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Stereoselective homonucleophilic substitution of 3-*O*-methyl and 3-*O*-ethyloxazepam enantiomers by chiral stationary phase high-performance liquid chromatography*

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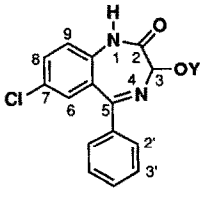
Abstract: Enantiomers of 3-*O*-methyloxazepam and 3-*O*-ethyloxazepam were resolved by chiral stationary phase high-performance liquid chromatography (CSP-HPLC). Temperature-dependent and acid-catalysed racemization of 3-*O*-methyloxazepam enantiomers in methanol and 3-*O*-ethyloxazepam enantiomers in ethanol were studied by quenching reaction products at various times by neutralization. Enantiomeric contents of reaction product were determined by CSP-HPLC. Thermodynamic parameters in the formation of the activated complex (E_{act}, $\Delta H\ddagger$, $\Delta S\ddagger$ and $\Delta G\ddagger$) were consistent with those determined by a spectropolarimetric method. A nucleophilically solvated and transient C3 carbocation intermediate resulting from an N4-protonated enantiomer is proposed to be an intermediate and responsible for the acid-catalysed stereoselective homonucleophilic substitution and the resulting racemization.

Keywords: *Oxazepam; 3-O-methyloxazepam; 3-O-ethyloxazepam; stereoselective homonucleophilic substitution; racemization; chiral stationary phase high-performance liquid chromatography.*

Introduction

3-*O*-methyloxazepam (MeOX) and 3-*O*-ethyloxazepam (EtOX) are pharmacologically active derivatives of oxazepam (OX) [1] (see structure and numbering system in Fig. 1). OX is an active metabolite of diazepam [2]. OX and diazepam are among the most frequently prescribed anxiolytic/hypnotic drugs [2, 3]. A recent report indicated that concurrent ingestion of OX and ethanol may form EtOX in the acidic environment of the stomach [4].



OX, Y = H
MeOX, Y = CH_3
EtOX, Y = C_2H_5

Figure 1
Structures of oxazepam (OX), 3-*O*-methyloxazepam (MeOX), and 3-*O*-ethyloxazepam (EtOX).

Hence it is of interest to understand the chemical properties of 3-*O*-alkyl derivatives of OX in acidic media.

Acid-catalysed homonucleophilic substitution of 3-alkoxy-1,4-benzodiazepines is illustrated in equations (1)–(3), where B represents a 1,4-benzodiazepine lacking the C3-substituent and HB^+ represents a protonated 1,4-benzodiazepine. 1,4-Benzodiazepines are protonated at N4 in a strongly acidic medium [5]. In equation (2), the reaction is a *homonucleophilic* substitution reaction when R_1O = R_2O = alkoxy. The homonucleophilic substitution reaction was called either 'identity reaction', 'degenerate nucleophilic exchange', or 'degenerate nucleophilic substitution' by other investigators [6, 7]. When $R_1O \neq R_2O$ in equation (2), it is a *heteronucleophilic* substitution reaction.

$$BOR_1 + H^+ \rightleftharpoons HB^+OR_1 \qquad (1)$$

$$HB^+OR_1 + R_2OH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HB^+OR_2 + R_1OH. \qquad (2)$$

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In an anhydrous R_2OH,

$$\text{Rate} = k_1[HB^+OR_1][R_2OH] = k_1'[HB^+OR_1] = 0.693[HB^+OR_1]/t_{1/2} \quad (3)$$

where k_1' ($= k_1[R_2OH]$) and $t_{1/2}$ are the pseudo first order rate constant and half-time of the nucleophilic substitution reaction, respectively.

Because the leaving and attacking groups are identical in homonucleophilic substitution reactions, more specialized physicochemical techniques are required to monitor the progress of the reaction. Homonucleophilic substitution reactions of some 3-alkoxy-1,4-benzodiazepines have been studied by (1) NMR [6], (2) polarimetry [6, 8], (3) mass spectrometry [7, 9], and (4) spectropolarimetry [4, 10]. All the above-mentioned methods require the availability of a specialized instrument, which may not be accessible to some investigators. We have developed a relatively simple CSP-HPLC method to study the acid-catalysed homonucleophilic substitution reactions of MeOX enantiomers in methanol and EtOX enantiomers in ethanol. Racemization of enantiomers occurred as a result of stereoselective substitutions. At the present time, instrumentation for HPLC is commonly available in research laboratories and CSP columns are becoming less expensive. Hence the method described in this report may be readily adopted to study other stereoselective homonucleophilic and heteronucleophilic substitution reactions.

