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STUDIES ON THE PYRROLIZIDINE ANTITUMOR AGENT, CLAZAMYCIN: INTERCONVERSION OF CLAZAMYCINS A AND B[1]

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ABSTRACT.—Clazamycin, a novel pyrrolizidine antitumor antibiotic, exists in aqueous solution as a mixture of two epimers, clazamycins A and B [**1A, 1B**], the ratio of which is pH dependent. Several lines of evidence are presented, including the results of trapping experiments and a study demonstrating base promoted interconversion of the two forms, that implicate an azacyclooctenone species [**3**] as an intermediate in the interconversion process. This result supports a previous observation, that the C6a carbinolamidine hydroxyl of clazamycin is unreactive towards nucleophiles and may be significant in helping to elucidate the mechanism of action of this antibiotic.

Clazamycin [**1**] (Scheme 1) is a novel pyrrolizidine natural product, first isolated from *Streptomyces puniceus* in 1977 by Dolak and DeBoer (1-3), although an independent isolation was reported by Umezawa and co-workers in 1979 (4-5). Clazamycin has moderate in vivo activity towards L1210 cells, as well as antiviral activity against *Herpes simplex*, Types 1 and 2. It also possesses significant activity against several *Pseudomonas* species, particularly gentamycin resistant strains.

In addition to its biological activity, clazamycin has several other unique features. It is one of only two pyrrolizidines to have been isolated from microorganisms[3] (6), although many are found in plants (7), and is the only known pyrrolizidine to contain a chlorine substituent in the base portion. Clazamycin is also one of only two known examples of a carbinolamidine[4] (8). This is noteworthy as carbinolamine and related functionalities are important structural constituents of many potent, naturally occurring antitumor agents, such as the anthramycins (9) and saframycins (10), in which they are known to function as alkylators towards biological nucleophiles. Clazamycin is rich in functional groups for a molecule of its size and appears to contain several possible electrophilic sites. We recently reported the results of experiments concerning the reaction of clazamycin with model biological nucleophiles (11), demonstrating that sulfur and nitrogen nucleophiles react at the C1 position, via a novel Michael addition to the conjugated amidine system. Finally, in contrast to the well known hepatotoxicity of other pyrrolizidine natural products (12,13), clazamycin appears to show no evidence of toxic effects towards the liver, kidney, or spleen (3).

In aqueous solution clazamycin exists as a mixture of two diastereomers, clazamycins A and B [**1A, 1B**] (Scheme 1), epimeric at C6a, with the A isomer predominating (62%) at neutral pH. Dreiding models indicate the possibility of an unfavorable steric interaction between the C5 chlorine and C6a hydroxyl which are on the same face of the five-membered ring in the B isomer, perhaps accounting for the observed ratio.[5] The interconversion of the two isomers can occur through two possible mechanisms (Scheme

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[1]Taken in part from the M.S. Thesis of Douglas D. Buechter, University of Texas at Austin, 1986.

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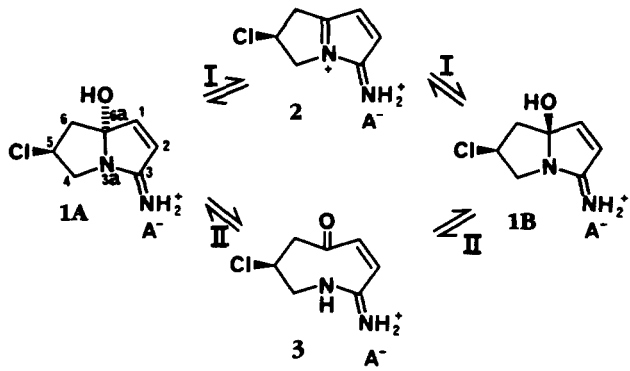
[3]Bohemamine is a pyrrolizidine natural product isolated from an *Actisporangium* species in 1980; however, it differs considerably in structure from clazamycin.

[4]2-Hydroxy-5-imino-1-azacyclopent-3-ene was isolated from *Streptoverticillium parvisporogenes* in 1979, and represents half of the clazamycin molecule (minus C4, C5 and C6).

[5]The authors thank a referee for this suggestion.



