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# Reactions of 7-Chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2,2-dioxide in Various pH Solutions

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Diazepam (**1**) is a frequently prescribed hypnotic/anxiolytic drug worldwide. 7-Chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2,2-dioxide (**2**) is an initial alkaline hydrolysis product of **1**. The mechanisms in the conversion of **2** to 2-methylamino-5-chloro-α-(phenylbenzylidene)glycinate (**3**), 2-methylamino-5-chlorobenzophenone (**4**), and **1** in aqueous solutions with pH ranging from 0 to 12.2 is the subject of this report. Results of temperature-dependent hydrolysis kinetics and product identification indicated that: (1) in solutions with pH between 7 and 12.2, **2** underwent a ring-opening reaction to form **3**; the rate decreased with increasing pH. (2) In solutions with pH between 2 and 7, **2** was rapidly converted to **3**, followed by a pH-dependent conversion to **4**; the rate increased with decreasing pH and became less sentitive to pH at pH ≤ 4.5. (3) In solutions with pH between 0 and 2, **2** was rapidly converted to **4** and **1**; the percentage of **1** increased with decreasing pH. (4) A **2** containing one oxygen-18 atom lost 50% of its oxygen-18 following conversion to **1** in 1 M HCl. In addition to understanding the mechanism in the transformations of **2** in various pH solutions, this study established a simple and efficient method in the quantitative conversion of **1** to **4** and in the preparation of an oxygen-18-containing **1** at C2 position.

## INTRODUCTION

Diazepam (**1**, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one, Fig. 1) is a frequently prescribed hypnotic/anxiolytic drug worldwide. Compound **1** is hydrolyzed in aqueous alkaline solution to form 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2,2-dioxide (**2**) as the initial product.[1] In a pH 11 solution, **2** is converted to form 2-methylamino-5-chloro-α-(phenylbenzylidene)glycinate (**3**).[2] Glycinate **3** is then slowly converted to form 2-methylamino-5-chlorobenzophenone (**4**) and 2-methylamino-5-chlorobenzophenone imine (**5**); the ratio of **5**:**4** increases with increasing NaOH concentration.[2]

This report describes the results on the reactions of **2** in various pH solutions. The results provided additional support for the proposed mechanism of alkaline hydrolysis of **1**,[1] established a simple and efficient method in the quantitative conversion of **1** to **4**, and provided a method to prepare an oxygen-18-containing **1** at C2 position.

## EXPERIMENTAL SECTION

### Materials

Crystalline **2** was prepared from **1** as previously described.[1] Compound **1** was synthesized from *N*-desmethyldiazepam by methylation with dimethyl sulfate.[3] *N*-desmethyldiazepam was prepared from clorazepate (Sigma Chemical Co., St. Louis, MO) by acid hydrolysis.[4] Compound **4** was prepared by methylation of 2-amino-5-chlorobenzophenone (**6**) (Aldrich Chemical Co., Milwaukee, WI).[5] Oxygen-18 water (99 atom % $^{18}O$) was purchased from Cambridge Isotope Laboratories (Andover, MA). Britton-Robinson buffers (0.04 M in acetic acid, boric acid,

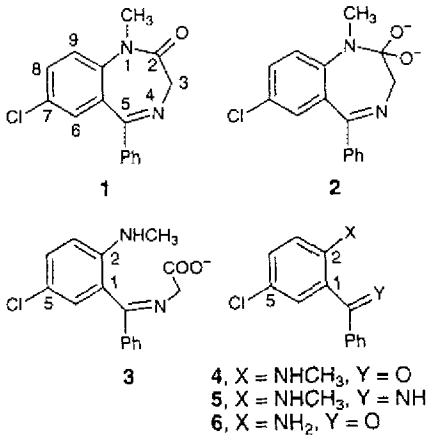



Fig. 1. Structure and abbreviation of diazepam (**1**), 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2,2-dioxide (**2**), 2-methylamino-5-chloro-α-(phenylbenzylidene)glycinate (**3**), 2-methylamino-5-chlorobenzophenone (**4**), 2-methylamino-5-chlorobenzophenone imine (**5**), and 2-amino-5-chlorobenzophenone (**6**).

and phosphoric acid; pH 2-12.2) were prepared as described by Barrett et al.[6] In this study, solutions with 1:3 volume ratios of ethanol-aqueous solution were used. The indicated pH was the pH of the aqueous portion of the solvent mixture.

For kinetic studies, 10 mg of **2** was dissolved in 4 mL of ethanol-1 M NaOH (1:3, v/v). The solution was stored at 4 °C. Upon prolonged storage, **2** was slowly converted to **4**. Greater than ≥ 95% of **4** in **2** could be removed by extraction twice with hexane. The presence of a minute amount of **4** did not interfere with the kinetic measurements.