Materials and Methods

Materials

Demoxepam (7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide; Ro 5-2092) was generously provided by Hoffmann-La Roche (Nutley, NJ). 3-*O*-Acyloxazepam was prepared from demoxepam according to Bell and Childress [11]. MeOX and EtOX were prepared by acid-catalysed methanolysis and ethanolysis of 3-*O*-acyloxazepam, respectively. Enantiomers of MeOX and EtOX were isolated by CSP-HPLC using a Pirkle type 1-A column (10 mm i.d. × 25 cm) packed with spherical particles of 5 μm diameter γ-aminopropylsilanized silica to which R-*N*-(3,5-dinitrobenzoyl)phenylglycine was bonded covalently [12, 13]. This was a 'Hi-Chrom Pirkle covalent phenylglycine' preparative HPLC column marketed by Regis Chemical (Morton Grove, IL). Dioxane–ethanol–acetonitrile–hexane (20:3.33:1.67:75, v/v/v/v) was used as the mobile phase at a flow rate of 2.5 to 3 ml min^{-1} [4, 10].

HPLC

HPLC was performed using a Waters (Millipore, Milford, MA) Model M45 solvent delivery system and a Kratos (Kratos Analytical Instruments, Ramsey, NJ) Model Spectraflow 757 UV–vis variable wavelength detector. Samples were injected via a Shimadzu (Shimadzu, Kyoto, Japan) Model SIL-9A automatic sample injector. The detector signal was recorded via MacIntegrator (a hardware and software package from Rainin Instruments, Emerysville, CA) on a Macintosh Classic II computer (Apple Computer, Cupertino, CA).

Analytical CSP-HPLC

Enantiomeric contents of either MeOX or EtOX of samples in the racemization studies were determined by CSP-HPLC using an analytical Pirkle column (4.6 mm i.d. × 25 cm) packed with spherical particles of 5 μm diameter γ-aminopropylsilanized silica to which S-*N*-(3,5-dinitrobenzoyl)leucine was bonded covalently. This was a 'Rexchrom Pirkle covalent L-leucine' HPLC column marketed by Regis Chemical (Morton Grove, IL). Dioxane–ethanol–acetonitrile–hexane (20:10:5:65, v/v/v/v) was used as the mobile phase at a flow rate of 1.5 ml min^{-1}.

A quench method for racemization kinetic studies

A quench method to 'freeze' the enantiomeric content in an acid-catalysed homonucleophilic substitution reaction of an enantiomerically pure MeOX (or EtOX) was developed. The method was based on the observation that enantiomers of MeOX and EtOX did not undergo racemization in neutral solutions of an acetonitrile–water mixture.

A typical experiment is described as follows. A neutralization buffer was prepared by adding 1 ml of 10 M NaOH to 99 ml of 0.1 M Tris-HCl buffer (pH 7.5). A test tube containing 125 nmoles of dried residue of an enantiomeric MeOX (or EtOX), maintained at a temperature with a constant temperature water bath, had 1 ml of MeOH (or EtOH) added to it. A 50-μl aliquot was taken as the 'zero time'

sample. To the remaining mixture was added with 30 μl conc. H_2SO_4, followed by immediate mixing. A 50-μl aliquot was subsequently taken at various times and added to a test tube containing 0.5 ml of the neutralization buffer. This was followed by the addition of chloroform (2 ml). Samples were each vortexed for ~30 s, followed by centrifugation to separate the aqueous and chloroform phases. Chloroform (1.5 ml) was taken from each sample and evaporated to dryness with a gentle stream of nitrogen. The residue was redissolved in 0.1 ml of acetonitrile for CSP-HPLC analysis.