SCHEME 1

1): iminium ion [2] formation through loss of the C6a hydroxyl or ring opening to the azacyclooctenone [3]. The mechanism by which the two epimers interconvert has not been previously studied and, apart from its interesting mechanistic features, may be significant with respect to the biological activity of the two isomers. We describe here the results of investigations based upon ^{1}H nmr and optical rotation that implicate the azacyclooctenone species [3] as an intermediate in the interconversion process. A complete assignment of the ^{1}H nmr of clazamycin was required for these studies and is also described.

RESULTS AND DISCUSSION

The ^{1}H-nmr spectrum of clazamycin sulfate in D_2O was first described in a 1978 Upjohn patent (2); the resolution was sufficient to show the presence of the minor isomer (clazamycin B) as a multiplet at 2.5 ppm[6] (for the H6 protons) and as shoulders on the H1 and H2 olefinic signals (6.37 and 7.2 ppm) of the major isomer. Although not completely assigned, ^{1}H-nmr data for the hydrochloride of clazamycin were reported in a later publication of Horiuchi and co-workers (4), with sufficient resolution to allow signals for all protons of both the A and B isomers to be observed. We anticipated that ^{1}H-nmr would be a useful tool for investigating the interconversion of the two epimers, providing a means of determining the A:B ratio under various conditions or for perhaps directly observing the presence of an intermediate in the interconversion. In addition, a detailed assignment of the ^{1}H nmr (Figure 1), with the specific goal of as-

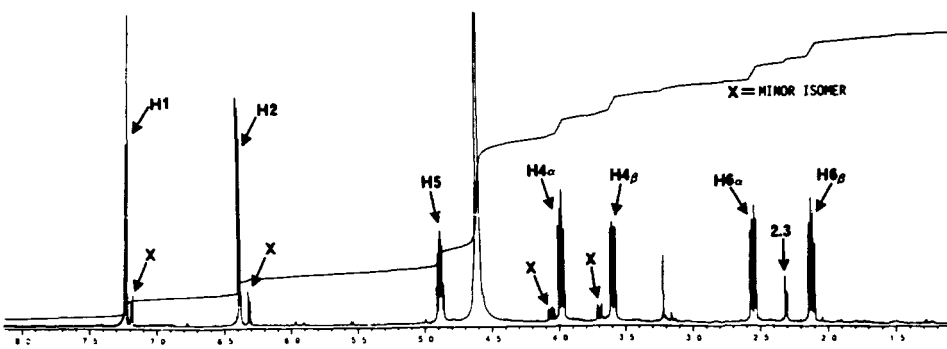


FIGURE 1. ^{1}H Nmr of clazamycin A and B sulfates in D_2O (360 MHz)

[6]The two H6 protons of clazamycin B sulfate are reported (2) to have a chemical shift of 2.5 ppm. However, in the spectrum shown here (Figure 1), these protons resonate at 2.3 ppm, with external reference to dioxane.

signing the α- or β-configuration to the H4 and H6 protons, was crucial for our studies on the reaction of clazamycin with nucleophiles.

It is known from the crystal structure of clazamycin A that the H5 proton is in the α-configuration, and Dreiding models indicate that this proton is closest in space to the neighboring α-H4 and α-H6 protons (Figure 2). ^{1}H-nmr experiments showed an nOe from the H5 proton into one H4 proton at 3.97 ppm and one H6 proton at 2.54 ppm, and on this basis these protons were assigned the α-configuration. Additional confirmation of these assignments was obtained by demonstrating an nOe from the other H6 proton at 2.12 ppm into the olefinic H1 proton at 7.2 ppm. It is clear from Dreiding models that this H6 proton has to be in the β-configuration in order to be in close proximity to H1. These experiments allowed us to assign the configuration of the H4 and H6 protons of the major isomer (clazamycin A) and the H4 protons of the minor isomer (clazamycin B). Additionally, cosy and hetcor experiments were performed which clearly supported the assignment (4) of the multiplet at 2.3 ppm to the two H6 protons of clazamycin B. Although it has not been possible to separate these signals in D_2O even at 500 MHz, they do separate into the expected pair of doublet of doublets (2.35 and 2.50 ppm) in CD_3OD at 360 MHz.

