

Research Papers

## Stability of Carmethizole hydrochloride (NSC-602668), an experimental cytotoxic agent

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### Summary

The solution stability and degradation mechanism of Carmethizole HCl (**1**, NSC-602668), an experimental anti-cancer drug, were studied. An apparent  $pK_a$  value of 4.2 at 25 °C and ionic strength = 0.15 M (NaCl) was determined for **1** by potentiometric titration. The degradation **1** in aqueous solution proceeds by two consecutive hydrolysis reactions of the bis-carbamate moieties at C4' and C5', with hydrolysis at C5' hydrolyzing approximately 8 times faster than hydrolysis at C4'. The rates of degradation of **1** in aqueous solution were determined as a function of pH, temperature, buffer concentration, ionic strength, and co-solvent concentration. The pH of maximum stability was at pH < 2 for the degradation of **1** at 25 °C. Analysis of the hydrolysis products by electron impact mass spectrometry showed that one <sup>18</sup>O atom was incorporated into the first degradation product, **2**, when the reaction was run in <sup>18</sup>O-enriched water. This is consistent with alkyl C-O bond cleavage rather than an addition-elimination reaction at a carbamate group. The entropy of activation ( $\Delta S^\ddagger$ ) was  $-6.71 \pm 0.05$  e.u. at pH 9.90, indicating a unimolecular mechanism. All observations were consistent with an S<sub>N</sub>1 mechanism.

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### Introduction

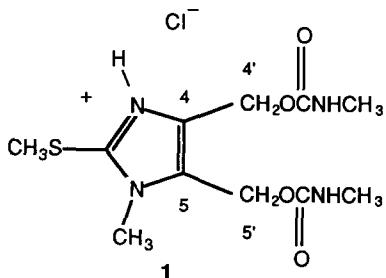
Carmethizole HCl (**1**, NSC-602668) is a new member of a class of anti-tumor agents that are bis-carbamoyloxyethyl heterocyclic derivatives which have undergone evaluation for anti-tumor activity at the National Cancer Institute (Anderson, 1982; Anderson et al., 1982). The formulation

of a related antitumor agent [(1-methylethyl)-[5-(3,4-dichlorophenyl)-2,3-dihydro-1H-pyrrolizine-6,7-diy] bis(methylene) ester (NSC-278214)] has been reported (El-Sayed et al., 1983). Difficulties were encountered in the formulation of NSC-278214 due to poor aqueous stability ( $t_{50} = 13$  min in CH<sub>3</sub>CN-water, T=25 °C) and poor water solubility ( $1.26 \times 10^{-6}$  M). A parenteral formulation of NSC-278214 was developed by incorporation of the drug into a commercial fat emulsion, dissolved in dimethylacetamide-cremophor-EL® solution. The resulting formulation was 0.7 mg/ml, and

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was approximately 100-fold more stable than in aqueous solution.



Carmethizole (**1**) has significantly improved water solubility (580 mg/ml) as its hydrochloride salt and improved aqueous stability relative to NSC-278214. While evaluating Carmethizole HCl as a potential anti-tumor agent, additional information was required regarding its physicochemical properties and stability. Numerous publications describing the synthesis of various bis-carbamoyloxymethyl heterocyclic antitumor agents have appeared, however, detailed information regarding the stability of these compounds is not currently available. In particular, (1) assessing the kinetics of Carmethizole HCl hydrolysis and (2) establishing the mechanism of hydrolysis, were of interest.

## Experimental Section

Carmethizole HCl (**1**) (NSC-602668, lot no, JA13-68-1) was obtained from the National Cancer Institute, Bethesda, MD, and was used without any further purification. Degradation products **2** and **3** and a possible degradation product, **4** (see Scheme 1), were supplied by Professor Wayne Anderson (SUNY, Buffalo, NY) and were used without further purification. All materials were homogeneous when analyzed by HPLC. All other chemicals, including buffer components, were ACS reagent grade and were used without further purification. The water used was deionized and glass distilled (Mega-Pure system model MP-1, Corning). Enriched [<sup>18</sup>O]water was purchased from Aldrich and was 97 atom%. Deutrium oxide (D<sub>2</sub>O) was obtained from Bio-Rad and was 99.8 mol%. All pH measurements were made at room temperature using a digital pH meter (Corning model 155; Medfield, MA).

