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Is ICRF-187 [(+)-1,2-Bis(3,5-dioxopiperazinyl-1-yl)propane] Unusually Reactive for an Imide?

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The hydrolysis of ICRF-187 and two model compounds, 4-methylpiperazine-2,6-dione (4-MP) and 3-methylglutarimide (3-MG), was investigated over the neutral to alkaline pH range at 25°C and an ionic strength of 0.5 (sodium chloride). The purpose of the study was to correlate the influence of molecular changes to the reactivity of these imides. Additionally, an improved chromatographic resolution of all the components of the degradation and NMR confirmation of the identity of the degradation products are presented. Based on the study of 4-MP, which is essentially half of an ICRF-187 molecule, and 3-MG, which has a carbon in place of the piperazine nitrogen, several conclusions can be drawn with regard to the stability of ICRF-187. The tertiary piperazine nitrogen/s of 4-MP and ICRF-187 contributed to the base-catalyzed hydrolysis of these compounds above pH 7 and caused a significant decrease in the pK_a values of the imide moiety of ICRF-187 and 4-MP compared with 3-MG. One 2,6-piperazinedione ring of ICRF-187 was shown to affect only minimally the rate of hydrolysis of the second ring. ICRF-187 hydrolyzes by parallel consecutive pathways forming two monoacids with one ring opened and, subsequently, the diacid with both rings hydrolyzed.

KEY WORDS: hydrolysis; imide; ICRF-187 (ADR-529); pH dependency; glutamides; cardioprotectant.

INTRODUCTION

ICRF-187 (Fig. 1), the *d*-enantiomer (1) of the racemic compound ICRF-159, was developed in order to circumvent the poor water solubility of the anticancer agent ICRF-159 (2). More recently, ICRF-187, apart from its anticancer activity, has been shown to be effective in reducing the level of cumulative damage to the myocardium caused by doxorubicin (3–5). Prior to the recent publication of Hasinoff (6), the chemical stability of ICRF-187 had not been extensively studied. Hasinoff studied the stability at pH values above 6.5 using a spectrophotometric technique in an attempt to relate the stability and the degradation product/s of ICRF-187 to the *in vivo* cardioprotectant action of this agent. A study (7) on the stability of ICRF-187 between pH 1 and pH 5 at 25°C was reported, while others reported some stability data for ICRF-59 (8). These results were based on polarimetric and TLC methods.

Chemical stability studies on structurally similar compounds such as succinimide (9–11), substituted succinimides (12), phthalimide (13), substituted phthalimides (13), and glutethimide (14,15) have shown the imide moiety to be susceptible to base-catalyzed hydrolysis. Accordingly, ICRF-187 has also been shown to be subject to base-catalyzed hydrolysis (6). The simple cyclic imides appear to exhibit greater stability than ICRF-187 (7) in the neutral and acidic pH ranges and, in the case of glutethimide, at elevated temperatures (14).

The greater base instability of ICRF-187 may be hypothesized to be due to contributions from the second nitrogen (the piperazine nitrogen in the 4 position) in the 2,6-piperazinedione rings and/or the presence of a second ring system in the molecule. To test these hypotheses, the chemical stability of ICRF-187 and two model compounds, 4-methylpiperazine-2,6-dione (4-MP) and 3-methylglutarimide (3-MG) (Fig. 1), was investigated. The two model compounds were chosen because 4-MP is a 2,6-piperazinedione that contains two heterocyclic nitrogens and is very similar to half of an ICRF-187 molecule, while 3-MG is a 2,6-piperidinedione that contains one heterocyclic nitrogen.

The purpose of the study, therefore, was to correlate the influence of molecular changes to the reactivity of these imides. Additionally, an improved chromatographic resolution of all the components of the degradation and NMR confirmation of the identity of the degradation products is presented.

MATERIALS AND METHODS

Materials

ICRF-187 (NSC 169780) was obtained from the National Cancer Institute, Bethesda, MD. 3-Methylglutarimide was received from ICN Pharmaceuticals, Plainview, NY. 4-MP was synthesized by the fusion of methyliminodiacetic acid with urea (16). Melting behavior, elemental analysis, mass spectrum, and ^1H NMR data were consistent with the structure. Methanol, phosphoric acid, and monobasic potassium phosphate were HPLC grade and purchased from Fisher Scientific. Chloroform-*d* 99.8 atom% D and methyliminodiacetic acid were obtained from Aldrich, and urea was obtained from Sigma. All other chemicals were reagent grade, used without further purification, and were obtained from Fisher Scientific. Water was deionized and charcoal filtered prior to distillation from glass (Mega-Pure System, Model MP-1, Corning).

