Qualitative Analysis of the Stability of the Oxazine Ring of Various Benzoxazine and Pyridooxazine Derivatives with Proton Nuclear Magnetic Resonance Spectroscopy

GERARD P. MOLONEY, DAVID J. CRAIK[x], AND MAGDY N. ISKANDER

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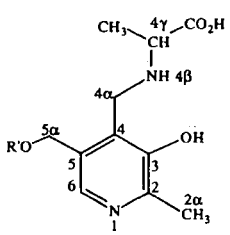
Abstract □ A series of 3,4-dihydro-1,3-benzoxazine and 3,4-dihydro-1,3-pyridooxazine derivatives was synthesized, and the hydrolysis of the derivatives was studied with proton nuclear magnetic resonance spectroscopy. The oxazine derivatives underwent various degrees of hydrolysis when H_2O was added to dimethyl sulfoxide solutions of the compounds. The rates and extents of decomposition of the oxazine ring systems depended on the electronic effects of substituents within the molecules. Examination of the proton nuclear magnetic resonance spectra that were generated during decomposition of the oxazines and trends in stability of the oxazine derivatives suggest the formation of an intermediate in the hydrolysis mechanism.

Benzoxazines have long been recognized for their wide range of biological activities (from bactericides[1,2] and fungicides[3] to antitumor agents[4]), and recently, there have been reports of their uses as both herbicides[5,6] and microbiocides.[7,8] However, their mode of action has not been established. We have recently developed[9,10] and synthesized a series of oxazine-based compounds as potential inhibitors of the pyridoxal-5-phosphate-dependent enzymes γ-aminobutyric acid aminotransferase,[11] alanine racemase,[12] and dihydroxyphenylalanine decarboxylase.[13] Potential inhibitors of alanine racemase of the general structures I and II (see structures) were synthesized as transition-state analogues[12] for the racemization of L-alanine to D-alanine.

Results of a study[12] of the antibacterial activity and proton nuclear magnetic resonance (^{1}H NMR) spectra of the proposed inhibitors in aqueous solution suggested that the oxazine rings for series II were hydrolyzing according to eq 1 to release formaldehyde, a known antimicrobial agent.[14]

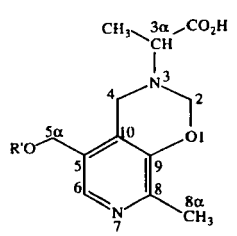
$$\mathbf{IIa} + H_2O \rightleftarrows \mathbf{Ia} + CH_2O \qquad (1)$$

This work led to the present study, involving the incorporation of oxazine rings for prodrugs and chemical delivery systems. The cyclic oxazine moiety may, in principle, be used in the delivery of drugs incorporating a γ-amino moiety (e.g., drugs of the *N*-aryl-*o*-hydroxybenzylamine type, which are useful anti-inflammatory agents).[15] In this approach, the masking of the phenolic oxygen and the amino group with formaldehyde would have the advantage of increasing lipophilicity, which would assist, for example, dermal absorption. This labile system could also be used in the delivery of various carbonyl drugs.[16–19]



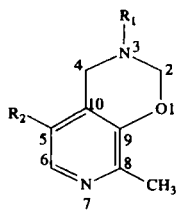
I(a-d)

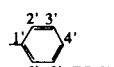
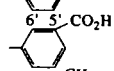
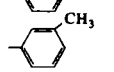
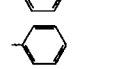
Ia (4γ-R), R' = H,
Ib (4γ-R), R' = PO_3H
Ic (4γ-S), R' = H
Id (4γ-S), R' = PO_3H



II(a-d)

IIa (3α-R), R' =H
IIb (3α-R), R' = PO_3H
IIc (3α-S), R' = H
IId (3α-S), R' = PO_3H



Compound Number	R_1	R_2
1	CH_3-CH-CO_2H	CH_2OH
2	CH_3-CH-CH_3	CH_2OH
3		CH_2OH
4		CH_2OH
5		CH_2OH
6		CH_3

To explore the potential of oxazines as both prodrugs and chemical delivery systems, information is needed about the structural factors that influence the stability and reactivity of oxazines. A diverse range of oxazine-based compounds (1–22; see structures) has been synthesized, and examination of substituent effects on the stability of the oxazine ring system was done with ^{1}H NMR spectroscopy.