**HPLC analysis** The HPLC system consisted of an Altex model 110 A pump, a Reodyne 7125 injector fitted with a 20- $\mu$ l loop, a Waters Model 440 detector with fixed wavelength detection at 254 nm, and a Shimadzu C-R3A integrator. The HPLC analysis utilized a reverse-phase C<sub>8</sub> column (Shandon 5  $\mu$ m MOS Hypersil, 150 mm  $\times$  4.6 mm), and the mobile phase was either 5% or 15% acetonitrile with 0.01 M acetate buffer, 1 mM tetrabutylammonium hydrogen sulfate, pH 4.4. A standard solution of Carmethizole HCl (**1**) was prepared in aqueous 0.01 M HCl solution, which is near the pH of maximum stability. The standard solution was kept cool on ice water during calibration of the HPLC in order to increase its stability, and was found to be stable for at least 3

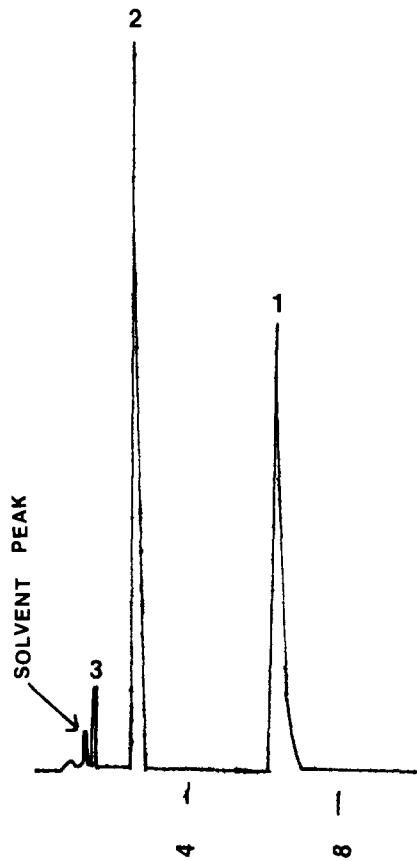


Fig. 1. Representative chromatogram of a partially degraded sample of **1** (1 mg/ml in water) showing the formation of the first degradation product, **2**, and the second degradation product, **3**.

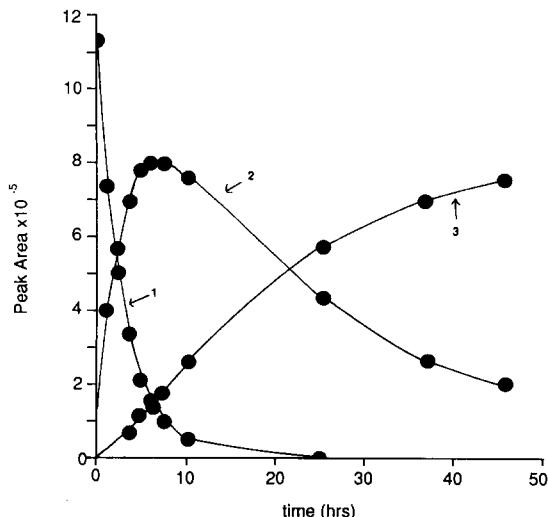


Fig. 2. Plot showing the loss of peak area for **1**, the appearance of **2**, and the eventual formation of **3**, as a function of time in carbonate buffer (0.01 M) at pH 9.90,  $T = 25^\circ\text{C}$ , ionic strength = 0.15 M (NaCl).

h. A quantitative dilution of the standard solution into the HPLC mobile phase was made immediately prior to analysis by HPLC. Once the stock solution of **1** was diluted into the mobile phase, degradation was relatively rapid. Two injections were made from one dilution, and the second injection was made immediately following the first. The peak area resulting from the second injection was slightly lower than the first, due to the rapid rate of degradation of **1** in the mobile phase. A standard curve of peak area vs concentration of **1** was linear up to at least 0.5 mg/ml.

A typical chromatogram of a partially degraded sample of **1** is shown in Fig. 1. A plot of the disappearance of **1** and the appearance of the first degradation product, **2**, and the second degradation product, **3**, as a function of time, is shown in Fig. 2. Compounds **2** and **3** had previously been identified as the principle degradation products by Professor Wayne Anderson (personal communications).

**$pK_a$  determinations** The apparent  $pK_a$  of **1** was determined by potentiometric titration, as described in the literature (Albert et al., 1971). In a typical titration, 212.9 mg of **1** (acidic pH, **1** quite

stable) was accurately weighed and dissolved in 50 ml of 0.15 M NaCl in a thermal-jacketed beaker equilibrated at  $25^\circ\text{C}$ . The drug solution was rapidly titrated with a 0.02 M standard NaOH solution, maintaining the ionic strength at 0.15 with NaCl. The pH was measured, and the  $pK_a$  was determined to be 4.2 at  $25^\circ\text{C}$  and ionic strength 0.15 (NaCl).