Instrumentation

The HPLC separations were conducted isocratically using a modular HPLC system that included a Kratos Spectroflow 400 solvent delivery system, a Kratos Spectroflow 480 fixed-loop injector fitted with a 20-μl loop, and a Kratos 783 multiwavelength detector operated at 208 nm. The separations were accomplished using a reversed-phase ODS Hypersil (C_{18}) column (15 cm × 4.6 mm, 5-μm particle size; Shandon). Quantification was by peak area measurement with either a Shimadzu CR-6A integrator or a Hewlett-Packard Model 86 personal computer and Nelson Analytical Chromatographic software.

Separations of the hydrolysis products of ICRF-187 were performed by TLC using Merck 20 × 20-cm aluminum sheets precoated to a thickness of 0.2 mm with silica gel 60 F_{254}. The solvent system was chloroform, methanol, 99% formic acid (9:3:1, v/v/v) (8).

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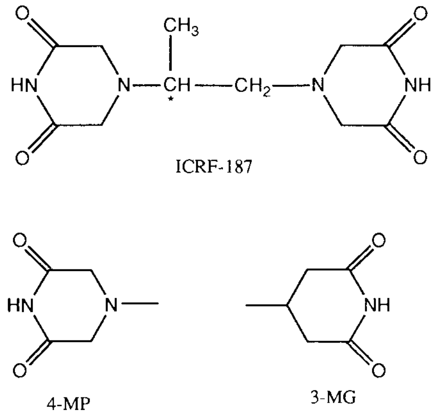


Fig. 1. Structures of ICRF-187, 4-methyl-2,6-piperazinedione (4-MP), and 3-methylglutarimide (3-MG).

The ^1H and ^{13}C NMR spectra were measured either at 300 MHz with a Varian XL-300 or at 500 MHz with a Bruker AM-500 fourier transform nuclear magnetic resonance spectrometer. Chemical shifts (δ ppm) were obtained for band centers relative to the internal standard, tetramethylsilane (δ 0.00 ppm) or dimethyl sulfoxide-d_6 (δ 2.50 ppm) for ^1H NMR and dimethyl sulfoxide-d_6 (δ 39.5 ppm) for ^{13}C NMR. Mass spectra were obtained with a Varian CH-5 or a Ribermag R-10-10 quadrapole mass spectrometer operated in either the electron impact (EI) or the chemical ionization (CI) mode. A Perkin-Elmer Lambda 1 UV-vis spectrophotometer, equipped with a Perkin-Elmer Super Sipper, was used for the spectrophotometric determination of the dissociation constants. All other UV-vis measurements were performed using a Perkin-Elmer 555 spectrophotometer. Elemental analysis was performed by Midwest Microlab, Ltd., Indianapolis, IN.

Miscellaneous

The pH of the buffers was measured with a Corning 155 pH meter equipped with a combination electrode (Corning). The pH in some experiments was controlled by the Metrohm pH-stat system, which included a 614 Impulsomat, a 632 pH meter equipped with a combination Ross electrode (Corning), a 655 Dosimat, and an E649 magnetic stirrer. The temperature for most of the kinetic studies and pK_a determinations was controlled at 25.0 ± 0.1°C with a circulating water bath (either a Forma Scientific Model 2059 or a Haake Model D-2) unless indicated otherwise. Freeze-drying was achieved using an Ace Thred Design Freeze-Drying Apparatus.

Nonlinear curve fitting was accomplished with the Simplex algorithm in the program MULTI [by Yamaoka *et al.* (17)] or "RS/1" (BNN Software Products Corp.) for personal computers. Linear regression analysis was performed with the program StatWorks (Data Metrics, Inc.).

Methods

Dissociation Constants of ICRF-187, 4-MP, and 3-MG

The pK_a values of the imide nitrogens of ICRF-187 and the two model compounds were determined spectrophotometrically at 225 nm (18) under solvent conditions identical to those for the kinetic studies, at 25°C and an ionic strength of 0.5 maintained with sodium chloride. Under strongly alkaline conditions, 3-MG underwent rapid hydrolysis. Therefore, at pH values above 11.0, the decrease in the absorbance of the 3-MG solutions was monitored with time. A plot of the logarithm of the absorbance versus time gave a straight line, with the intercept equal to the absorbance of the 3-MG solution at zero time, which was used in the calculation of the pK_a value. The pK_a values for the piperazine nitrogens in ICRF-187 and 4-MP were determined by potentiometric titration (18) under the same conditions.

Hydrolysis of ICRF-187, 4-MP, and 3-MG

The HPLC mobile phases required to monitor the degradation kinetics of each of the compounds were mixtures of 0.01 *M* potassium phosphate buffer at pH 6.0 and methanol. The methanol content of the mobile phases was 10% for ICRF-187, 0% for 4-MP, and 3% for 3-MG. The flow rate in all cases was 1.5 ml/min. The kinetics were followed by monitoring the decrease in peak area with time.