**Kinetic Analysis**

The transformation of **2** in various pH solutions was conducted by adding 15 μL of **2** (2.5 mg/mL) in ethanol: 1 M NaOH (1:3, v/v) into an ice-cold test tube containing 1.2 mL of ethanol-W (1:3, v/v) solutions, where W was either a buffered aqueous solution or an aqueous HCl solution. The resulting solution was thoroughly mixed for ~10 sec and immediately transferred into a water-jacketed cuvette. The temperature of the cuvette was maintained by passing constant-temperature water from a thermostated water circulator. The actual temperature of the solution in the cuvette was measured with a portable digital thermometer fitted with a detachable probe (Thomas Scientific, Swedesboro, NJ). After reaching temperature equilibrium in 0.5 to 3 min (depending on the temperature under study), absorbance changes of the sample were either continuously monitored at a fixed wavelength or scanned from 200 to 500 nm repetitively at a fixed interval. The pH of the aqueous portion of the reaction medium was determined at ambient temperature by adding 112.5 μL of 1 M NaOH to 9 mL of a Britton-Robinson pH buffer, in the absence of ethanol.

Pseudo-first order reaction half-time ($t_{1/2}$) was determined from the $A_{\lambda,t}$ vs. time plot by a curve-fitting computer software (SigmaPlot, a product of Jandel Scientific, Corte Madera, CA). The equation used in curve-fitting for the conversion of **2** to **1** at 290 nm was $A_{\lambda,t} = a \times \exp(-0.693 \times t/t_{1/2}) + A_{\lambda,0}$. The equation used in curve-fitting for the conversion of **2** to **3** at 380 nm in solutions with pH > 10 and the conversion of **2** to **4** at 420 nm in solutions with pH between 1 and 7 was $A_{\lambda,t} = a \times [1 - \exp(-0.693 \times t/t_{1/2})] + A_{\lambda,0}$. The equation used in curve-fitting for the conversion of **2** to **3** at 380 nm in solutions with pH < 10 was $A_{\lambda,t} = a \times [1 - \exp(-0.693 \times t/t_{1/2})] + b \times t + A_{\lambda,0}$. In the above equations, $A_{\lambda,t}$ is the absorbance value of the sample at time t and wavelength λ, a is the net absorbance change of the initial pseudo-first order reaction at wavelength λ, and $A_{\lambda,0}$ is the absorbance value at time zero. When **2** was dissolved in solutions with pH between 7 and 9, in addition to the **2** to **3** conversion, a slow and pseudo-first order conversion of **3** to **4** was detectable. Hence, the term b × t was added in the equation to account for the slow increase of absorbance at 380 nm due to pseudo-first order conversion of **3** to **4**. The initial phase of absorbance increase (or decrease) in a very slow pseudo-first order reaction can be approximated by a straight line.

**pH-Dependent Conversion of 2 to 1 and 4**

Crystalline **2** (10 mg) was dissolved in 4 mL of ethanol-0.01 M NaOH (1:3, v/v), and the resulting solution was kept at 4 °C. A 0.1 mL of the solution was added to test tubes, each containing 3 mL of either a Britton-Robinson buffer (pH 2, 3, 4, 5, 6, and 7) or a HCl solution (0.05 M, 0.1 M, 0.25 M, 0.5 M, 0.75 M, and 1 M HCl). Experiments were carried out in triplicate for each pH. Reaction mixtures with pH 6 and 7 were heated at 50 °C for 1 and 22 h, respectively. Other reaction mixtures were heated at 50 °C for 10 min. Compound **6** (50 ng in 0.1 mL acetonitrile) was added to each reaction mixture as an internal standard for chromatography, followed by 4 mL of dichloromethane. Following thorough mixing and phase separation, the organic layer was washed with water (3 × 2 mL). The residue in the organic layer was analyzed by reversed-phase HPLC.

**Oxygen-18 Incorporation into 1 via 2**

Unlabeled **1** (5 mg) was dissolved in 0.9 mL ethanol and 0.8 mL $H_2{}^{18}O$ (99 atom % $^{18}O$), followed by the addition of 10 M NaOH (0.1 mL). The mixture was heated at 50 °C. An aliquot (0.1 mL) was taken at various intervals and immediately added to a test tube containing 0.1 mL of 2 M HCl; all test tubes were kept on ice. After the last aliquot was taken, all mixtures were heated at 50 °C for 10 min. To each test tube, 2 mL of saturated $Na_2HPO_4$ solution (pH 8.9) was added, followed by 3 mL of dichloromethane. Following thorough mixing and phase separation, the organic layer was washed with water (3 × 2 mL). The residue in the organic layer, containing predominantly **1**, was analyzed by GC-MS.

**Spectral Analysis**

Uv-vis absorption spectra were determined using a 1-cm path length quartz cuvette on a Model DW2000 spectrophotometer (SLM Instruments, Urbana, IL). GC-MS analysis was performed on a Hewlett-Packard 5890 instrument with a 5971 mass selective detector and an HP Vectra QS/20 PC computer using HP-G1030A MS ChemStation (DOS series) software. A Heliflex (Deerfield, IL) AT-1 silica capillary column (30 cm × 0.25 mm × 1 μm film) was used. Sam-

ples were dissolved in dichloromethane (~0.5 μg/mL) and 1 to 5 μL was injected via an autosampler for analysis. Ionization was induced by electron impact (70 eV). The carrier gas was helium and a temperature program of 13.3 min (30 °C/min from 50 to 150 °C and then 15 °C/min from 150 to 300 °C) was used. Compound **1** was eluted from the GC column at 17.3 min.