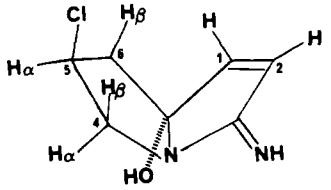


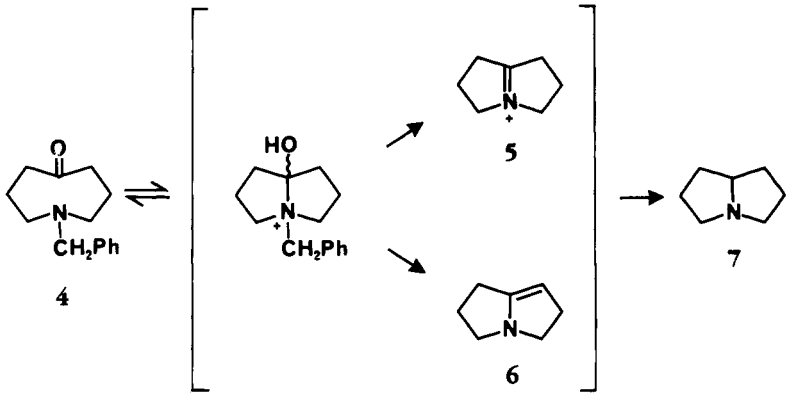
FIGURE 2. Configurational structure of clazamycin A [**1A**]

On a reverse phase C-18 hplc column (Waters, μ-Bondapak) using sodium phosphate buffer (pH 7.4)/CH_3CN (70:30) as mobile phase (1 ml/min), clazamycins A and B can be resolved into two peaks with retention times of 14.4 and 13.6 min, respectively. However, numerous attempts to isolate the individual isomers were unsuccessful, as epimerization occurred readily under these conditions. The two possible mechanisms that could account for this interconversion are outlined in Scheme 1. Pathway I involves formation of the iminium species **2**, which could occur by expulsion of hydroxide ion or, after initial protonation, a molecule of H_2O. In pathway II, the electrons on the C6a hydroxyl oxygen move inward towards C6a, forming a carbonyl with concomitant heterolytic cleavage of the C6a-N3a bond. The resultant azacyclooctenone [**3**] may then close by the reverse process to give a mixture of the A and B epimers. An S_N2-type mechanism involving direct displacement of the carbinolamidine hydroxyl via direct attack of hydroxide ion or H_2O at the C6a position has also been considered. However, this mechanism appears unlikely as the C6a hydroxyl is tertiary, and other nucleophiles such as MeOH do not appear to react in this manner (see below). Although the ^{1}H nmr of clazamycin has been studied in DMSO-d_6, CD_3OD, and in D_2O at several different pHs, we have never detected the presence of the iminium ion or the azacyclooctenone.[7] Only signals corresponding to the A and B epimers have been observed, suggesting that the intermediate or intermediates are too transient to be observed on the nmr time scale. ^{1}H nmr was, however, useful in providing a convenient

[7] ^{13}C-nmr data for clazamycins A and B were reported by Horiuchi and co-workers in 1979 (4). Both isomers may be distinguished by ^{13}C nmr, although as noted in the text, an intermediate in the conversion of the A and B forms has never been observed.

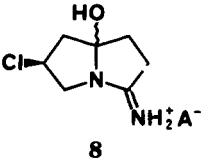
method (integration of the respective olefinic signals) for determining the ratio of the two epimers under different conditions.

Several lines of evidence suggest that the interconversion occurs through the azacyclooctenone intermediate **3**, as shown in Scheme 1. First, the pK_a of clazamycin is approximately 13,[8] implying that at neutral pH the amidine moiety should be protonated with the charge delocalized throughout the amidine system. This should limit the availability of the N3a electron pair to participate in formation of a double bond between N3a and C6a, as required for iminium ion formation. Second, Leonard and Sato (14) have postulated the intermediacy of the iminium ion **5** or enamine **6** in the catalytic hydrogenation of the *N*-benzyl substituted azacyclooctanone **4** to the parent saturated pyrrolizidine **7** (Scheme 2). However, in our hands, catalytic hydrogenation of