The hydrolysis at 25°C of ICRF-187, 4-MP, and 3-MG was studied at pH values greater than 7. The various components, concentration ranges, and pH range of the buffer solutions used in the kinetic studies were pH 7.0–8.0 (0.025–0.1 *M* phosphate), 9.0–10.0 (0.06–0.1 *M* borate), 10.9–12.0 (0.025–0.075 phosphate), and 12.0–13.2 (sodium hydroxide). All buffers were adjusted to an ionic strength of 0.5 with sodium chloride. The effects of buffer concentration were examined by varying the concentration of buffers while maintaining a constant pH. Either three or four concentrations of buffer were used at each pH.

Hydrolysis Products of ICRF-187

ICRF-187 and its hydrolysis products were analyzed using a mobile phase that consisted of 3 parts methanol and 97 parts 0.01 *M* tetrabutylammonium phosphate, 0.01 *M* dibasic potassium phosphate, and 0.01 *M* sodium tetraborate aqueous solution adjusted to pH 7.0 with 1.0 *M* phosphoric acid. The product analysis study was carried out at pH 10.0 maintained with a pH-stat.

To isolate the products, an aqueous solution of ICRF-187 (3.7×10^{-2} *M*) was degraded at 65°C and pH 7.0. The sample solution was held at 65.0°C while the pH was maintained with a pH-stat and periodically analyzed by HPLC. Once the maximum areas for the initial degradants were reached, the reaction solution was frozen in an acetone/dry ice bath and then lyophilized. The resulting white fluffy material was purified by preparative TLC. The final product was a light yellow, flaky, mica-like solid.

In another study, an aqueous solution of ICRF-187 (3.7×10^{-2} *M*) was degraded in 0.1 *M* borate buffer at pH 10.25 and 25.0°C for approximately 3 hr. Preparative HPLC with a mobile phase that consisted of 0.01% formic acid in water was used to isolate the compound, which eluted with a retention time of 1.8 min. The collected fractions were frozen in an acetone/dry ice bath and then lyophilized. The result was a white fluffy solid material.

RESULTS AND DISCUSSION

The Ionization of ICRF-187, 4-MP, and 3-MG

The pK_a of the weakly acidic imide moiety of 3-MG was found to be 11.45 ± 0.02. This is comparable to a pK_a of 11.2 for glutarimide and actidione (19), which are analogous to 2,6-dioxopiperidines. 4-MP contains two sites capable of ionization. The value of pK_{a1} for the protonation of the tertiary piperazine nitrogen was determined to be 2.68 ± 0.05. The value of pK_{a2} for the imide functionality in 4-MP of 9.62 ± 0.01 was similar to the value of 10.03 reported for ICRF-187 by Hasinoff (6).

ICRF-187 has four potential sites of ionization, two tertiary piperazine nitrogens and two imide functionalities. Although there are four possible ionizable groups, only two pK_a values were observed experimentally. The value of pK_{a1} was determined to be 2.47 ± 0.06, which is comparable to the approximate value of 2.4 determined previously by optical rotation for ICRF-187 (8) and to the value of pK_{a1} (2.68) for 4-MP. One equivalent of acid was required to reach the end point, an indication of the addition of one proton to the molecule.