In electron impact mass spectral analysis, the mass ion intensity ratio of unlabeled **1** for mass ions at *m/z* 283, 284, and 285 was found to be 1.000:0.922:0.481 (average of 7 determinations). The same mass ion intensity ratio (1.000:0.922:0.481) was assumed to be the same for mass ions at *m/z* 285, 286, and 287 of **1** containing an oxygen-18 atom at C2. The oxygen-18 content of **1** with various degrees of oxygen-18 incorporation was calculated by equation 1 below.

$$^{18}\text{O Content (\%)} = 100 \times (RI_{285} - 0.481 \times RI_{283})/[RI_{283} + (RI_{285} - 0.481 \times RI_{283})] \quad (1)$$

Where $RI_{285}$ and $RI_{283}$ are the relative intensities of mass ions at *m/z* 285 and 283, respectively.

### High-Performance Liquid Chromatography (HPLC)

Reversed-phase HPLC was performed using a Waters Associates (Milford, MA) Model 6000A solvent delivery system and a Spectraflow 757 variable wavelength absorbance detector (Kratos Analytical Instruments, Ramsey, NJ) set at 232 nm. Samples were injected via a Waters Associates Model 717 autosampler. A Zorbax SB-C18 column (3.5 μm particles, 4.6 mm i.d. × 15 cm, Mac-Mod Analytical Inc., Chadds Ford, PA) was used. The mobile phase was acetonitrile-0.02 M phosphate pH 7 (13:7, v/v) at a flow rate of 1 mL/min. HPLC analysis was conducted at ambient temperature.

## RESULTS AND DISCUSSION

### Absorption Spectral Changes of 2 in Various pH Solutions

Fifteen μL of **2** in ethanol-1 M NaOH (1:3, v/v; containing 2.5 mg of **2** per mL) was diluted into 1.2 mL of ethanol-pH 11 buffer (1:3, v/v; final pH of the aqueous portion of the solution was 11.6), and the absorption spectra were taken every 10 min at 50 °C (Fig. 2A). The absorption spectrum at the completion of the reaction (Fig. 2A) was identical to that of the glycinate **3**, which was previously characterized via its methyl ester by mass and proton NMR spectral analyses.[2] The absorption spectra in Fig. 2A indicated that the kinetics in the conversion of **2** to **3** could be continuously monitored at either 255 or 380 nm; the reaction was found to be of a pseudo-first order. The reaction $t_{1/2}$ in the conversion of **2** to **3** at 50 °C in ethanol-pH 11.6 buffer (1:3, v/v), monitored at 380 nm, was found to be 36.5 ± 0.3 min (n = 3).

When 15 μL of **2** in ethanol-1 M NaOH (1:3, v/v; containing 2.5 mg of **2** per mL) was diluted into 1.2 mL of ethanol-pH 6 buffer (1:3, v/v; final pH of the aqueous portion of the solution was 6.83), the absorption spectrum taken at 1 min and 50 °C was identical to that of the glycinate **3** (Fig. 2B). The absorption spectrum at the completion of the reac-

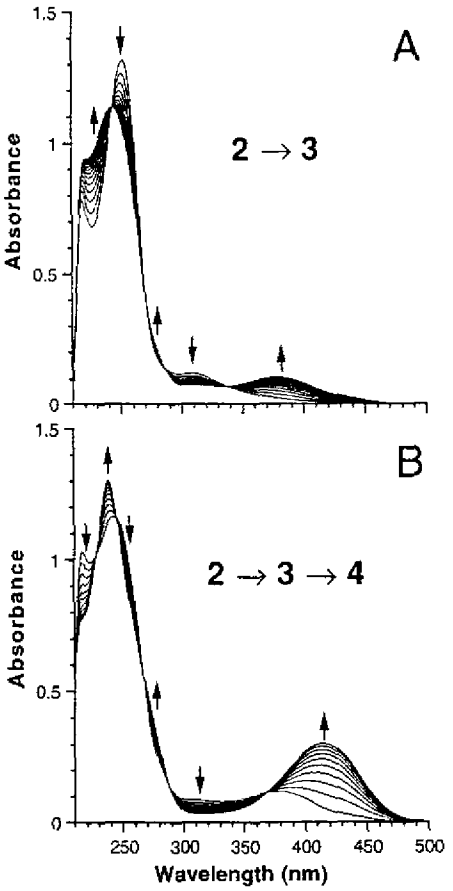



Fig. 2. Time-dependent absorption spectra in the conversion of (A) **2** to **3** in ethanol-Britton-Robinson buffer pH 11.6 (1:3, v/v) at 50 °C and (B) **2** to **4** via **3** in ethanol-Britton-Robinson buffer pH 6.83 (1:3, v/v) at 50 °C. In both experiments, the first scan was conducted at 1 min following transfer of sample into the cuvette thermostated at 50 °C. Thereafter, scans were conducted every 10 min for 13 times. Wavelength scan rate was 2 nm/s. Direction of arrows indicates the direction of absorbance change.