**$^{18}\text{O}$  labeling mass spectrometry studies** High resolution mass spectral analyses were carried out with a Nermag R10-10 Quadrupole mass spectrometer. Electron impact mass spectrometry at 70 mV was performed by direct probe injection.

Into two screw-top vials, each containing 0.4 mg of **1**, were placed 250  $\mu\text{l}$  of  $^{18}\text{O}$ -enriched water or 250  $\mu\text{l}$  of unlabeled water. Degradation of **1** was monitored by HPLC until the first degradation product **2** reached a maximum in concentration. At this point, the samples were freeze-dried, then redissolved in methanol immediately prior to analysis by mass spectrometry. Direct mass spectrometry of pure **1** and **2** was used as controls.

**Kinetics** Apparent first-order rate constants for the degradation of **1** at  $25^\circ\text{C}$  were determined in aqueous solutions of HCl, NaOH, and dilute (0.01 M) aqueous buffer solutions at various pH values ranging from 1 to 13. The ionic strength was adjusted to 0.15 M with the addition of NaCl. For these studies, an accurately weighed quantity of the drug was dissolved in water to give a 0.05 M stock solution. Degradation reactions were initiated by diluting 100  $\mu\text{l}$  of the stock solution into 25 ml of an aqueous buffered solution equilibrated at  $25^\circ\text{C}$  in a water bath. Aliquots were withdrawn periodically and diluted quantitatively into the mobile phase immediately prior to analysis by HPLC. Plots of  $\ln C$  vs time were linear, where  $C$  is the concentration of **1** remaining at time  $t$ . Reactions were followed for at least 3–4 half-lives. Apparent first-order rate constants ( $k_{\text{obs}}$ ) were calculated from the slopes of these plots. The degradation of **1** was not catalyzed by acetate or phosphate buffers. A similar study was performed for the degradation of **2** to **3** over a limited pH range. An Arrhenius study was performed at pH 9.90 for the degradation of **1** by following the decomposition at 0, 5, 15, 25 and  $37^\circ\text{C}$  in 0.01 M carbonate buffer.

## Results and Discussion

### Degradation pathway

The hydrolysis of a 1 mg/ml solution of **1** led to the appearance of two hydrolysis products **2** and **3**, as demonstrated by the representative HPLC chromatogram shown in Fig. 1. The retention times for **2** and **3** were compared to standards and for peak symmetry after co-injection of **2** and **3**. Fig. 2 shows the loss in peak area for **1**, the appearance of **2**, and the eventual formation of **3** at pH 9.90. These kinetics are characteristic of a degradation scheme that involves two consecutive irreversible hydrolysis reactions. The degradation of **1** involves two consecutive hydrolysis reactions of the bis-carbamate moieties at C5' and C4'. Fig. 3 shows that the first degradation product, **2**, is the C5' alcohol/monocarbamate, resulting from hydrolysis of C5' carbamate of **1**, rather than **4**, the C4' alcohol/monocarbamate. The degradation pathway is shown in Scheme 1.

### <sup>18</sup>O-labeling mass spectrometry

An S<sub>N</sub>1 mechanism has been assigned for the hydrolysis of the related anti-cancer agent NSC-278214 (El Sayed et al., 1983). In view of the similarity in structure between **1** and NSC-278214, an S<sub>N</sub>1 mechanism has also been proposed for the hydrolysis of **1** as shown in Scheme 2. Alternatively, the mechanism of hydrolysis could involve hydrolysis of the carbamate moiety by an addition-elimination mechanism at the carbonyl carbon (Christenson, 1964; Dittert et al., 1963; Bender et al., 1965; Williams et al., 1975), also shown in Scheme 2. These two mechanistic possibilities may be distinguished based on the site of C–O bond cleavage, as illustrated in Scheme 2.