Results and Discussion

The acid concentration (0.57 M H_2SO_4) chosen for this study was much higher than the K_a values of MeOX in methanol [10] and EtOX in ethanol [4], respectively. At 0.57 M H_2SO_4, ~98% of MeOX in methanol and ~90% of EtOX in ethanol were protonated, respectively. The racemization $t_{½}$ of enantiomeric MeOX and EtOX were not significantly different among repeated experiments; standard deviations were 1–4% of the mean.

Racemization of MeOX enantiomers

CSP-HPLC analysis of time-dependent changes in the racemization of an (*S*)-MeOX in methanol containing 0.57 M H_2SO_4 at 37°C is shown in Fig. 2. The data in Fig. 2 indicated that the racemization progressed with time at 37°C. A plot of enantiomeric excess [i.e. the difference between the percentages of (*S*)-MeOX and (*R*)-MeOX] vs time (Fig. 3) yielded a straight line with a $t_{½}$ of 30.6 min. Results of experiments conducted at four other

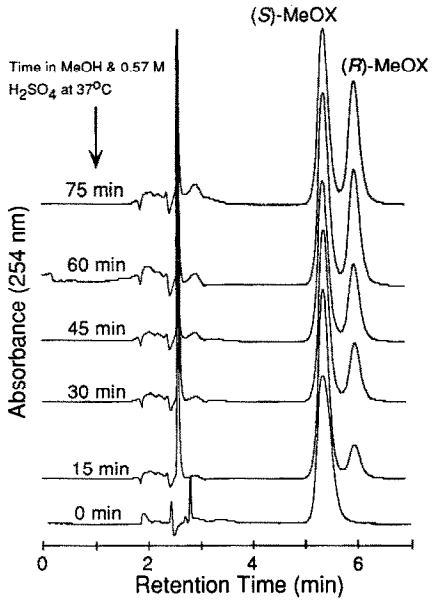

Figure 2
Time-dependent changes of the enantiomeric content in the acid-catalysed homonucleophilic substitution of (*S*)-MeOX and the resulting racemization. (*S*)-MeOX (0.125 mM) in methanol containing 0.57 M H_2SO_4 was incubated at 37°C and aliquots were taken at indicated times. Samples were processed and analysed as described in 'Materials and Methods'.

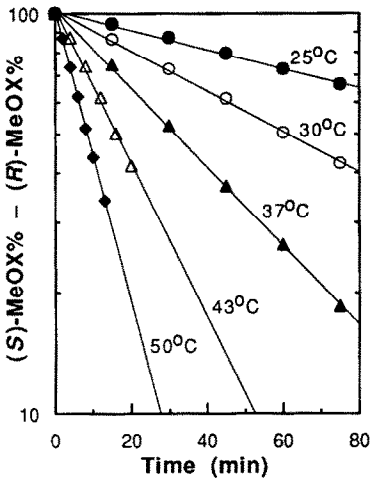

Figure 3
Temperature-dependent racemization of (*S*)-MeOX (0.125 mM) in methanol containing 0.57 M H_2SO_4. Each data point represents an enantiomeric excess [difference between the percentage of (*S*)-MeOX and the percentage of (*R*)-MeOX] determined at the indicated time and temperature. Experiments were performed and samples were processed and analysed as described in 'Materials and Methods'.

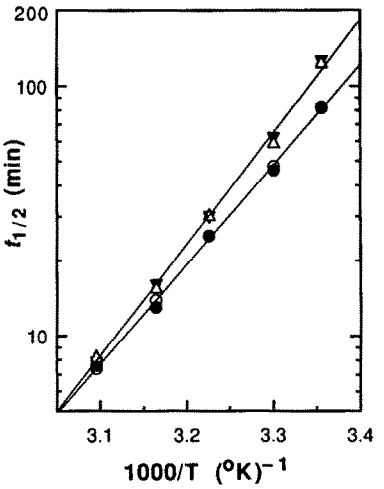

Figure 4
Arrhenius plots for temperature dependence of $t_{½}$ in acid-catalysed racemization of enantiomeric MeOX in methanol [Δ for (*S*)-MeOX and ▼ for (*R*)-MeOX] and EtOX in ethanol [○ for (*S*)-EtOX and ● for (*R*)-EtOX]. Thermodynamic parameters derived from these data are shown in Table 1.