*tion was that of* **4** (Fig. 2B). The absorption spectra in Fig. 2B indicated that the kinetics in the conversion of **2** to **4** via **3** could be continuously monitored at 420 nm. The reaction was found to be pseudo-first order. The reaction $t_{1/2}$ in the conversion of **2** to **4** via **3** at 50 °C in ethanol-pH 6.83 buffer (1:3, v/v), monitored at 420 nm, was found to be 32.1 ± 0.4 min (n = 3).

When 15 μL of **2** in ethanol-1 M NaOH (1:3, v/v; containing 2.5 mg of **2** per mL) was diluted into 1.2 mL of ethanol-0.1 M HCl buffer (1:3, v/v; final pH of the aqueous portion of the solution was 1.06), the absorption spectrum taken at 1 min and 50 °C was probably that of a reaction intermediate (Fig. 3). The absorption spectrum taken at later time intervals at 50 °C was a composite spectrum of **1** and **4** (Fig. 3). The conversion of **2** to a mixture of **1** and **4** in a solution with pH 1 was confirmed by reversed-phase HPLC analysis (see Fig. 5 below). The absorption spectra in Fig. 3 indicated that the kinetics in the conversions of **2** to **1** and of **2** to **4** could be continuously monitored at 290 nm and 420 nm, respectively. Both reactions were found to be pseudo-first order. The reaction $t_{1/2}$ in the conversion of **2** to **1** at 50 °C in ethanol-0.1 M HCl (1:3, v/v), monitored at 290 nm, was found to be 0.56 ± 0.01 min (n = 3). The reaction $t_{1/2}$ in the conversion of **2** to **4** at 50 °C in ethanol-0.1 M HCl (1:3, v/v), monitored at 420 nm, was found to be 0.50 ± 0.02 min (n = 3). It appeared that, although mechanistically different, there were no significant differences in the kinetics of **2** to **1** and **2** to **4** conversions in ethanol-0.1 M HCl (1:3, v/v).

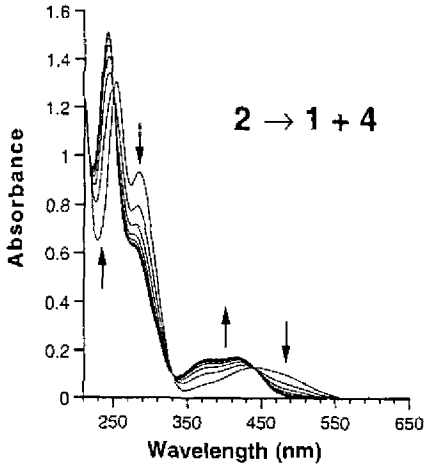



Fig. 3. Time-dependent conversion of **2** to **1** and **4** in ethanol-0.1 M HCl (1:3, v/v) at 20 °C. The first scan was conducted at 1 min following transfer of sample into the cuvette thermostated at 20 °C. Thereafter, scans were conducted every 4 min for 7 times. Wavelength scan rate was 2 nm/s. Direction of arrows indicates the direction of absorbance change.

**pH-Dependent Kinetics**

The reaction $t_{1/2}$ in the conversion of **2** to **3** increased with increasing pH in the alkaline region (Fig. 4). The slopes of the $t_{1/2}$ vs. pH plots at 25 °C and 50 °C were 0.73 and 1.03, respectively. The results suggested that the **2** to **3** conversion was first-order with respect to hydrogen ion. The rate of **2** to **3** conversion in solutions with pH < 9 occurred very fast, and its reaction $t_{1/2}$ could not be reliably determined. A slow increase of absorbance at 420 nm was observed corresponding to the **3** to **4** conversion.

A 15 μL of **2** in ethanol-1 M NaOH (1:3, v/v; containing 2.5 mg of 2 per mL) was diluted into 1.2 mL of ethanol-pH 8 buffer (1:3, v/v; final pH of the aqueous portion of the solution was 8.93), and the change in absorbance was monitored at 380 nm and 50 °C. The initial pseudo-first order increase in absorbance was relatively fast ($t_{1/2}$ = 0.36 min), owing to **2** to **3** conversion. This was followed by a very slow increase in absorbance and the process was too slow to reliably determine its reaction $t_{1/2}$. This slow process was due to **3** to **4** conversion because prolonged heating of the solution resulted in the formation of exclusively **4** (data not shown). The slow **3** to **4** conversion was also observed in solutions with pH > 9 and the rate of absorbance increase became progressively slower with increasing pH.

In solutions with pH between 5 and 7, the reaction $t_{1/2}$ corresponding to the **3** to **4** conversion could be determined and the slopes of the $t_{1/2}$ vs. pH plots at 25 °C and 50 °C were similar to those in the **2** to **3** conversion in the alkaline region (Fig. 4). The results suggested that the **3** to **4** conver-

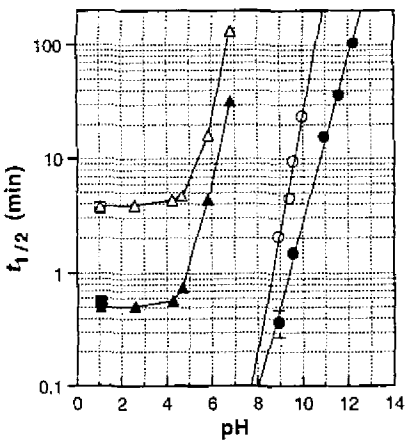



Fig. 4. pH-Dependent reaction half-times in the conversion of **2** to **3** at 25 °C (○, monitored at 380 nm) and 50 °C (●, monitored at 380 nm), **2** to **4** at 25 °C (△, monitored at 420 nm) and 50 °C (▲, monitored at 420 nm), and **2** to **1** at 25 °C (□, monitored at 290 nm) and 50 °C (■, monitored at 290 nm).