In order to determine the site of C–O bond cleavage, **1** was hydrolyzed in <sup>18</sup>O-enriched water and unlabeled water. The extent of hydrolysis was monitored by HPLC. Analysis of the unlabeled water reaction mixture by HPLC and electron impact mass spectrometry (EI-MS) indicated that the mixture components consisted primarily of the intermediate **2** ( $M^+ = 245$ ,  $M^+ - 58 = 187$ ) and a small amount of unreacted **1** ( $M^+ = 302$ ,  $M^+ - 74 = 228$ ). Electron impact mass spectrometry of the <sup>18</sup>O-enriched water reaction mixture indicated that

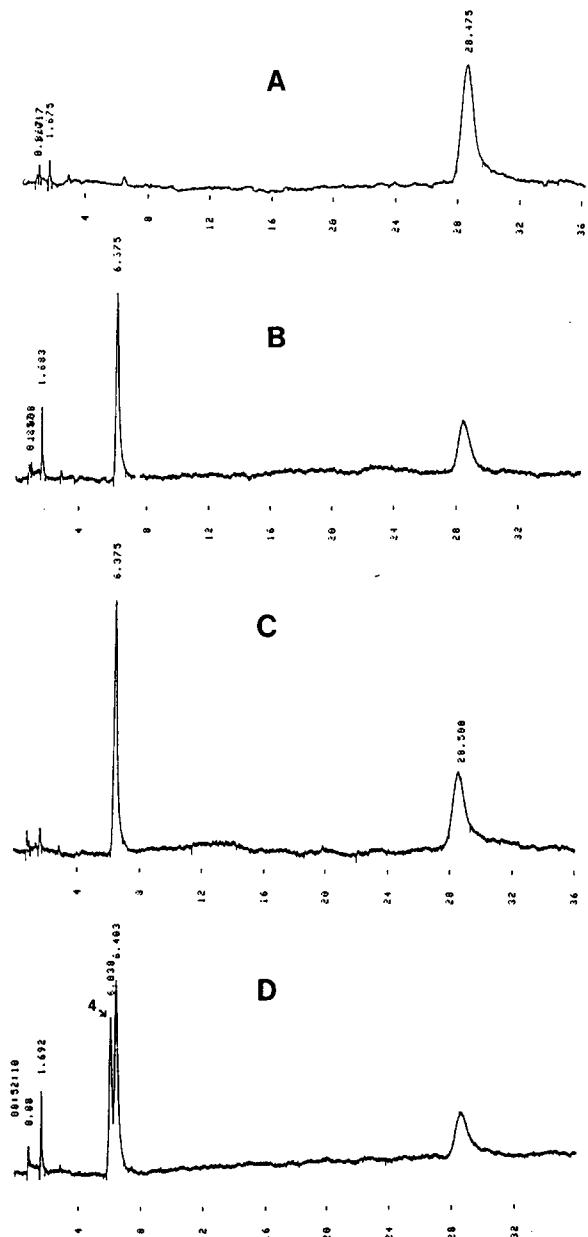


Fig. 3. Identification of the second degradation product by HPLC. (A) **1**. (B) Partially degraded **1**. (C) Partially degraded **1**, spiked with **2**, showing that **2** coelutes with the first degradation product. (D) Partially degraded **1** spiked with **4**. The arrow points to **4**, showing that **4** is resolved from the observed first degradation product, **2**.

the mixture components consisted primarily of the intermediate **2** with incorporation of one <sup>18</sup>O-labeled atom ( $M^+ = 247$ ,  $M^+ - 58 = 189$ ), and a

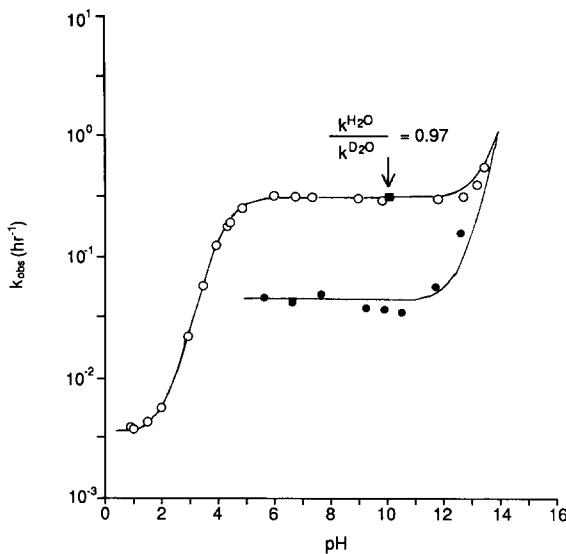


Fig. 4. pH-rate profile for the hydrolysis of **1** (○) and the first degradation product **2** (●) at 25 °C and ionic strength = 0.15 M (NaCl). Solid lines drawn according to a fit to Eqn 1.

small amount of unreacted **1** ( $M^+ = 302$ ,  $M^+ - 74 = 228$ ). The incorporation of  $^{18}\text{O}$  into the hydrolysis product **2** with no incorporation into unreacted