Experimental Section

^{1}H NMR Spectra—^{1}H NMR spectra were recorded in $(CD_3)_2SO$ or $(CD_3)_2SO$–D_2O solutions, as outlined in the next section on stability studies, with a Bruker AM-300, wide-bore spectrometer operating at 300.13 MHz. Chemical shifts were expressed in parts per million relative to tetramethylsilane but were measured relative to the solvent peak at 2.49 ppm. All spectra were recorded in 5-mm tubes at 300 K. The conditions for Fourier transform measurements were as follows: spectral width, 4500 Hz; pulse width, 3.0 μs (30° flip angle); acquisition time, 1.8 s; repetition time, 2.0 s; number of data points 16 384; and number of transients, 32.

All oxazine compounds (1–22) were synthesized by the general procedure shown in Scheme I.

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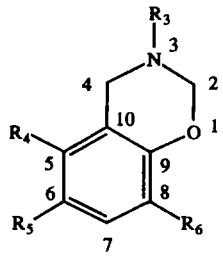
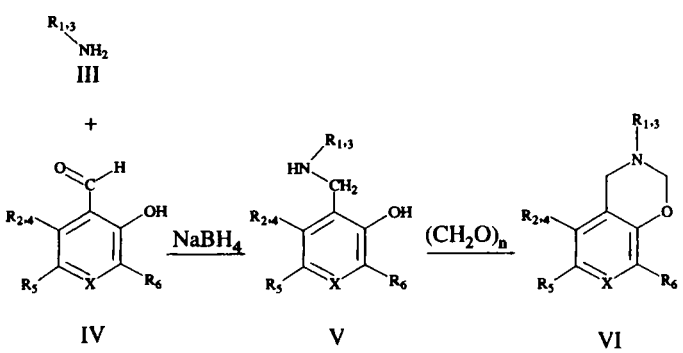


Compound Number	R_3	R_4	R_5	R_6
7	CH_3-CH-CO_2H	H	H	H
8	CH_3-CH-CH_3	H	H	H
9	CH_3-CH-CH_3	CH_3	H	CH_3
10	CH_3-CH-CO_2H	CH_3	H	CH_3
11		H	H	H
12	m-$CH_3C_6H_4$	H	H	H
13	m-$CO_2HC_6H_4$	H	H	H
14	p-$CH_3C_6H_4$	H	H	H
15	p-$OCH_3C_6H_4$	H	H	H
16	p-$NO_2C_6H_4$	H	H	H
17	p-CNC_6H_4	H	H	H
18	$C_6H_5CH_2$-	H	H	H
19	p-$N(CH_2CH_3)_2C_6H_4$	H	H	H
20	C_6H_5	H	OCH_3	H
21	C_6H_5	H	NO_2	H
22	p-BrC_6H_4	H	Br	H

A solution of an amine (III; 10 mM) in dry methanol (35 mL) was added in a dropwise manner to a solution of an aldehyde (IV; 10 mM) in dry methanol (30 mL). After 30 min of continuous stirring, the yellow solution obtained was treated with sodium borohydride (10 mM) at 5 °C (added in portions). The reaction mixture was acidified with glacial acetic acid to pH 5 and then concentrated under reduced pressure. The residue (V) obtained was recrystallized from ethanol–water. To a suspension of paraformaldehyde (10 mM) in dry methanol (50 mL) was added potassium hydroxide (20 mg) to dissolve the paraformaldehyde. The secondary amine V (5 mM) was then added, and the reaction mixture was refluxed. After 2 h, the reaction mixture was cooled and then concentrated under reduced pressure. The resultant residue (VI) was recrystallized from dry ethanol.

^{1}H NMR Stability Studies—Solubility problems were encountered with many of the compounds in D_2O, especially the *N*-phenylbenzoxazine derivatives. However, those that dissolved underwent spontaneous hydrolysis in D_2O to produce formaldehyde and the corresponding secondary amine compounds. Therefore, $(CD_3)_2SO$ was chosen as the solvent to study the relative stability of the oxazine rings of the different compounds. This choice allowed the amount of water added to be controlled. All stability experiments were performed in $(CD_3)_2SO$ obtained from freshly opened ampules. Reaction mixtures were kept at 300 K during the course of the experiments.