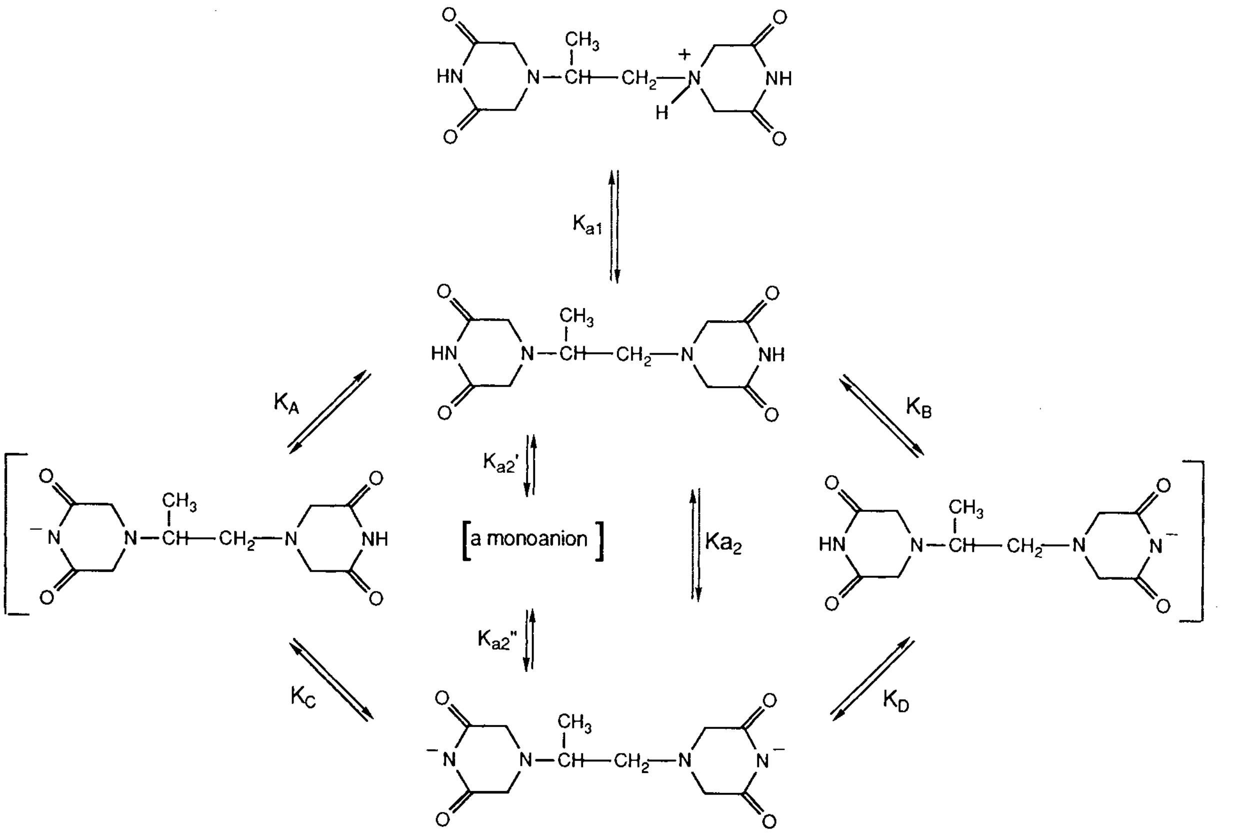
The value of pK_{a2} for ICRF-187 was determined to be 9.70 ± 0.01. This is in agreement with the pK_{a2} of 4-MP of 9.62 and similar to that reported by Hasinoff (6). ICRF-187 has two ionizable imide nitrogens but only one observed pK_a. This indicates that pK_{a2} is a complex term that consists of overlapping pK_a values. Since the imide functionalities are well separated in the molecule, the overlapping pK_a values are expected to be similar, differing by approximately 0.6 of a pK_a unit (equal to log 4), the statistical factor for two equivalent ionization sites on a molecule (20). Using the statistical correction, the estimated value of $pK_{a2'}$ for the loss of a proton from the neutral species to form the monoanionic species is 9.40 and the estimated value of $pK_{a2''}$ for the loss of the second imide proton to form the dianionic species is 10.0. Scheme I outlines the proposed ionization of ICRF-187 including the probable microscopic ionization constants (18).

The pK_{a2} values of ICRF-187 and 4-MP are significantly lower than the pK_a of 3-MG, which has a carbon atom in place of the piperazine nitrogen. It appears, therefore, that the piperazine nitrogen has an acid-strengthening (decrease the pK_a) effect on the imide functionality.

The Hydrolytic Stability of ICRF-187, 4-MP, and 3-MG

The kinetics of the hydrolytic degradation of ICRF-187, 4-MP, and 3-MG followed pseudo-first-order kinetics under all conditions studied. Buffer catalysis was observed for the hydrolysis of all three compounds, although minimally in some instances, and depended on the compound, buffer system, and pH. For the instances where the rate of hydrolysis exhibited buffer dependence, reasonably linear plots were obtained when the observed pseudo-first-order rate constants were plotted against the total buffer concentration at constant pH. The criterion used to indicate if the data exhibited buffer dependence was whether the slope was significantly different from zero.

The pH–rate profiles above neutrality for ICRF-187,



Scheme I. Ionization scheme for ICRF-187.

4-MP, and 3-MG are shown in Fig. 2. The profile for 3-MG is consistent with the pH–rate profiles of other cyclic imides with similar pK_a values such as succinimide and substituted succinimides (5–8,21,22), glutethimide (14), phthalimide (13), and 3-phenyl-imidazoline-2,4-dione (23). A plateau exists at the maximum rate of hydrolysis at very high pH. As the pH decreases, a concomitant decrease in the hydrolysis rate occurs. The degradation at pH values less than 8.0 was too slow to measure conveniently at 25°C. The profiles for ICRF-187 and 4-MP are similar to that of 3-MG over the pH range of 7 to 10. The profile for ICRF-187 above pH 7 is similar to that reported by Hasinoff (6). The hydrolysis of 3-MG was also studied at pH 7.0 and 2.0 over a period of approximately 6 weeks. No apparent hydrolysis occurred at these pH values, an indication that water attack on the neutral species and acid-catalyzed hydrolysis of the neutral species were not important hydrolytic pathways at 25°C over this time frame.