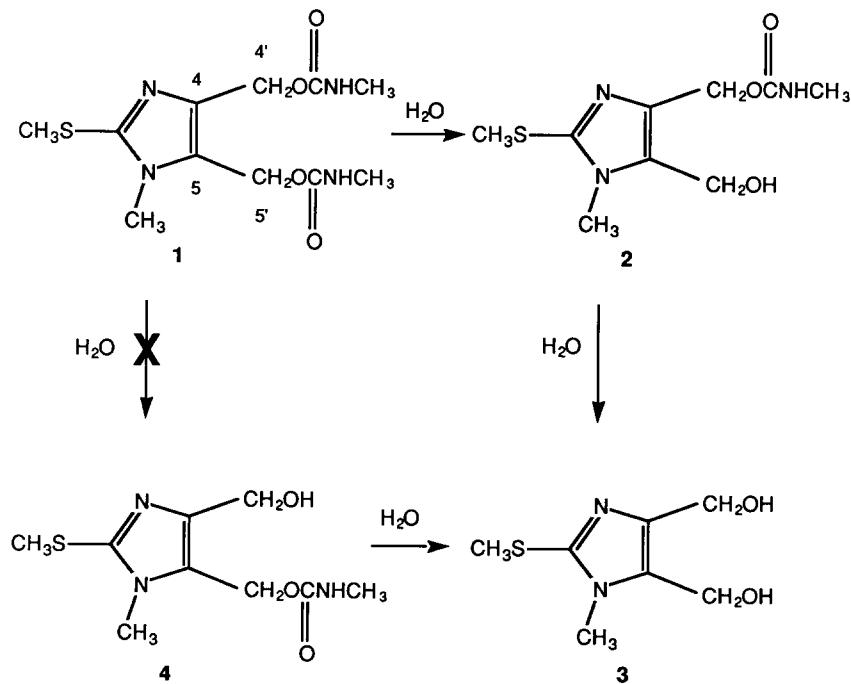
**1**, is strong evidence for alkyl carbon-oxygen bond cleavage rather than carbonyl carbon-oxygen bond cleavage. Based on the prior history of NSC-278214, this observation is also consistent with the hydrolysis occurring via an  $\text{S}_{\text{N}}1$  mechanism.

#### pH-rate profile

The apparent rate constants for the degradation of **1** were determined at various pH values in aqueous solutions of HCl, NaOH, and dilute (0.01 M) aqueous buffer solutions ranging from pH 1 to 13 at constant ionic strength (0.15 M with NaCl and  $T = 25^\circ\text{C}$ ). The apparent first-order rate constants ( $k_{\text{obs}}$ ) for the degradation of **1** are summarized in Table 1, and a plot of  $\log k_{\text{obs}}$  vs pH is shown in Fig. 4. The dependence of  $\log k_{\text{obs}}$  on pH was interpreted according to Eqn 1.

$$k_{\text{obs}} = \frac{K_a}{K_a + a_{\text{H}^+}} (k + k_{\text{OH}^-} a_{\text{OH}^-}) + \frac{a_{\text{H}^+}}{K_a + a_{\text{H}^+}} k' \quad (1)$$