Scheme I

Oxazine solutions (0.03–0.05 M) in $(CD_3)_2SO$ were prepared, and the resulting ^{1}H NMR spectra indicated that the oxazine rings were stable in this solvent. Preliminary experiments were then carried out in which increasing aliquots of D_2O were added. Examination of the spectra after each addition showed a gradual decrease in the intensity of peaks from the oxazine derivative, with a proportional increase in peak intensity from the corresponding secondary amine derivative and formaldehyde. Control of the extent of oxazine hydrolysis was achieved by addition of controlled amounts of D_2O to the oxazine–$(CD_3)_2SO$ solutions. Eventually, the conditions were such that approximately equal intensities of peaks from both the oxazine starting material and the corresponding secondary amine could be observed. Once these conditions were reached, another oxazine–$(CD_3)_2SO$ solution was prepared, and the previously determined amount of D_2O required to achieve ~50% decomposition was added. ^{1}H NMR spectra were then recorded at various intervals over several days. The degree of hydrolysis of the oxazine ring was determined by measuring the relative intensities of peaks from the oxazine derivative and the decomposition products (i.e., formaldehyde and the secondary amine derivative). From these ^{1}H NMR stability studies, qualitative equilibrium constants were calculated and used to compare substituent effects on oxazine hydrolysis.

The equilibrium constant (K_e) was calculated according to eq 2, which is readily derived from eq 1:

$$K_e = \frac{S_I S_I}{S_{II}(S_{II} + S_I)} \cdot \frac{[II]_i}{[D_2O]_i} \qquad (2)$$

In eq 2, S_I and S_{II} are the equilibrium signal intensities of the oxazine (II) and open compounds (I), respectively, $[II]_i$ is the initial concentration of the oxazine, and $[D_2O]_i$ is the added concentration of D_2O.

To further compare relative stabilities, a second experiment was carried out in which a fixed amount of D_2O was added to each compound. In this experiment, a solution of each oxazine derivative (0.04 M) in $(CD_3)_2SO$ was prepared [2.0×10^{-5} mol of each oxazine dissolved in dry $(CD_3)_2SO$, 500 μL], 280 μL of D_2O was added to the solution, and a series of ^{1}H NMR spectra was recorded at various intervals over several days. Most oxazine hydrolysis occurred in the first hour so that several spectra were initially recorded at 3–5-min intervals. Spectra were then recorded after several hours and, finally, after several days. The hydrolysis of the oxazine rings was followed by comparing the relative intensities of peaks from the benzoxazines and pyridooxazines to their corresponding "open" secondary amine derivatives. The percentage of breakdown of the oxazine rings was plotted against time.

Results and Discussion

A series of spectra showing the progressive breakdown of the oxazine ring for the hydrolysis of *p*-(3,4-dihydro-1,3-benzoxazin-3-yl)toluene (14; Figure 1) revealed peaks corresponding to the open secondary amine derivative (14′) and formaldehyde and their gradual increase in intensity (peaks corresponding to the benzoxazine showed a proportional decrease in intensity). A spectrum of the pure secondary amine derivative *p*-*N*-(*o*-hydroxybenzyl)toluidine (14′) is shown in Figure 1 to clarify the assignment of the new peaks.

As evidenced by the K_e values (Table I), the structure of the oxazine derivative has a pronounced effect on the degree of hydrolysis of the oxazine ring. K_e values vary by more than three orders of magnitude from 0.1×10^{-4} to 124.7×10^{-4}, with 9 being the most stable and 17 the least stable under the conditions used. The compounds may be broadly divided into those containing aliphatic substituents (R_1) on the oxazine nitrogen and those containing aromatic substituents.

Aliphatic R_1 Substituents—Because 9 is the most stable, it provides a convenient starting point for analysis of structural and electronic effects on the stability of the oxazine ring.