The pH–rate profile of 3-MG is adequately described by Eq. (1):

$$k'_{obs} = \frac{k_{OH^-}K_w}{[H^+] + K_a} \qquad (1)$$

where k'_{obs} is the buffer-independent rate constant, k_{OH} is the bimolecular rate constant for hydroxide ion-catalyzed hydrolysis of the neutral species of 3-MG, K_w is the dissociation constant of water, and K_a is the dissociation constant for 3-MG.

Quantitatively, the data for k'_{obs} generated at various pH values were fit to Eq. (1) by a nonlinear curve-fitting program. A weighting factor of $1/y^2$ was used to fit the data. From the fit, the rate constant k_{OH^-} was determined to be $2.38 \times 10^3\ M^{-1}\ hr^{-1}$. The ionization constant, K_a, was determined to be 7.81×10^{-13}, which corresponds to a pK_a of 12.1, somewhat higher than the spectrophotometrically determined value of 11.45. This difference may be attributed to the error introduced by the instability of 3-MG when the spectrophotometric pK_a determination was conducted.

The shape of the observed pH–rate profile between pH 8 and pH 13.3 for 4-MP (Fig. 2) was consistent with that seen for 3-MG. There was evidence of an increase in the hydrolysis rate at pH >12 from the hydroxide ion-catalyzed hydrolysis of the anionic species that was not observed for 3-MG. Equation (2), for the observed first-order rate constant of the hydrolysis of 4-MP, adequately describes the pH-rate profile from pH 8 to pH 13.3:

$$k'_{obs} = \frac{k_{OH}K_w + k'_{OH}\left(\frac{K_w}{[H^+]}\right)K_{a2}}{[H^+] + K_{a2}} \qquad (2)$$

Values for the various constants that adequately described the profile were $1.54 \times 10^4\ M^{-1}\ hr^{-1}$ for k_{OH} and $7.93 \times 10^{-1}\ M^{-1}\ hr^{-1}$ for k'_{OH}, where k_{OH} and k'_{OH} are the bimolecular rate constants for the hydroxide-ion catalyzed hydrolysis of the neutral and anionic species, respectively. The ionization constant, K_{a2}, was determined to be 2.31×10^{-10}, corresponding to a pK_a of 9.63, which is in excellent agreement with the spectrophotometrically determined value of 9.62.

As expected, the shape of the pH–rate profile for the hydrolytic degradation of ICRF-187 over the pH range of 7.8 to 13.3 mirrors the pH–rate profile for 4-MP and 3-MG. The importance of the ionization of ICRF-187 in this pH region becomes apparent in the development of an equation for k'_{obs} to describe this portion of the profile. If the observable complex macroscopic pK_{a2} of 9.70 for ICRF-187 is treated as a single pK_a, then the pH–rate profile described earlier by Hasinoff (6) results. Since the macroscopic pK_{a2} probably involves two overlapping pK_a values and therefore results in the release of two protons (see earlier pK_a discussion), the profile and equation are slightly more complex than those proposed by Hasinoff (6).

Equation (3) appears to describe adequately the pH–rate profile from pH 7.8 to pH 13.3.

$$k'_{obs} = \frac{k_{OH}K_w[H^+] + k'_{OH}K_wK'_{a2} + k''_{OH}\left(\frac{K_w}{[H^+]}\right)K'_{a2}K''_{a2}}{[H^+]^2 + [H^+]K'_{a2} + K'_{a2}K''_{a2}} \qquad (3)$$

where k_{OH}, k'_{OH}, and k''_{OH} are the bimolecular rate constants for the hydroxide ion-catalyzed hydrolysis of the neutral, monoanionic, and dianionic species, respectively. The data for k'_{obs} generated at various pH values between 7.8 and 13.3 were fit to Eq. (3). From the fit, k_{OH} was determined to be $4.08 \times 10^4\ M^{-1}\ hr^{-1}$, k'_{OH} was $2.99 \times 10^4\ M^{-1}\ hr^{-1}$, and k''_{OH} was $4.83\ M^{-1}\ hr^{-1}$. The ionization constants, K'_{a2} and K''_{a2}, were determined to be 2.29×10^{-10} and 1.34×10^{-10}, respectively, corresponding to pK'_{a2} of 9.64 and pK''_{a2} of 9.87, which are in reasonable agreement with the estimated overlapping macroscopic pK_{a2} values described earlier.

The likely mechanism of hydrolysis for ICRF-187, 3-MG, and 4-MP is via an addition/elimination reaction principally involving attack by hydroxide ion on the neutral imide to form a tetrahedral intermediate which collapses to the product. Not distinguishable, with the evidence collected, is rate-controlling breakdown of the tetrahedral intermediate.