sion was first-order with respect to hydrogen ion. In solutions with pH between 1 and ~4.5 the reaction $t_{1/2}$ was less sensitive to the change of pH (Fig. 4). Between pH 1 and 7, the overall pH-dependent rate of the **3** to **4** conversion was similar to that in the hydrolysis of the extensively studied *N*-substituted imines.[7] The breaking point occurred at pH ~4.5 and appeared to correspond to the $pK_a$ of the *N*-substituted imine **3**.

In solutions with pH < 1, **2** was predominantly converted to **1** and the amount of **4** formed, determined by uv-vis absorption spectra, became progressively lower as the pH decreased. In strongly acidic solutions, uv-vis absorption spectral analysis indicated that **3** was not formed as an intermediate. The dioxide **2** was probably protonated at the oxygens and quickly dehydrated (via loss of a water molecule) to form **1**. The rate of **2** to **1** conversion was relatively fast and the reaction $t_{1/2}$ could not be reliably determined in solutions with pH < 0.6.

**Temperature-dependent Kinetics**

The kinetics of **2** to **1** conversion in 0.1 M HCl monitored at 290 nm, **3** to **4** conversion in 0.1 M HCl monitored at 420 nm, **3** to **4** conversion in pH 4.26 and 5.83 buffers monitored at 420 nm, and **2** to **3** conversion in pH 9.56 buffer monitored at 420 nm were studied at four temperatures (Table 1). Arrhenius plots yielded thermodynamic parameters for each reaction (Table 1).

Two independent reactions occurred when **2** was dissolved in ethanol-0.1 M HCl (1:3, v/v). One reaction, monitored at 420 nm, was the formation of **4** via **3**. The **3** to **4** conversion involved the formation of an activated complex resulting from the addition of a water molecule to the imine bond of **3** and this was reflected by the relatively large negative $\Delta S^{\neq}$ (-23.3 cal/K/mol). A second reaction was the formation of **1** from **2**. This reaction was probably caused by the loss of a water molecule following protonation of **2**. An activated complex involving the loss of a water molecule was consistent with the relatively large negative $\Delta S^{\neq}$ (-27.6 cal/K/mol).

In pH 4.26 and 5.83 solutions, **2** was essentially instantaneously converted to **3**; the subsequent conversion of **3** to **4** could be conveniently monitored at 420 nm. The relatively large negative $\Delta S^{\neq}$ values of these two reactions (Table 1) were consistent with the hydrolysis of the imine bond of **3**. However, it was not apparent why the reaction in pH 4.26 solution had larger values of $E_{act}$ and $\Delta H^{\neq}$ than those in pH 5.83 solution (Table 1).

In a pH 9.56 solution, the reaction $t_{1/2}$ in the conversion of **2** to **3** was monitored at 380 nm. This was followed by a **3** to **4** conversion, the rate of which was too slow to determine reliably. The negative $\Delta S^{\neq}$ value (-17.7 cal/K/mol) suggested that an activated complex (presumably pro-

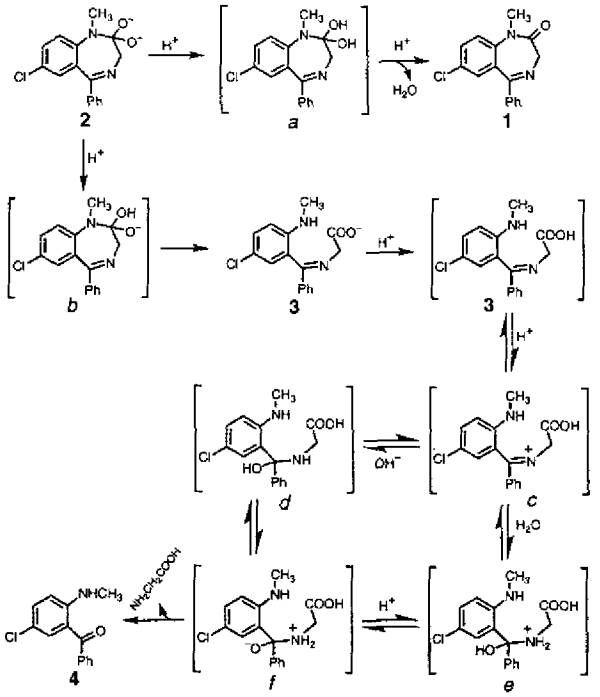
Table 1. Kinetic and Thermodynamic Parameters of the Reactions of **2** in Various pH Solutions[a]