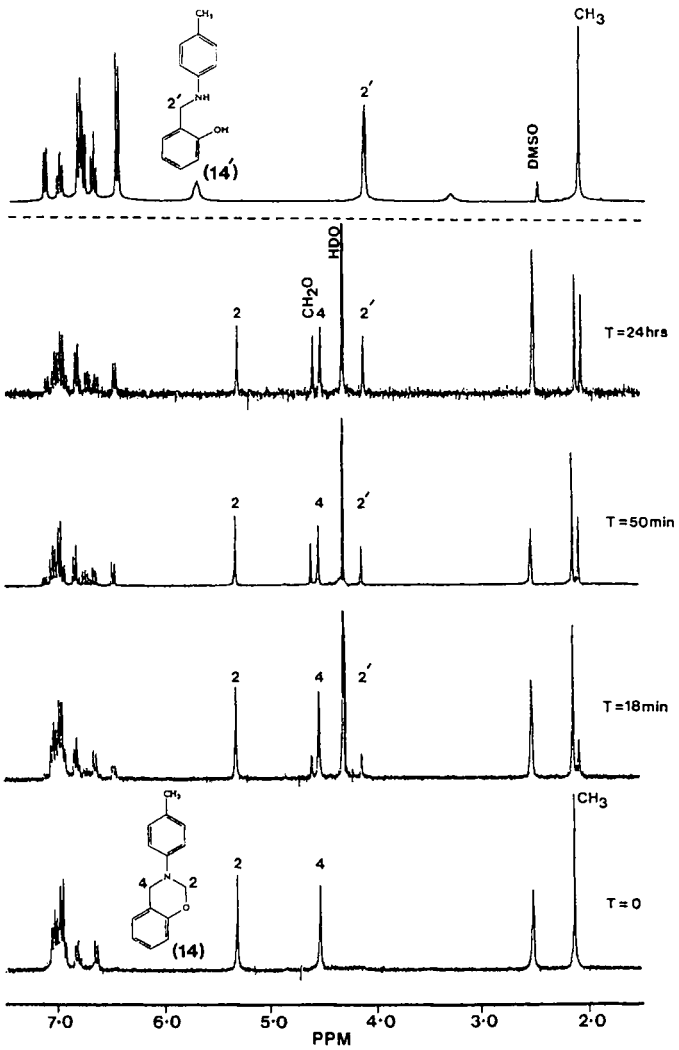


Figure 1—^{1}H NMR spectra recorded at various time intervals after the addition of 280 μL of D_2O to a 0.04 M solution in $(CD_3)_2SO$. The spectrum of the corresponding open secondary amine derivative (**14'**) in $(CD_3)_2SO$, seen in the top trace, is also included to assist spectral assignment. Time intervals of the spectra recording are indicated.

Table I—Values of K_e[a] for Oxazine Ring Opening

Compound	$K_e \times 10^4$	Compound	$K_e \times 10^4$
1	10.5	**12**	10.7
2	12.5	**13**	20.7
3	15.0	**14**	6.4
4	24.5	**15**	4.0
5	6.3	**16**	31.1
6	0.3	**17**	124.7
7	18.0	**18**	0.6
8	1.9	**19**	6.2
9	0.1	**20**	11.7
10	31.8	**21**	101.1
11	11.6	**22**	85.2

[a] Calculated with eq 2 based on equilibrium peak heights in ^{1}H NMR spectra; all values are averages of at least two concordant determinations.

Compound **10** differs only in the replacement of a methyl of the isopropyl substituent by a carboxyl to form an alanyl R_1 group. However, this change produces a nearly 400-fold decrease in K_e. Because the two R_1 groups do not interfere sterically with any other part of the molecule, steric effects do not account for this change; rather, it is the electron-withdrawing effect of the carboxyl function that most likely promotes breakdown of the oxazine ring. The carboxyl group presumably results in a more electron-deficient oxazine ring, which would thus be more prone to nucleophilic attack from water.

Similarly, **7** is less stable than **8**, although in this case the difference is only 10-fold. By contrast, the same change in substituents in **1** and **2** produces no significant change in stability. It appears that electron withdrawal is less important in the pyridooxazine series than in the benzoxazine compounds.

The rates of approach to equilibrium for the compounds discussed so far are indicated in Figure 2, which shows the degree of breakdown of the oxazine derivatives as a function of time after addition of a fixed amount of D_2O. Most derivatives exhibit the expected exponential approach to equilibrium. However, **9** shows a rapid initial breakdown followed by an apparent recovery of the closed form. This behavior is probably due to solubility problems associated with this compound in the presence of D_2O. As D_2O was added to the solution of oxazine (**9**) in $(CD_3)_2SO$, the NMR reaction mixture became extremely turbid because of partial precipitation of the oxazine derivative. (The same results were obtained with several of the oxazine compounds having a phenyl group at position 3.) In all cases, however, the oxazine compounds eventually dissolved to give clear solutions for NMR measurements at equilibrium.

Another oxazine derivative (**18**) incorporating a benzyl substituent at position 3 was also tested. The results indicate that the electron-donating benzyl group stabilizes the oxazine ring to hydrolysis to a greater extent than does a phenyl substituent.