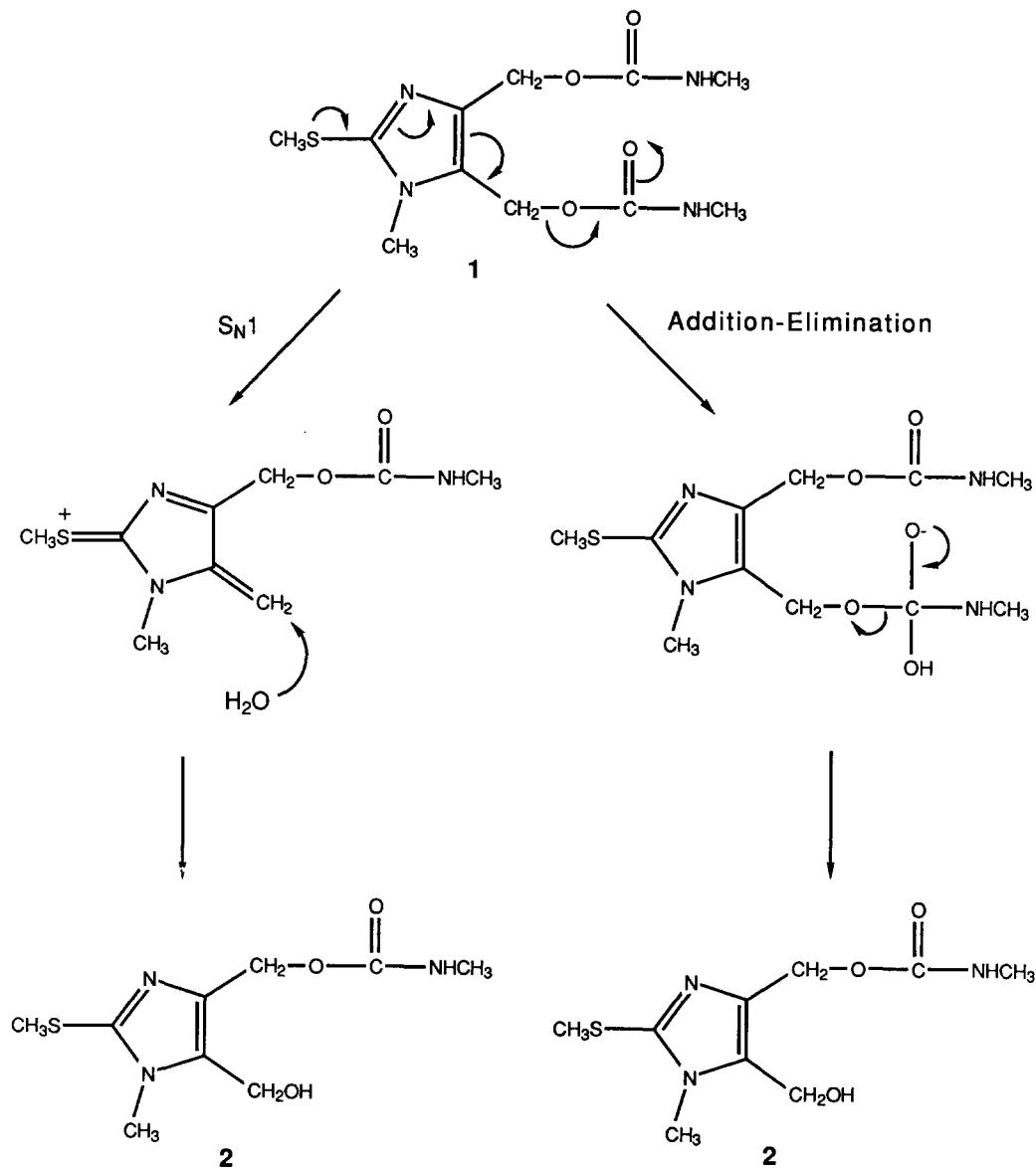
The reaction shown in Scheme 3 describes rate-de-



Scheme 1.

terminating *O*-alkyl bond fission ( $S_N1$  hydrolysis) of both protonated ( $\mathbf{1H}^+$ ,  $k'$  pathway) and unprotonated ( $\mathbf{1}$ ,  $k$  pathway) Carmethizole. Eqn 1 was derived for the reaction shown in Scheme 3, and was fit to the experimental data using the values  $K_a = 6.31 \times 10^{-5}$  ( $pK_a = 4.2$ ),  $k' = 3.5 \times 10^{-3}$   $\text{h}^{-1}$ ,  $k = 3.2 \times 10^{-1}$   $\text{h}^{-1}$ , and  $k_{\text{OH}} = 8.0 \times 10^{-1}$   $\text{M}^{-1} \text{ h}^{-1}$ . The value of  $pK_a = 4.2$  determined from

the hydrolysis kinetics of  $\mathbf{1}$  is excellent agreement with the  $pK_a$  for  $\mathbf{1}$  as determined by potentiometric titration. Fig. 4 shows a break in the pH-rate profile at pH 4.2, which corresponds to the  $pK_a$  of  $\mathbf{1}$ . The pH of maximum stability occurs at pH < 2. The small increase in  $k_{\text{obs}}$  at pH > 13 is attributed to hydrolysis of the carbamate moieties by a nucleophilic reaction with hydroxide ( $k_{\text{OH}}$  term),



Scheme 2.

TABLE 1

Summary of apparent first-order rate constants for the degradation of Carmethizole HCl (**1**) in 0.01 M aqueous buffer solutions, ionic strength = 0.15 (NaCl),  $T = 25^\circ\text{C}$  <sup>a</sup>