SCHEME 2

clazamycin leads to the previously reported (4) dihydroclazamycin [**8**] in nearly quantitative yield, suggesting that in the case of clazamycin, the iminium ion does not readily form. Third, if the iminium ion is involved in the interconversion of the two isomers, it should be possible to trap it as, for example, the carbinolamidine methyl ether or to label the C6a position with ^{18}O. Experiments of this type were performed by allowing the epimers to equilibrate in the presence of either MeOH or $H_2{}^{18}O$ at high pH (11.2), since we had already established that the interconversion of clazamycins A and B is rapid at this pH (see below). Equilibration in $H_2{}^{18}O$ (50% isotopic purity) led to 15% ^{18}O incorporation, as determined by ms. While this does not necessarily rule out iminium ion formation, it is also consistent with formation and subsequent hydration (15) of the azacyclooctenone. Additionally, no trace of the carbinolamidine methyl ether could be observed by either ^1H nmr or ms after equilibration of the A and B epimers in MeOH. However, clazamycin does react with MeOH under these conditions, leading to loss of the C1-C2 double bond. Ms and nmr data of this product are consistent with reaction at C1 via 1,4-addition to the conjugated amidine system. We have previously shown (11) that clazamycin undergoes this reaction with several sulfur- and nitrogen-containing nucleophiles.



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[8]L.A. Dolak, personal communication, 1985.

Fourth and most significantly, information was obtained by investigating the effect of pH on the rate of interconversion of the two epimers. Base promotion would be expected to support the intermediacy of the azacyclooctenone, as deprotonation of the C6a hydroxyl should facilitate carbonyl formation and subsequent ring opening. Conversely, acidic conditions should favor protonation of the hydroxyl, leading to the expulsion of H_2O and subsequent iminium ion formation. To test this, clazamycin was dissolved in 50mM sodium phosphate buffers at several pHs (11.04, 7.91, 7.00, 5.65, and 2.80), followed by immediate monitoring of the change in optical rotation with time (Figure 3). When the rotation reached a constant value, an aliquot of each solution (2 ml) was lyophilized, stored at −78°, redissolved in exactly 2 ml of D_2O, and the final