Aromatic R_1 Substituents—All the remaining compounds have an aromatic substituent at R_1. Examination of K_e values in Table I shows that there are markedly different degrees of oxazine stability resulting from substituent variation in both

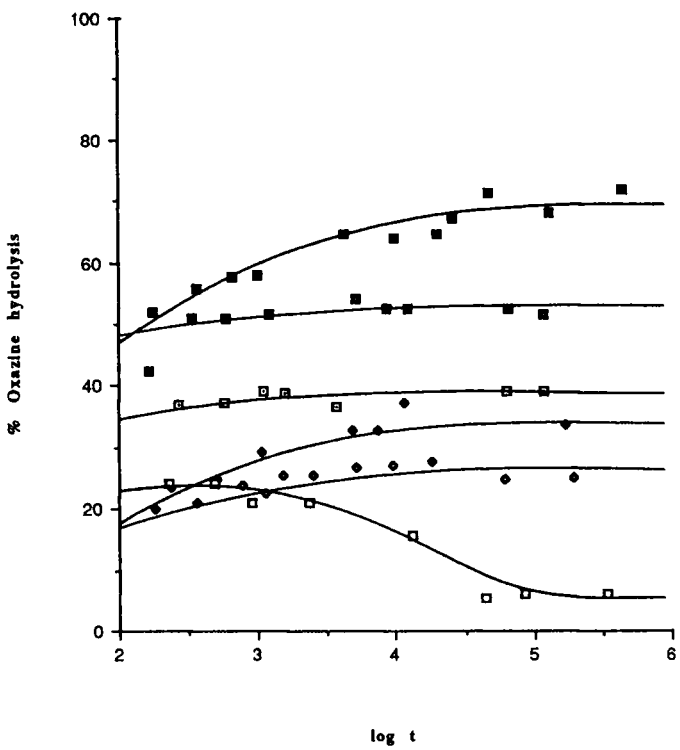


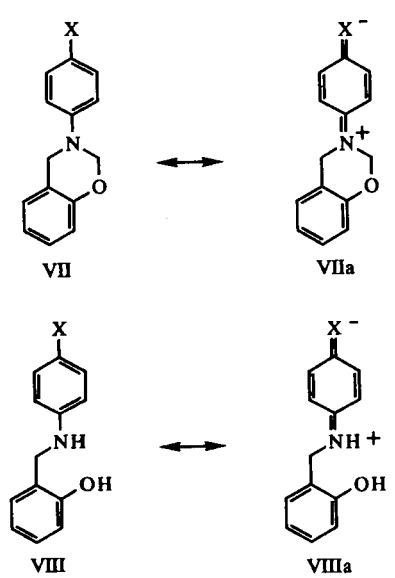
Figure 2—Plot of percentage of oxazine hydrolysis versus log time (s) for the pyridooxazines (⊡) **1** and (◆) **2** and the benzoxazine pairs (□) **7** and (◇) **8** and (□) **9** and (■) **10**.

the R_1 phenyl substituent and the fused benzene ring. Trends in oxazine ring stability can be explained in terms of the electronic nature of substituents incorporated within the benzoxazine molecule. Changes in substituents in the pyridine ring of the pyridooxazine also result in marked changes in oxazine ring stability.

Substituent Variation at Position 4'—Comparisons were made between the relative stabilities of benzoxazines with either electron-donating or electron-withdrawing substituents at position 4' of the phenyl ring on the oxazine nitrogen. Compounds **16** and **17** are two of the least stable oxazine derivatives, and it appears that the electron-withdrawing nature of the nitro and cyano substituents enhances the overall hydrolysis of the oxazines to the corresponding secondary amine compounds. (This result agrees with the breakdown trends observed for derivatives **8** and **9** relative to the less stable benzoxazine derivatives **7** and **10**, which have an electron-withdrawing carboxyl substituent in the aliphatic R_1 moiety.) The benzoxazine derivatives **11**, **14**, **15**, and **19**, which have proton, methyl, methoxy, and diethylamino substituents at position 4', respectively, all exhibited increased resistance to oxazine ring hydrolysis as a result of the neutral or electron-donating substituent.