The influence of one ring on the stability of the other in

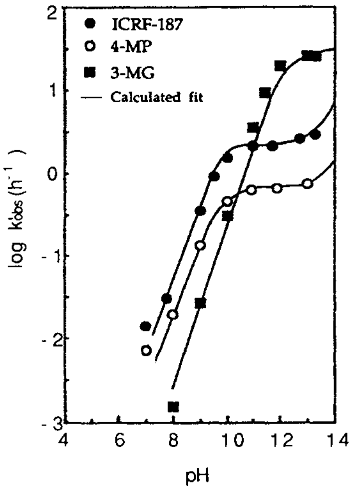


Fig. 2. Partial pH–rate profile for the hydrolysis of ICRF-187, 4-methyl-2,6-piperazinedione (4-MP), and 3-methylglutarimide (3-MG) at 25°C and ionic strength 0.5.

ICRF-187 can be seen by comparing the k_{OH} terms for 4-MP to ICRF-187 (see Table I). The values of k_{OH} were 1.54×10^4 M^{-1} hr^{-1} for 4-MP and 4.08×10^4 M^{-1} hr^{-1} for ICRF-187, respectively. ICRF-187 is hydrolyzed 2.6 times faster than 4-MP. Since 4-MP can be assumed to be half of an ICRF-187 molecule, and since ICRF-187 contains two essentially identical reaction sites, ICRF-187 would be expected to hydrolyze approximately two times faster than 4-MP. The ratio of 2.6 times faster is consistent with these effects and indicates that one 2,6-piperazinedione ring of ICRF-187 has only a minimal influence on the rate of hydrolysis of the second ring.

Comparison of the values of k_{OH} for 4-MP and ICRF-187 to that for 3-MG indicates that 3-MG is hydrolyzed approximately an order of magnitude slower than ICRF-187 and 4-MP. Table I summarizes the data for these rate constants and the pK_a values of the three compounds. The division of Δlog k_{OH} by ΔpK_a for the 4-MP and 3-MG data results in a value (slope) of -0.44. Similarly, for the ICRF-187 and 3-MG data, a value of -0.53 is obtained. These slopes indicate that the reactivity of the compound increases as the acidity of the compound increases. This observation is consistent with the tertiary heterocyclic nitrogens of ICRF-187 and 4-MP acting as electron withdrawing substituents to decrease the pK_a of the imide moiety relative to 3-MG and to increase the reactivity of ICRF-187 and 4-MP, since the carbonyl carbon becomes more electrophilic.

Further evidence linking the tertiary heterocyclic nitrogen to the hydrolytic instability of the dioxopiperazine compounds is also noted in the neutral and acidic pH regions of the profiles, which will be the subject of a future paper. When the stability of 3-MG at the neutral and acidic pH values of 7 and 2 was studied, no apparent hydrolysis occurred over approximately 6 weeks, an indication that water attack on the neutral species and acid-catalyzed hydrolysis of the neutral species were not major hydrolytic pathways for 3-MG.

The Hydrolytic Decomposition Products of ICRF-187

Several hydrolysis products of ICRF-187 have been reported in the literature (6,7,24,25). Earlier studies have used largely a TLC approach to follow the degradation. The products were reported to appear sequentially during the hydrolysis, an indication that ICRF-187 hydrolyzes by a consecutive reaction pathway. Recently, Burke *et al.* (25) were able to isolate and identify the primary degradation products of ICRF-187 using HPLC and mass spectral analysis.

Table I. A Summary of pK_a Values and Values for the Second-Order Rate Constants for Hydroxide Ion Attack on the Neutral Species for ICRF-187, 4-MP, and 3-MG

Compound	$k_{OH} \times 10^{-4}$ (M^{-1} hr^{-1})	log k_{OH} (M^{-1} hr^{-1})	pK_a
4-MP	1.54	4.19	9.62[a]
ICRF-187	4.08 (2.04)[b]	4.61 (4.31)[b]	9.70[c]
3-MG	0.24	3.38	11.45

[a] This value corresponds to pK_{a2}.

[b] This value is an approximate corrected value for the hydrolysis of one of the two rings of ICRF-187 obtained by dividing the value of k_{OH} by 2.

[c] This value corresponds to the complex macroscopic pK_{a2}.