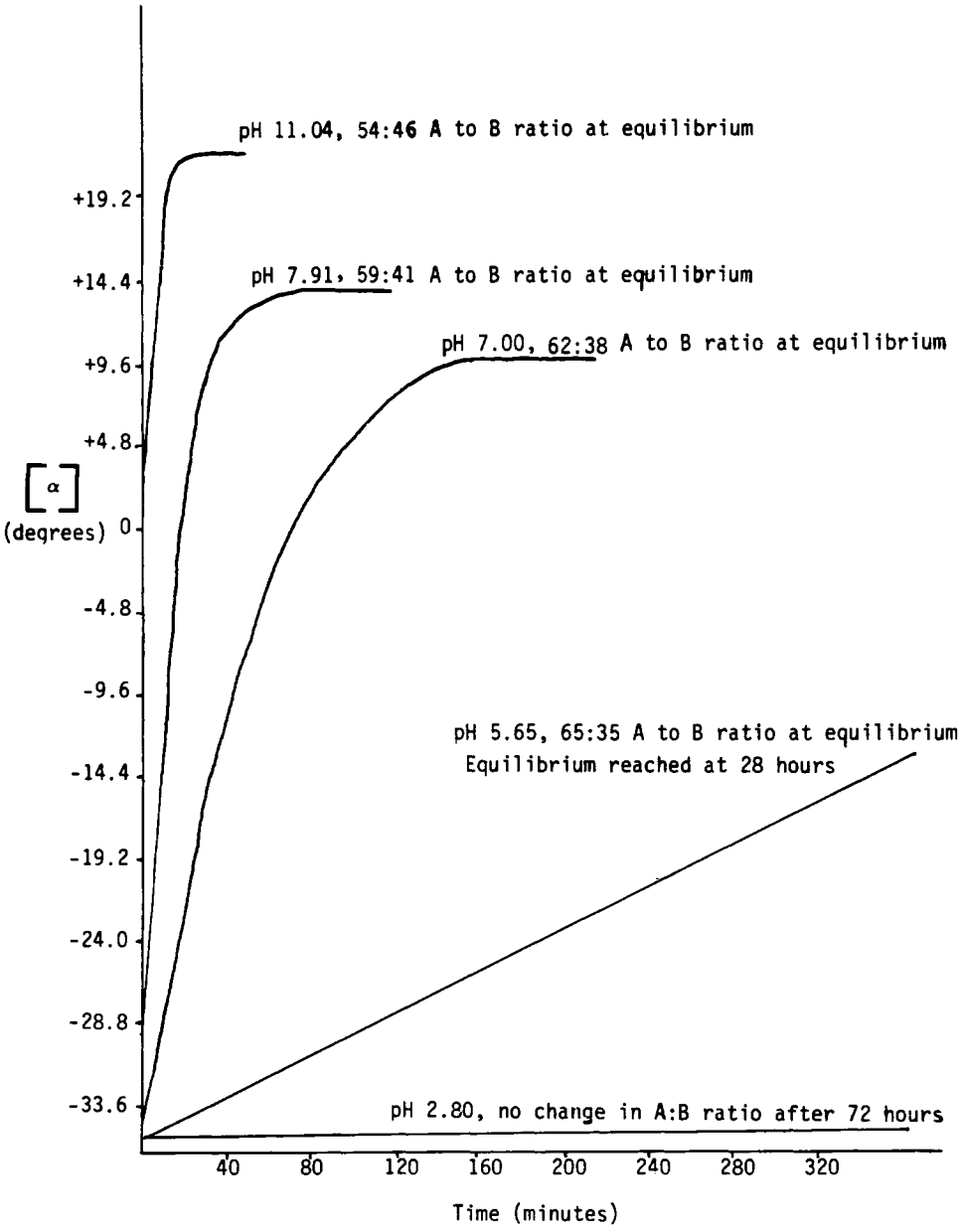

FIGURE 3. Optical rotation vs. time for clazamycin A and B sulfates at different pH values

ratio of the A and B isomers determined by ^{1}H nmr. As the contribution to the optical rotation from each of the two diastereomers is additive, the determination at two different pHs of both the optical rotation and the ratio of the two isomers at equilibrium allowed calculation of the specific rotation of each individual isomer, as well as the concentration of each isomer at any particular time point. These calculations gave $[\alpha]^{25}$ 579.1 (*c* 1.0, H_2O) values of $-44°$ and $+100°$ for clazamycins A and B, respectively.[9]

Based upon our results, the interconversion of the A and B isomers can be represented by the following model:

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B$$

where k_1 and k_2 are first-order rate constants for the interconversion. At any specific time (t) the change in the concentration of A ([A]) is given by:

$$\frac{d[A]}{dt} = -k_1[A] + k_2[B]$$

By appropriate substitution and integration, the following equation relating the concentration of the A isomer and time may be obtained:

$$\ln([A]_t - [A]_{eq}) = \ln([A]_0 - [A]_{eq}) - (k_1 + k_2)t$$

where $[A]_t$, $[A]_{eq}$ and $[A]_0$ are the concentrations of A at time=t, at equilibrium and at time=0, respectively. A plot of $\ln([A]_t - [A]_{eq})$ vs. time will then give a straight line of slope $-(k_1 + k_2)$.

Shown in Table 1 are k_1 and k_2 values obtained by drawing such plots for each pH value, and several features are apparent. First, k_2 is greater than k_1 (the A isomer predominates) for all pH values investigated. Second, both k_1 and k_2 increase with pH but not at the same rate as would be expected for true base catalysis (neither k_1 nor k_2 shows a simple linear or log-linear dependence upon pH). Third, as the pH is increased, k_1 increases at a faster rate than k_2, causing the concentration of the B isomer at equilibrium to increase. It is not known at this time why the ratio of the two isomers changes with pH, although it is possible that hydrogen bonding is disrupted in the A isomer as a re-

TABLE 1. Forward (k_1) and Reverse (k_2) Rate Constants for the Interconversion of the A and B Epimers of Clazamycin:

$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B$$

	pH			
	5.65	7.00	7.91	11.04
k_1(min.$^{-1}$)	8.86×10^{-4}	1.03×10^{-2}	3.17×10^{-2}	1.27×10^{-1}
k_2(min.$^{-1}$)	1.61×10^{-3}	1.72×10^{-2}	4.67×10^{-2}	1.52×10^{-1}

[9]Horiuchi and co-workers have reported (4) separation of clazamycin A and B hydrochlorides by chromatography on Amberlite XAD-2 resin, using 0.5N HCl as eluent. Optical rotation values of $[\alpha]^{25}D-56°$ and $[\alpha]^{24}D+96°$ (*c* 1.0, H_2O) were noted for the A and B forms, although it is not clear whether separation was complete.