The relative stabilization of the 4-X-phenyl benzoxazines by electron-withdrawing X groups can be explained in terms of delocalization of the basic pair of electrons on the oxazine nitrogen. Such delocalization is favored by electron-withdrawing X groups (**VII**↔**VIIa**; Scheme II). The observed trend in K_{eq} can be explained if the degree of stabilization brought about by this delocalization is greater in the open secondary amines (**VIII**↔**VIIIa**; Scheme II) than it is in the closed oxazines (**VII**↔**VIIa**). The oxygen atom of the oxazine ring is responsible for the reduced importance of this electron delocalization in the closed, relative to the open, compounds.

Evidence for an enhanced contribution of the protonated resonance forms (**VIIa** and **VIIIa**) for electron-withdrawing substituents is obtained from carbon-13 chemical shifts reported earlier.[19] For X substituents at C-4', acceptors produce upfield shifts and donors produce downfield shifts at both the C-2 and C-4 positions in the oxazine ring. In other series, protonation of a nitrogen usually produces an upfield shift at the adjacent carbon-13 site.[20] Therefore, by analogy, acceptor substituents induce a greater preference for the protonated resonance form (**VIIa**; Scheme II). There is likely to be decreased electron density at C-2 and C-4 in the presence of acceptor X substituents because of the increased preference for the protonated nitrogen. Although the upfield shift for acceptors appears at first to contradict this expectation, evidence from other series[20] again shows that, for sites adjacent to nitrogen, decreased electron density may produce an upfield shift.



Scheme II

Figure 3 illustrates the time course of breakdown of the benzoxazine systems **11**, **14**, **15**, **16**, and **17**. Because of the logarithmic time axis, the exponential nature of the breakdown is somewhat distorted. Although the *p*-NO_2 and *p*-CN compounds were the least stable and thus had the greatest percentage of hydrolysis at equilibrium, they initially decomposed at a slower rate than did the other benzoxazines.

Substituent Variation at Position 3'—A series of benzoxazine compounds (**11**–**13**) and pyridooxazine derivatives (**3**–**5**) incorporating substituents of different electronic effect at position 3' were also examined. Although a diminished electronic effect would be expected for substituents *meta* to the oxazine nitrogen, the benzoxazine compound **13** with a carboxyl group at position 3' underwent hydrolysis to a greater extent than did the corresponding oxazine derivatives **11** and **12** with a hydrogen atom and a methyl group at position 3', respectively. A similar trend also was observed for the pyridooxazine derivatives **3**–**5**. The electron-withdrawing character of the carboxyl substituent *meta* to the oxazine nitrogen renders the oxazine ring more liable to hydrolysis; this fact is in agreement with the pattern of stability of the benzoxazines incorporating substituents at position 4'.

Substituent Variation at Position 6—The two benzoxazines **20** and **21** differ only in their substituents at position 6 of the fused phenyl ring. The K_e values indicate that an electron-donating methoxyl substituent at position 6 stabilizes the oxazine ring to a greater extent than does a nitro substituent.

Comparison of Pyridooxazines 3 and 6—(5,8-Dimethyl-3,4-dihydropyrido[4,3-*e*]-1,3-oxazine-3-yl)benzene (**6**) differs from the pyridooxazine derivative **3** only in the incorporation at

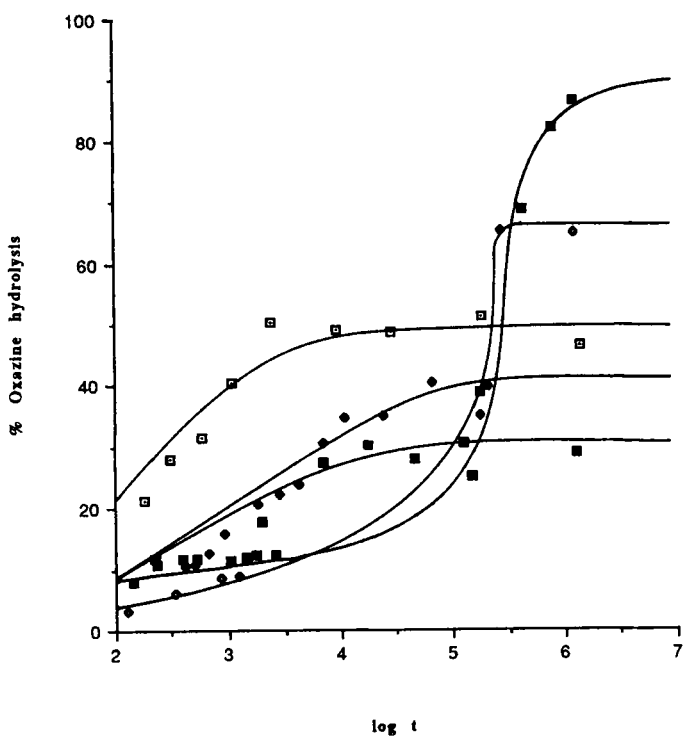


Figure 3—Plot of percentage of oxazine hydrolysis versus log time (s) for the benzoxazines (⊡) **11**, (◆) **14**, (□) **15**, (◇) **16**, and (■) **17**.