| Parameter | 0.1 M HCl (420 nm)[b] | 0.1 M HCl (290 nm)[b] | pH 4.26 (420 nm)[b] | pH 5.83 (420 nm)[b] | pH 9.56 (380 nm)[b] |
|---|---|---|---|---|---|
| Reaction & Product | **2** (→**3**) → **4** | **2** → **1** | **2** (→**3**) → **4** | **2** (→**3**) → **4** | **2** → **3** |
| $t_{1/2}$ at 10 °C[a] | 13.7 ± 2.9 (3) | 11.8 ± 0.2 (3) | 19.3 ± 0.6 (3) | – | – |
| $t_{1/2}$ at 15 °C[a] | 8.2 ± 0.7 (3) | 8.2 ± 0.1 (3) | 11.1 ± 0.4 (3) | – | – |
| $t_{1/2}$ at 20 °C[a] | 5.2 ± 0.3 (4) | 5.3 ± 0.1 (4) | 6.5 ± 0.2 (4) | 21.0 ± 0.4 (4) | – |
| $t_{1/2}$ at 25 °C[a] | 3.8 ± 0.3 (5) | 3.7 ± 0.2 (5) | 4.3 ± 0.1 (5) | 13.9 ± 0.8 (3) | 9.5 ± 0.5 (3) |
| $t_{1/2}$ at 30 °C[a] | – | – | – | 9.9 ± 0.8 (3) | 7.1 ± 0.9 (3) |
| $t_{1/2}$ at 37 °C[a] | – | – | – | 5.7 ± 0.6 (3) | 3.3 ± 0.2 (3) |
| $t_{1/2}$ at 43 °C[a] | – | – | – | – | 2.0 ± 0.1 (3) |
| Slope[c] | 3.172 | 2.889 | 3.699 | 3.010 | 3.656 |
| $r^2$[d] | 0.9930 | 0.9984 | 0.9973 | 0.9987 | 0.9881 |
| $E_{act}$ (kcal/mol)[e] | 14.5 | 13.2 | 16.9 | 13.8 | 16.7 |
| $\Delta H^{\neq}$ (kcal/mol)[f] | 13.9 | 12.6 | 16.3 | 13.2 | 16.1 |
| $\Delta S^{\neq}$ (cal/K/mol)[f] | -23.3 | -27.6 | -15.5 | -28.7 | -17.7 |
| $\Delta G^{\neq}$ (kcal/mol)[f] | 20.9 | 20.9 | 21.0 | 21.7 | 21.4 |

[a] All solvents contained 1:3 volume ratios of ethanol-W, where W was either HCl or an apueous Britton-Robinson buffer.
[b] Half-time ($t_{1/2}$, mean ± SD from 3 to 5 determinations) of pseudo-first order reaction, determined at the indicated wavelength.
[c] Slope in Arrhenius plot (log $t_{1/2}$ vs. 1000/T).
[d] Correlation coefficient in Arrhenius plot.
[e] Determined from the slope of Arrhenius plot.
[f] Values calculated for temperature at 25 °C.

tonated **2**, intermediate b in Scheme I) preceded the ring-opening of **2**. If the ring-opening step were rate-determining, a positive $\Delta S^{\neq}$ would have been observed.

**Scheme I** Proposed mechanism in the conversion of **2** to **3**, **4**, and **1**. See text for discussion



**pH-Dependent Conversion of 2 to 1 and 4**

The products formed from crystalline **2** in solutions with pH ranging from 0.014 to 7 at 50 °C were determined by reversed-phase HPLC (see representative chromatograms in Fig. 5). In a strongly acidic solution (pH 0.014), **1** was formed predominantly (the area ratio of **1**:**4** at 232 nm was ~12.1, Fig. 5A). In a pH 1 solution, **2** was converted to form comparable amounts of both **1** and **4** (Fig. 5B). In a pH 7 solution, **2** was converted to form exclusively **4** (Fig. 5C). It should be pointed out that, although a crystalline **2** was employed, it was dissolved in 0.01 M NaOH prior to carrying out the experiment described. This dissolution process caused the formation of a small amount of **4**. Compound **4** observed in Fig. 5A might have been derived from the dissolution of **2** in 0.01 M NaOH.

In this experiment, sufficient time was allowed for a complete conversion of **2** in various pH buffers. The length of time was chosen on the basis of kinetic data shown in Fig. 4. The relative amounts of **1** and **4** formed from **2** were dependent on the pH of the solution (Fig. 6). In solutions with pH > 3, **2** was converted exclusively to **4**. The formation of **1** increased with decreasing pH in solutions with pH < 3. In a pH 1 solution, both **1** and **4** were formed, a result consistent with that observed by spectrophotometry (Fig. 3).