In the present study, the appearance and disappearance kinetics of the two primary hydrolytic degradation products of ICRF-187 were studied at pH 10.25 and 25°C by HPLC following the methodology outlined earlier. Four major peaks were observed over the course of the study. ICRF-187 was eluted from the column in 3.4 min ($k' = 2.0$) and three hydrolysis products were eluted from the column in 1.1, 4.1, and 5.1 min ($k' = 0.0$, 2.7 and 3.6), denoted D3, D1, and D2, respectively. Figure 3 shows a representative chromatogram of a partially degraded sample of ICRF-187. Figure 4 shows a plot of the peak area of D1, D2, and D3 versus time. Three observations can be made from this figure. First, D1 forms to a greater extent than D2. A comparison of the peak areas where D1 and D2 exist at maximum concentration shows that D1 is formed preferentially by a ratio of approximately 3/2. This comparison is based on the assumption that the response factors (i.e., the chromophoric properties) of D1 and D2 are equal. This is a reasonable assumption (25) based on the proposed structures of D1 and D2 shown in Fig. 5. Second, D1 and D2 reach their maximum concentration at approximately the same time, and finally, after a short lag time, the peak area of D3 increases with time.

These observations led to the proposed hydrolytic pathway of ICRF-187 shown in Scheme II. This scheme, also proposed by Hasinoff (6) and consistent with the observations of Burke *et al.* (25), illustrates that ICRF-187 hydro-

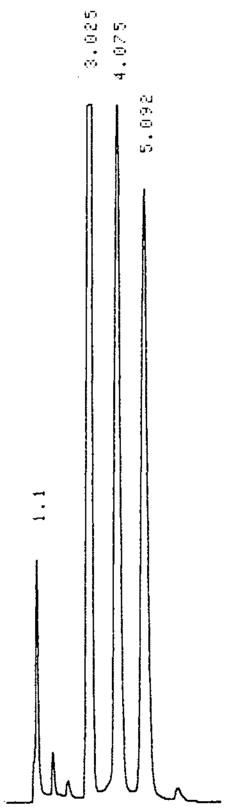


Fig. 3. Sample chromatogram of a partially degraded sample of ICRF-187 maintained at pH 10.25 and 25°C. ICRF-187 eluted at 3.4 min, while D1, D2, and D3 eluted at 4.1, 5.1, and 1.1 min, respectively.

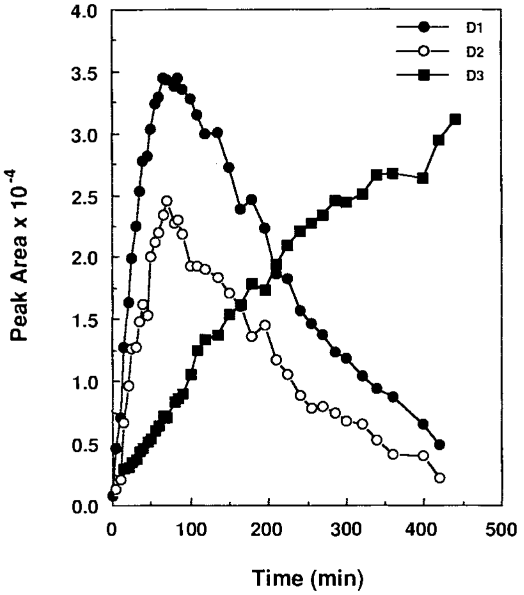


Fig. 4. A plot of peak area versus time of the immediate aqueous degradation products of ICRF-187, D1 and D2, which correspond to ICRF-187 with one imide ring cleaved, and D3, the product resulting from hydrolytic cleavage of both imide rings at pH 10.25 (25°C).

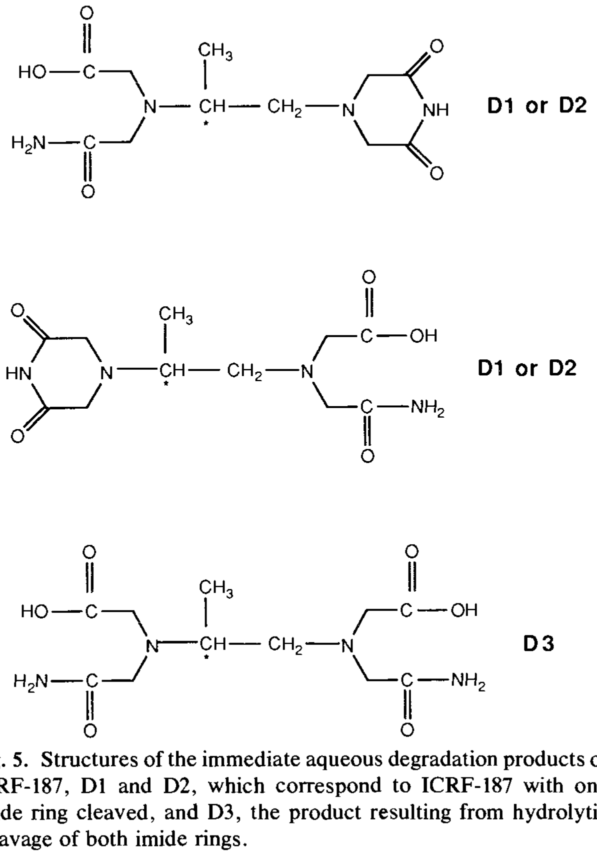
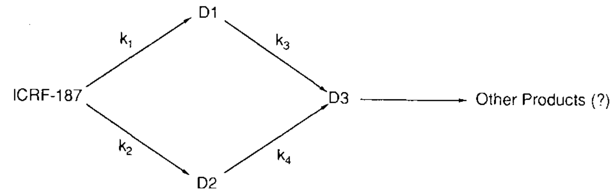


Fig. 5. Structures of the immediate aqueous degradation products of ICRF-187, D1 and D2, which correspond to ICRF-187 with one imide ring cleaved, and D3, the product resulting from hydrolytic cleavage of both imide rings.