[10]The interconversion might also be a function of the concentration of the unprotonated species which, although present in only low concentration over the pH ranges investigated (e.g., 0.0001% at pH 7.0), should increase towards 50% as the pH approaches the pKa value of 13.

sult of the pH increase.[10] However, it is clear that the interconversion is base promoted with both k_1 and k_2 increasing with pH.

All of the above results are consistent with participation of the azacyclooctenone species and not the iminium ion in the interconversion of clazamycins A and B. Assuming that S_N2-type attack by a nucleophile at C6a is unlikely, then the only other reasonable pathway for nucleophilic substitution at C6a would involve the iminium ion. However, the fact that iminium ion formation does not appear to occur, at least under the conditions of these studies, suggests that in contrast to other carbinolamine-containing natural products such as the anthramycins, the carbinolamine moiety of clazamycin is relatively inert towards biological nucleophiles. This result supports and complements our previous observation (11) that the C1 position of clazamycin is the exclusive reactive site (at least in vitro) towards nucleophiles.

EXPERIMENTAL

CLAZAMYCINS A [**1A**] AND B [**1B**].—A mixture of clazamycins A and B, obtained via fermentation, was kindly supplied by Dr. Lester Dolak of the Upjohn Company, Kalamazoo, Michigan.

^{1}H-NMR.—Nmr spectra were obtained at 360 MHz in D_2O on a Nicolet 1280 spectrometer.

HYDROGENATION.—Catalytic hydrogenation of clazamycin sulfate was based upon the method of Horiuchi (4). Clazamycin sulfate (100 mg) was dissolved in deionized H_2O (15 ml) and 10% palladium on carbon (13 mg, Aldrich) added. After hydrogenation at 20 psi for 1.5 h (Parr hydrogenator), the mixture was filtered (celite) and lyophilized to afford dihydroclazamycin sulfate [**8**] as golden yellow crystals (106 mg, 104% crude yield), mp 122° (dec.); ms (sims) *m/z* 175 (M^++1, 100%), 177 (M^++3, 32%); ^{1}H nmr (D_2O) 4.83 (m, H5), 3.87 (dd, J=5.9 and 12.7 Hz, H4), 3.61 (dd, J=4.7 and 12.7 Hz, H4), 3.25 (m, H2), 2.96 (dd, J=8.3 and 16.7 Hz, H2), 2.55 (dd, J=6.3 and 14.3 Hz, H6), 2.42-2.24 (m, H6 and 2H1); ir (Nujol, cm^{-1}, Perkin-Elmer 1330 Spectrophotometer) 3700-1990 (broad), 1710-1620 (broad), 1345, 1305, 1240, 1208, 1195-990 (broad), 970, 920, 720.

^{18}O AND MeOH INCORPORATION.—Clazamycin sulfate (12.0 mg) was dissolved in either $H_2{}^{18}O$ (1 ml, 50% isotopic purity, Alfa) or hplc grade MeOH (5 ml, Fisher). Each solution was adjusted to pH 11.2 with 5% NaOH (1-2 drops) and allowed to equilibrate at room temperature for 30 min. The solvents were removed in vacuo and the resulting residues subjected to secondary ion mass spectrometry (sims) and ^{1}H nmr.

OPTICAL ROTATION.—Clazamycin sulfate (12.0 mg) was dissolved in 50 mM sodium phosphate buffer (5.0 ml, pH 2.80, 5.65, 7.00, 7.91, or 11.04) and the optical rotation immediately determined (30-45 sec were required to place the solution into the polarimeter) with a Perkin-Elmer Model 241 MC polarimeter (Hg lamp, 579.1 nm, slit 1.4, energy 84 microamperes). A standard 1-decimeter cell was used, and the polarimeter was zeroed with the corresponding buffer before each experiment. The rotation at each pH was recorded at various time intervals, and when a constant value was reached, an aliquot (2.0 ml) of each solution was lyophilized and stored at −78°. ^{1}H nmr was performed by dissolving the lyophilized powders in precisely 2.0 ml of D_2O. The ratio of the A and B epimers was determined by integration of their olefinic signals.

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