For purposes of detection and structural confirmation, a 1,4-benzodiazepine contained in clinical samples such as blood and urine is often hydrolyzed under strongly acidic conditions (e.g., 6 M HCl at 100 °C for 1 hr) to form a benzophenone derivative.[8-13] The conversion of a 1,4-benzodiazepine to the corresponding benzophenone was not quantitative. For example, the yield of **4** in acid hydrolysis of **1** ranged from 74%[12] to 94%.[8] The non-quantitative yield

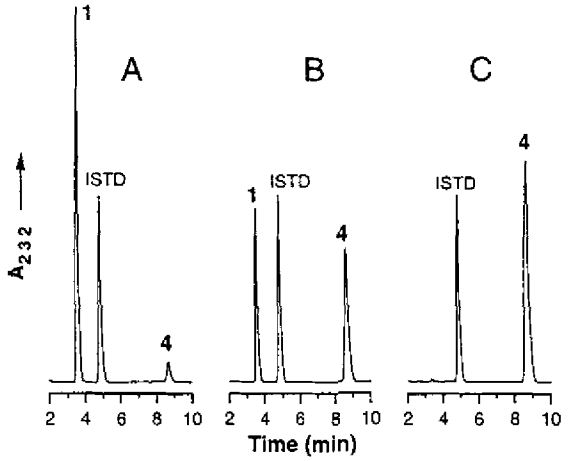



Fig. 5. Reversed-phase HPLC chromatograms in the conversion of **2** to **4** and/or **1** in ethanol-1 M HCl (1:3, v/v) at 50 °C for 10 min (A), in ethanol-0.1 M HCl (1:3, v/v) at 50 °C for 10 min (B), and in ethanol-Britton-Robinson pH 7 buffer (1:3, v/v) at 50 °C for 22 h (C), The internal standard (ISTD) was **6**.

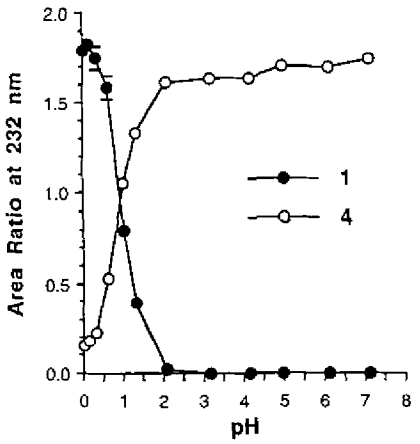



Fig. 6. Relative amounts of products formed in pH-dependent conversion of **2** at 50 °C. The amount of either **1** or **4** formed was expressed by area ratio at 232 nm relative to that of internal standard **6**. Product formations were analyzed by reversed-phase HPLC as shown in Fig. 5.

was probably due to incomplete conversion of **1** to **4**. The quantitative conversion of **1** to **4** via **2** and **3** in two experimental steps (hydrolysis of **1** to form **2** in alkaline solution, followed by conversion to **4** via **3** in pH 3-5 solution) is a considerably simpler method than the acid hydrolysis procedure extensively reported in the literature. The potential application of the mechanism-based procedure described in this report to detect **1** via **4** in clinical samples awaits investigation.

### Oxygen-18 Labeling

The finding that **2** was converted to **1** in strongly acidic solutions (Figs. 5 and 6) presented an opportunity to evaluate the reversibility of the **1** to **2** conversion in alkaline solution.[1] In an alkaline solution containing oxygen-18 water, a reversible **1** $\leftrightarrow$ **2** reaction should result in a **2** containing two oxygen-18 atoms, while an irreversible **1** to **2** reaction should result in a **2** containing only one oxygen-18 atom. Thus by determining the oxygen-18 content of **1** derived from the oxygen-18-labeled **2** [formed by reaction of unlabeled **1** in ethanol-1.11 M $Na^{18}OH$ (1:1, v/v) at 50 °C] should provide a definitive proof for the reversibility of the **1** $\rightarrow$ **2** reaction in alkaline solution.

GC-MS analysis of **1** formed by acidification of an oxygen-18-labeled **2** indicated that the analyte (**1**) contained half of an oxygen-18 atom (Fig. 7). Hence only one of the two oxygen atoms in **2** was labeled with oxygen-18. The results indicated that the **1** $\rightarrow$ **2** reaction in alkaline solution was irreversible.

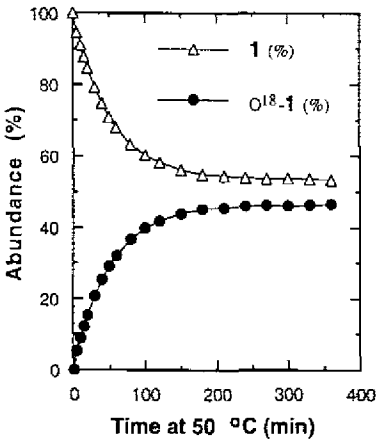



Fig. 7. Relative abundance of oxygen-18-labeled **1** formed from oxygen-18-labeled **2** in strongly acidic solution. Oxygen-18-labeled **2** was prepared from **1** in ethanol-1.11 M $Na^{18}OH$ (1:1, v/v; ~90 atom % $^{18}O$) and heated at 50 °C. Aliquots were taken at various times and the products were converted to **1** in 1 M HCl. Oxygen-18 content of **1** was determined by GC-MS.

Electron impact mass spectra (*m/z* 200-300) of unlabeled and an oxygen-18-labeled **1** (derived from the aliquot taken at 360 min as described in Fig. 7) are shown in Fig. 8. The results clearly indicated that the base ion at *m/z* 256 in the mass spectrum of unlabeled **1** was derived by the loss of $NCH_2$ and the mass ion at *m/z* 221 in the mass spectrum of unlabeled **1** was derived from mass ion at *m/z* 256 by the loss of a chlorine atom. The observed $[M - 28]^+$ ion due to the loss of $NCH_2$ was consistent with that proposed by Sadée[14] and in disagreement with the loss of CO proposed by Benz et al.[15]

A bonus of the experimental observation described above was the discovery of a method to prepare **1** containing an oxygen-18 atom at C2 position. An oxygen-18-labeled **1** containing >90 atom % $^{18}O$ has been prepared in our laboratory by performing the above-described oxygen-18-labeling procedure four successive times. The availability of an oxygen-18-labeled **1** should facilitate a more detailed study on the fragmentation pattern in electron impact mass spectral analysis.