Scheme II. Degradation scheme for ICRF-187.

lyzes by parallel pathways to the two primary hydrolysis products D1 and D2. The two primary products then hydrolyze further to form D3, which may itself go on to form other products. The hydrolysis products from the degradation of other imides (10,11,26,27), previous studies on ICRF-187 degradation by TLC (8,24), and chemical intuition suggest that the primary hydrolytic degradation products D1 and D2 are the two possible monoacids resulting from the hydrolysis of one of the rings of ICRF-187, and D3 is the diacid from the hydrolysis of the second intact 2,6-dioxopiperazine ring of D1 and D2 (Fig. 5).

The primary hydrolysis products of ICRF-187, D1 and D2, were isolated as a mixture as described in the experimental section. The mixture was a light yellow, flaky, mica-like solid. Chromatography of a portion of this sample by HPLC indicated two major peaks that corresponded to the retention times of D1 and D2 (4.8 and 5.8 min, respectively) and a minor peak that corresponded to the retention time of D3 (1.1 min). ICRF-187 was not observed in the HPLC chromatogram of this mixture.

The chemical ionization mass spectrum of the mixture exhibited a peak at *m/e* of 287, which corresponds to the M + 1 peak for the monoacids. The base peak of the spectrum occurred at *m/e* of 269, which corresponds to a mass loss of 18 and is indicative of the loss of water from the molecule. Two other peaks at *m/e* of 141 and 127 were noted. Fragmentations of this type have been previously reported (28).

A comparison of the ^{1}H NMR of ICRF-187 and the hydrolysis mixture spectra reveals some differences. First, the spectrum of ICRF-187 does not contain any peaks between δ 7.0 and 7.7 ppm. The spectrum for D1 and D2 has several doublets in this region that are assigned to the two sets of amide protons ($-\mathrm{CONH_2}$) in the hydrolysis products. Second, both spectra exhibit two peaks between δ 11.0 and δ 11.1 ppm. These are assigned to the protons on the imide nitrogens ($-\mathrm{NH}-$). The ratio of these peaks in the spectrum of the mixture is approximately 3/2, the same as the ratio of products observed previously in the study of the appearance kinetics of the hyidrolysis products. Finally, a doublet is observed at δ 0.9 ppm in the ICRF-187 spectra which is assigned to the methyl group ($-\mathrm{CH_3}$) on the chiral carbon. In the mixture, a doublet of doublets is observed between δ 0.85 and δ 0.9 ppm, which corresponds to two methyl groups in slightly different environments. The observations in the ^{1}H NMR spectra support the monoacid structure of the primary hydrolysis products D1 and D2.

A comparison of the ^{13}C NMR spectra of ICRF-187 and the hydrolysis mixture also reveals distinct differences. The primary difference is that the spectrum for ICRF-187 has a single peak in the region of δ 12–13 ppm. This peak, δ 12.6 ppm, is assigned to the methyl group ($-\mathrm{CH_3}$) on the chiral carbon. The spectrum of the hydrolysis products has two peaks in this region, one at δ 11.9 ppm and the second at δ 12.9 ppm. This indicates two methyl groups in slightly different environments. The downfield areas of the spectra are also different. The spectrum of ICRF-187 has two peaks between δ 171 and δ 172 ppm that are assigned to the imide carbonyl carbons. The spectrum for the hydrolysis products has at least six peaks between δ 170 and δ 172 ppm that are attributed to the various carbonyl carbons in the monoacids.

The third hydrolysis product, D3, was also isolated. The chemical ionization mass spectrum of this sample exhibited a peak at *m/e* of 303. This peak represents a mass of M + 1 − 2, which corresponds to the M + 1 molecular ion, 305, of the diacid with loss of the two acid protons, to give a *m/e* of 303. This result suggests that the isolated product is the diacid hydrolysis product.

Although Burke *et al.* (25) made an assignment for the two degradation products, it would be interesting to speculate on the specific structures of D1 and D2 in this study. However, since the chromatographic conditions used by Burke *et al.* were sufficiently different from those in this study, no ion-pairing versus ion-pairing agent, we are not comfortable making the assignments at this time.

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