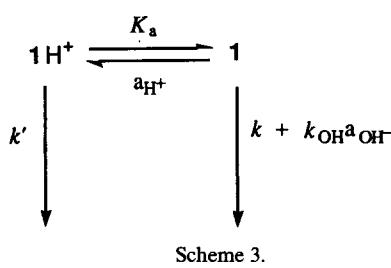
Buffer	pH	$k_{\text{obs}}$ ( $\text{h}^{-1}$ )	$t_{50}$ (h)	$t_{90}$ (h)
0.15 M HCl	0.89	$3.89 \times 10^{-3}$	178	27.0
0.10 M HCl	1.05	$3.75 \times 10^{-3}$	185	28.0
0.03 M HCl	1.55	$4.33 \times 10^{-3}$	160	24.2
0.01 M HCl	2.03	$5.78 \times 10^{-3}$	120	18.2
0.01 M Formate	2.99	$2.24 \times 10^{-2}$	30.9	4.69
0.01 M Formate	3.46	$5.83 \times 10^{-2}$	11.9	1.80
0.01 M Acetate	3.96	$1.29 \times 10^{-1}$	5.37	0.81
0.01 M Acetate	4.39	$1.83 \times 10^{-1}$	3.79	0.57
0.04 M Acetate	4.47	$2.01 \times 10^{-1}$	3.45	0.52
0.01 M Acetate	4.92	$2.64 \times 10^{-1}$	2.63	0.40
0.01 M Phosphate	6.05	$3.35 \times 10^{-1}$	2.07	0.31
0.01 M Phosphate	6.74	$3.34 \times 10^{-1}$	2.08	0.31
0.01 M Phosphate	7.42	$3.25 \times 10^{-1}$	2.13	0.32
0.01 M Borate	9.04	$3.11 \times 10^{-1}$	2.23	0.34
0.01 M Carbonate	9.91	$3.04 \times 10^{-1}$	2.28	0.35
0.01 M NaOH	11.86	$3.12 \times 10^{-1}$	2.22	0.34
0.10 M NaOH	12.77	$3.21 \times 10^{-1}$	2.16	0.33
0.50 M NaOH	13.26	$4.09 \times 10^{-1}$	1.69	0.26
1.00 M NaOH	13.50	$5.69 \times 10^{-1}$	1.22	0.18

<sup>a</sup>  $[\mathbf{1}]_0 = 2.36 \times 10^{-4}$  M.

and was not investigated further since this was outside the pH range of interest.

The hydrolysis kinetics of **2** is also shown in Fig. 4. The solid line in this case was fit to only the first term in Eqn 1. This data confirms the slower rate of degradation of the carbamate moiety at C4'. The hydrolysis at C5' carbamate group was approximately 8 times faster than hydrolysis at C4'. No attempt was made to study the hydrolysis mechanism of the C4' carbamate group.

To fully understand the possible mechanism of degradation of the carbamate group of C5', a number of techniques were used.



### Kinetic solvent isotope effect

The solvent isotope effect for the hydrolysis of **1** was determined. The hydrolytic rate constants were measured in  $\text{H}_2\text{O}$  and  $\text{D}_2\text{O}$  solutions buffered with 0.01 M carbonate at  $T = 25^\circ\text{C}$  and ionic strength = 0.15 with NaCl. In  $\text{H}_2\text{O}$ ,  $k_{\text{obs}} = 3.04 \times 10^{-1}$   $\text{h}^{-1}$  at pH 9.91. In  $\text{D}_2\text{O}$ ,  $k_{\text{obs}} = 3.14 \times 10^{-1}$   $\text{h}^{-1}$  at  $\text{pD} = 10.16$ . These values of pH and  $\text{pD}$  are within a region where  $k_{\text{obs}}$  is independent of pH, as shown in Fig. 4. The ratio  $k_{\text{H}_2\text{O}}/k_{\text{D}_2\text{O}}$  was found to be 0.97, indicating that water is probably not involved in the transition state. This result is consistent with the  $\text{S}_{\text{N}}1$  mechanism proposed in Scheme 2.

### Salt effect

There was no observable effect of NaCl concentration on the apparent first-order rate constants ( $k_{\text{obs}}$ ) for the hydrolysis of **1** at pH 9.90 in 0.01 M carbonate buffers. The degradation of the antineoplastic drug, clomesome ([2-chloroethyl (methylsulfonyl)methanesulfonate], NSC-338947), which took place via an early transition state  $\text{S}_{\text{N}}2$  mechanism, was accelerated by added halide ions ( $\text{F}^-$ ,  $\text{Cl}^-$ , and  $\text{Br}^-$ ) due to nucleophilic catalysis (Kennedy et al., 1988).

