did not result in UV absorption, while following realkalization of the solution, UV absorption with λ_{max} at 258 nm appeared. An explanation for the absorption pattern observed in alkaline solution is that it is due to the formation of the keto form of formylacetic acid, while the disappearance of UV absorption in neutral or acidic solution is due to the formation of the enol form, as reported by Haginaka *et al.*[4] and Kemal and Knowles[5] for sulbactam and by

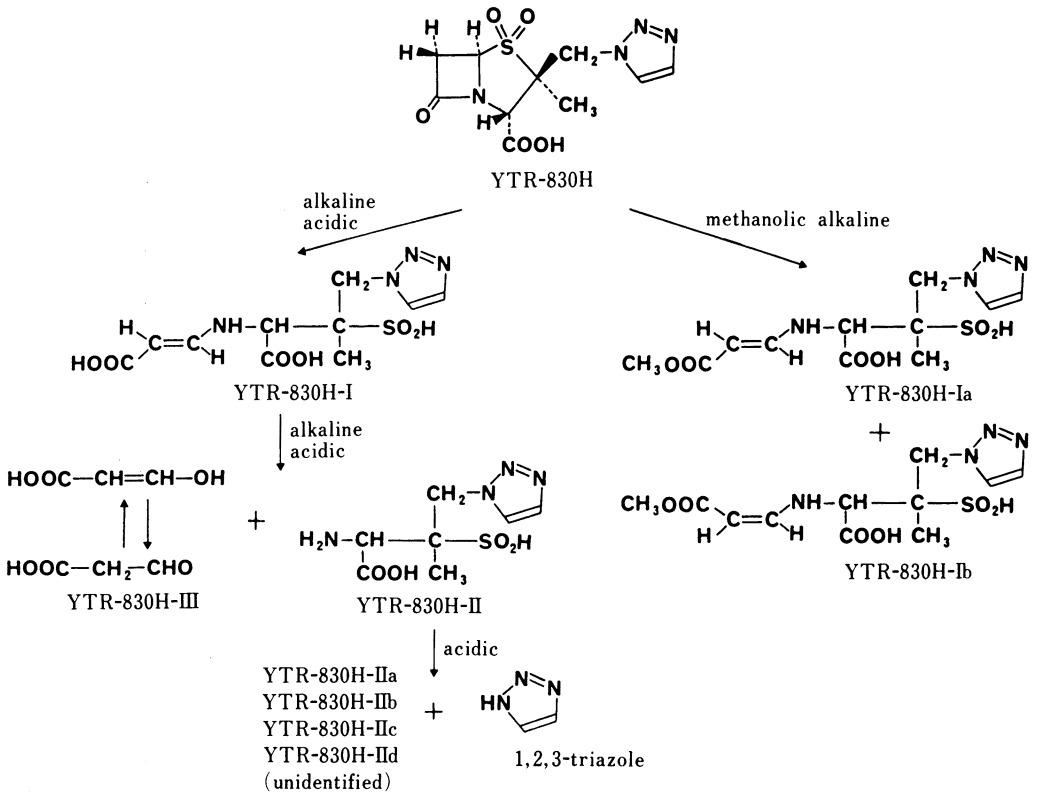

Chart 1. Degradation Pathways of YTR-830H

Cherry and Newall[7] for lithium clavulanate.

Thus, YTR-830H-III was identified as formylacetic acid.

Chemical Structure of 1,2,3-Triazole

1,2,3-Triazole as a degradation product could not be isolated in the present examination. It was identified by comparison of its t_R on an HPLC chromatogram with that of an authentic sample.

Presumed Degradation Pathways of YTR-830H

From the above results, the degradation pathways of YTR-830H in aqueous solution and alkaline methanol solution are assumed to be as illustrated in Chart 1.

In the initial step of degradation, YTR-830H is converted to YTR-830H-I as a intermediate, by hydroxide ions attacking the β-lactam ring, resulting in fission of the C–S bond. YTR-830H-I was observed only in alkaline solution, since this product is reasonably stable under alkaline conditions, but is very unstable under neutral or acidic conditions. The YTR-830H-I is then hydrolyzed to produce YTR-830H-II and -III, with product YTR-830H-II then proceeding to YTR-830H-IIa under neutral and alkaline conditions, and to 1,2,3-triazole, YTR-830H-IIa, -IIb, -IIc and -IId under acidic conditions. It was confirmed that the degradation products 1,2,3-triazole, YTR-830H-IIa, -IIb, -IIc and -IId are derived from YTR-830H-II. The formation of these products has not been reported for sulbactam.

The degradation of YTR-830H in NaOH-saturated methanol yielded a mixture of the *trans* and *cis* forms of YTR-830H-Ia and -Ib, which are the methyl ester forms of YTR-830H-I.

The degradation kinetics of YTR-830H will be reported elsewhere.

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