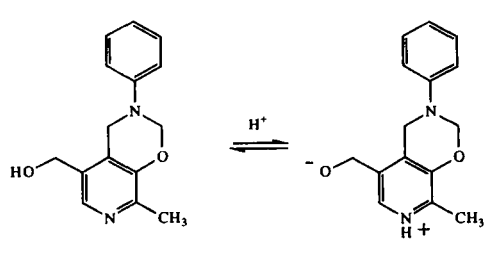
position 5 of a methyl group instead of a hydroxymethyl function. The introduction of a methyl group enhances the stability of the oxazine ring (Table I). This phenomenon can be explained in terms of the characteristics of the pyridine ring. The hydroxyl proton of the hydroxymethyl group at position 5 is acidic and can protonate the pyridine nitrogen (Scheme III). Thus, an electron sink is created and electron density is drawn from the oxazine ring. As a result, the ring system of the pyridooxazine 3 is more prone to nucleophilic attack.

Mechanism of Oxazine Hydrolysis—These results indicate that an electron-rich oxazine ring system is more stable than one that is electron deficient. In principle, trends in the kinetics of oxazine breakdown as a function of the electronic nature of substituents allow postulations to be made regarding the mechanism of hydrolysis. In the current case, however, a quantitative analysis of the reaction kinetics was not attempted because solubility variations were encountered for different compounds. The qualitative profiles (Figure 3), however, suggest that a somewhat complicated multistep mechanism may be involved.

Previous work by Fife and co-workers[21,22] and by Bundgaard and Johansen[23] on the mechanism of hydrolysis of various oxazolidines indicates that the formation of a Schiff base intermediate is involved in oxazolidine hydrolysis. A similar mechanism may be involved in the hydrolysis of the pyridooxazines and benzoxazines investigated in this study. A possible mechanism for oxazine breakdown through a cationic Schiff base intermediate is illustrated in Scheme IV.

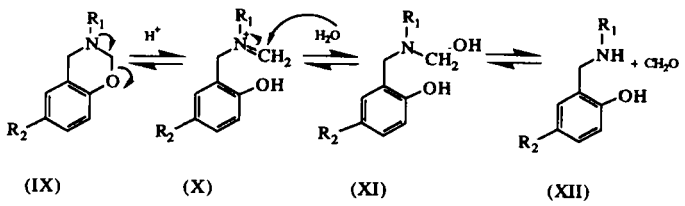
If the hydrolysis mechanism for the oxazine ring of the benzoxazine and pyridooxazine derivatives is as proposed, then resonances corresponding to the protons of either intermediate should be detectable in the ^1H NMR spectra. In most cases, only peaks pertaining to the protons of the oxazine and corresponding open, secondary amine structure could be detected during the ^1H NMR experiments; however, spectra of the pyridooxazine derivatives 3–5 also contained peaks corresponding to an intermediate involved in oxazine hydrolysis.

Spectra of the pyridooxazine 5 (Figure 4) have peaks that were easily assigned to both the parent oxazine and the corresponding secondary amine derivative after comparison with spectra of the pure derivatives in $(CD_3)_2SO$–D_2O mixtures. A third set of low-intensity peaks was also detected and is indicated. These peaks probably correspond to the primary alcohol intermediate (XI; Scheme IV). This proposal is based on the expected low stability of a Schiff base intermediate. In addition, the results of ^1H NMR stability studies on oxazolidine systems indicate that the Schiff base intermediates of these compounds are far less stable than the corresponding alkylhydroxy hydrolysis products (unpublished results). The chemical shifts for the third compound observed in the decomposition of 3–5 are not consistent with the stable existence of a cationic Schiff base. For example, the chemical shift of the singlet expected for the methylene CH_2-2α would be expected to be further upfield, because it is connected to an



3 3'

Scheme III



(IX) (X) (XI) (XII)