### Reaction Mechanisms

The proposed reaction mechanisms in the conversion of **2** to **1**, **3**, and **4** are summarized in Scheme I. The initial step in the **2** to **1** conversion was likely due to a diffusion-

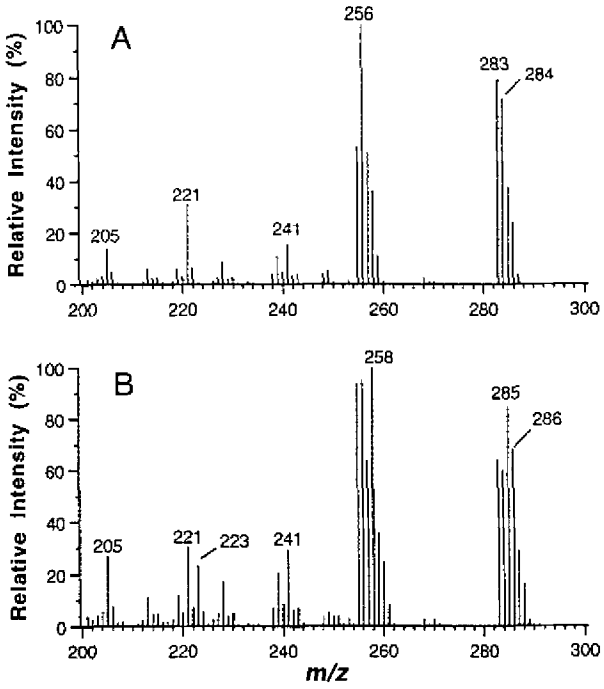



Fig. 8. Electron impact mass spectra of (A) authentic **1** and (B) a partially oxygen-18-labeled **1** derived from an aliquot taken at 360 min in the experiment described in Fig. 7.

controlled protonation (**2** to intermediate a, Scheme I), followed by an acid-catalyzed dehydration. The oxygens at C2 of **2** are equivalent. The proposed mechanism is consistent with both the thermodynamic parameters involved in the reaction (Table 1) and the results of the oxygen-18-labeling experiment (Figs. 5-7).

The **2** to **3** conversion, detectable in solutions with pH $\geq$ 7, was first-order with respect to hydrogen ion (Fig. 4). The negative $\Delta S^{\ddagger}$ involved in this reaction (Table 1) indicated that the rate-determining step was not a direct **2** to **3** conversion, and intermediate b was probably formed (Scheme I). Thus the rate-determining step was probably the conversion of intermediate b to **3**. It should be pointed out that, in solutions with pH > 7, **3** derived from **2** continued to undergo hydrolysis to form **4** at a very slow rate.

The ionic state of **3** should be dependent on the pH of the solution; predominantly anionic form in solutions with pH > $pK_a$ and primarily neutral form in solutions with pH < $pK_a$. Because **3** was quickly converted to **4** in solutions with pH < 6, the $pK_a$ of **3** could not be determined by titration. However, the pH-dependent rate of **3** to **4** conversion (Fig. 4) suggested that the $pK_a$ of **3** was approximately 4.5, which was consistent with the $pK_a$ of compounds containing a carboxylic acid functional group.

The mechanism in the hydrolysis of **3** to **4** in solutions with various acidities is expected to be similar to those of the extensively studied imines.[7] In solutions with pH >> $pK_a$, the rate-determining step is nucleophilic attack by hydroxide ion on the protonated C=N bond (intermediate c to intermediate d), followed by breakdown of the tetrahedral intermediate f (Scheme I). In solutions with pH $\approx$ $pK_a$, water replaces hydroxide as the dominant nucleophile, and the rate-determining step is intermediate c to intermediate e, followed by breakdown of the tetrahedral intermediate f (Scheme I). In solutions with pH << $pK_a$, the rate-determining step becomes the breakdown of the tetrahedral intermediate f (Scheme I).

## CONCLUSION

Compound **2**, the initial alkaline hydrolysis product of **1**, is quantitatively converted to **4** via **3** in solutions with pH values greater than the $pK_a$ of **3**. In solutions with pH << $pK_a$, **2** is converted to **1**. The results of this study not only confirmed the irreversibility of the **1** to **2** reaction in alkaline solution, but also established a simple and efficient method in the quantitative conversion of **1** to **4** and in the preparation of an oxygen-18-containing **1** at C2 position.

## ACKNOWLEDGMENT

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**Key Words**

Diazepam; 7-Chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2,2-dioxide; Hydrolysis; 2-Methylamino-5-chloro-α-(phenylbenzylidene)glycinate; 2-Methylamino-5-chlorobenzophenone; Oxygen-18 labeling.

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