### Temperature dependence

The effect of temperature on  $k_{\text{obs}}$  was investigated at pH 9.90, which lies within a region where  $k_{\text{obs}}$  is independent of pH. The data is fit according to the Arrhenius equation as shown in Fig. 5, with  $\ln A = 35.1 \pm 0.1$  and  $E_a = 21.5 \pm 0.2$  kcal  $\text{mol}^{-1}$ .

$$\ln k = \ln A - \frac{E_a}{T} \quad (2)$$

The thermodynamic activation parameters for the unimolecular  $\text{S}_{\text{N}}1$  rate constant  $k$  (see Scheme 3) were determined at pH 9.90 according to the Eyring equation (Eyring, 1935). The enthalpy of activation ( $\Delta H^\ddagger$ ) was  $20.9 \pm 0.2$  kcal  $\text{mol}^{-1}$ , and the entropy of activation ( $\Delta S^\ddagger$ ) was  $-6.71 \pm 0.05$  e.u. The small negative value for the entropy of activation is also consistent with a unimolecular  $\text{S}_{\text{N}}1$  mechanism.

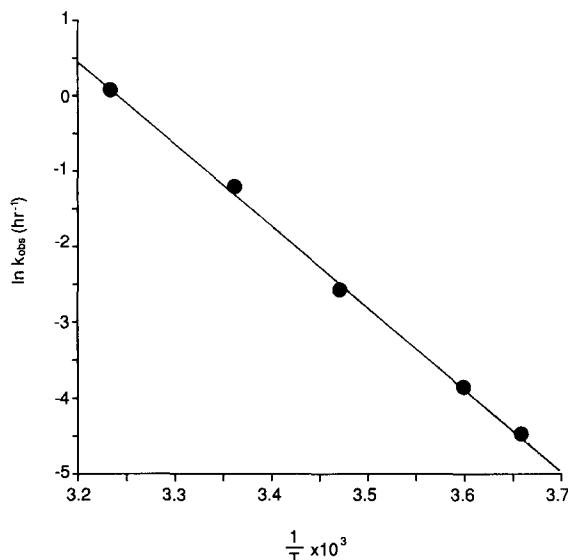


Fig. 5. Arrhenius plot for the hydrolysis of **1** at pH 9.90 and ionic strength = 0.15 M (NaCl).

#### Stability in organic co-solvents

The rate of hydrolysis of **1** is sensitive to solvent polarity in a manner that is typical for  $S_N1$  reactions (March et al., 1985). The effect of various organic co-solvents on the stability of **1** was studied, and the data is summarized in Table 2. **1** hydrolysis did not follow strict first order kinetics in the co-solvents. Relative stability is reported in

TABLE 2

Summary of apparent  $t_{90}$  values for the degradation of 0.2 mg/ml Carmethizole HCl (**1**) in various aqueous-organic solutions at 25°C <sup>a</sup>

Solution	Initial pH	$t_{90}$ (h)
100% water	3.90	0.86
10% <i>t</i> -BuOH; 90% H <sub>2</sub> O	3.74	1.66
20% <i>t</i> -BuOH; 80% H <sub>2</sub> O	3.64	4.19
40% <i>t</i> -BuOH; 60% H <sub>2</sub> O	3.52	9.9
10% PG; 2.5% EtOH; 87.5% H <sub>2</sub> O	3.75	1.4
10% PEG; 2.5% EtOH; 87.5% H <sub>2</sub> O	3.72	1.76
20% PG; 5% EtOH; 75% H <sub>2</sub> O	3.62	2.23
20% PEG; 5% EtOH; 75% H <sub>2</sub> O	3.75	3.46
40% PG; 10% EtOH; 50% H <sub>2</sub> O	3.53	6.91
40% PEG; 10% EtOH; 50% H <sub>2</sub> O	4.08	13.44

<sup>a</sup> PG, propylene glycol; PEG, polyethylene glycol 400.

terms of  $t_{90}$  values, i.e., the time for the first 10% of **1** to degrade. In general, the apparent  $t_{90}$  values for degradation increased as the solvent polarity decreased. This observation is again consistent with an  $S_N1$  mechanism.

#### Conclusion

The solution stability and degradation mechanism of Carmethizole HCl (**1**), an experimental anti-cancer drug, have been studied. The mechanism of degradation in aqueous solution was shown to involve rate-determining *O*-alkyl bond fission according to an  $S_N1$  mechanism, with prior ionization of the imidazole moiety ( $pK_a = 4.2$ ). Incorporation of <sup>18</sup>O into the degradation products was used as a probe of the reaction mechanism, and the results were consistent with the proposed mechanism. The pH-rate profile was interpreted and showed a pH of maximum stability at pH < 2. Carmethizole hydrochloride (**1**) has improved solubility and stability relative to NSC-278214, a related anticancer agent, as well as other bis-carbamoyloxymethyl heterocyclic derivatives in its class.

#### Acknowledgments

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