Scheme IV

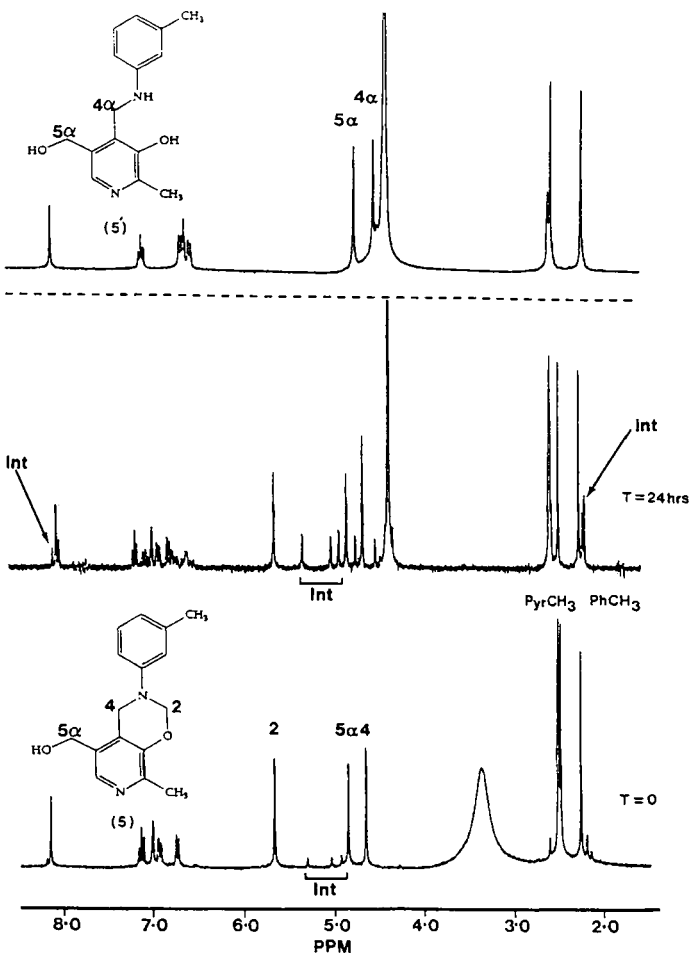


Figure 4—^1H NMR spectra of the pyridooxazine **5** and its corresponding open secondary amine derivative **5'**. The spectrum recorded at T = 24 h (middle panel) has peaks corresponding to the pyridooxazine **5**, the open derivative **5'**, and a third intermediate (designated "Int" and indicated by the arrows and bracket).

imine-type nitrogen. An upfield shift of the aromatic protons connected to the imine nitrogen would also be expected. The proton shifts do, however, correspond to expectations for the protons of the primary alcohol intermediate (XI).

The singlet corresponding to monomeric formaldehyde appears under the large HDO peak centered at 4.30 ppm. The pyridooxazine derivatives 3 and 5 were especially sensitive to initial oxazine hydrolysis, because when the compounds were dissolved in $(CD_3)_2SO$ from freshly opened ampules, peaks corresponding to an intermediate appeared, with oxazine ring hydrolysis resulting from residual moisture in the solvent. This phenomenon is seen in the lower spectrum in Figure 4. Addition of increasing amounts of D_2O resulted in the appearance of peaks corresponding to the open secondary amine derivative as further hydrolysis of the oxazine, and corresponding breakdown, occurred.

The formation of the Schiff base intermediate appears probable, on the basis that Buur and Bungaard[24] found that the cyclic compounds obtained from γ-aminoalcohols and ketones behave hydrolytically as the corresponding oxazolidines. However, the inherent instability of the compounds results in spontaneous hydrolysis to the primary alcohol derivative and the secondary amine compound. Thus, although the Schiff base intermediate is formed, further hydrolysis to the primary alcohol occurs too quickly for the compound to be detected by ^{1}H NMR methods.

Conclusions

The stability of oxazines under the conditions studied is markedly influenced by the electronic nature of substituents on both the phenyl group in position 3 and the fused benzene ring. Oxazine ring stability is enhanced by the inclusion of electron-donating substituents at position 3' and decreased by electron-withdrawing substituents at the same position. The inclusion of an alanyl substituent instead of an isopropyl group at position 3 in either a pyridooxazine or a benzoxazine derivative destabilized the oxazine ring.

References and Notes

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