Table 1
Thermodynamic parameters in the racemization of enantiomeric MeOX in acidic methanol and EtOX in acidic ethanol*

Substrate	(*S*)-MeOX	(*R*)-MeOX	(*S*)-MeOX†	(*S*)-EtOX	(*R*)-EtOX	(*R*)-EtOX§
Solvent	MeOH	MeOH	MeOH	EtOH	EtOH	EtOH
Method	CSP-HPLC	CSP-HPLC	CD	CSP-HPLC	CSP-HPLC	CD
$t_{1/2}$ (min)‖ at 25°C	124.7	124.7	92.9	81.3	82.4	73.1
30°C	59.7	61.5	57.4	47.7	46.0	49.4
37°C	30.6	29.8	28.2	25.0	25.1	26.3
43°C	15.7	15.9	16.9	13.9	13.0	16.3
50°C	8.3	7.8	7.7	7.4	7.6	8.0
Slope¶	4.468	4.565	4.179	3.993	3.992	3.729
r^{2}**	0.9964	0.9983	0.9993	0.9998	0.9979	0.9979
E_{act} (kcal mol^{-1})	20.5	20.9	18.8	18.3	18.3	16.7
$\Delta H\ddagger$ (kcal mol^{-1})††	19.9	20.3	18.3	17.7	17.7	16.1
$\Delta S\ddagger$ (cal K^{-1} mol^{-1})††	−10.4	−8.9	−16.8	−16.8	−16.9	−22.0
$\Delta G\ddagger$ (kcal mol^{-1})††	23.0	23.0	22.7	22.7	22.7	22.6

* Unless indicated otherwise, the solvent contained 0.57 M H_2SO_4.
† Data taken from ref. 10 for ready comparison ([H_2SO_4] = 0.5 M).
§ Data taken from ref. 4 for ready comparison ([H_2SO_4] = 0.5 M).
‖ Standard deviations ranged from 1 to 5% of the mean values shown.
¶ Slope in Arrhenius plot (log$t_{1/2}$ vs 1000/T).
** Correlation coefficient in Arrhenius plot.
†† Values calculated for temperature at 25°C.

temperatures are shown in Fig. 3. The nucleophilic substitution reaction was an apparent first order reaction. Racemization $t_{1/2}$ of repeated experiments were reproducible; standard deviations were within 5% of the mean values indicated in Fig. 4.

Arrhenius plots ($\log t_{1/2}$ vs $1000/T$) of data shown in Fig. 3 and the data obtained using enantiomerically pure (*R*)-MeOX are shown in Fig. 4. Experiments conducted using either (*S*)-MeOX or (*R*)-MeOX gave essentially the same results. These results further confirmed an earlier finding that enantiomers of MeOX had the same racemization $t_{1/2}$ in acidic methanol [10] although enantiomers differ in conformational preferences [14–17]. The thermodynamic parameters, calculated from the slope of the Arrhenius plot and rate constant at 25°C, involved in the formation of activated complex are shown in Table 1.

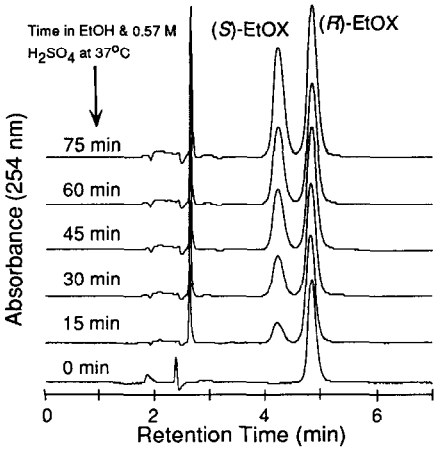

Figure 5
Time-dependent changes of the enantiomeric content in the acid-catalysed homonucleophilic substitution of (*R*)-EtOX and the resulting racemization. (*R*)-EtOX (0.125 mM) in methanol containing 0.57 M H_2SO_4 was incubated at 37°C and aliquots of samples were taken at indicated times. Samples were processed and analysed as described in 'Materials and Methods'.

Racemization of EtOX enantiomers

CSP-HPLC analysis of time-dependent changes in the racemization of an (*R*)-EtOX in ethanol containing 0.57 M H_2SO_4 at 37°C is shown in Fig. 5. The data in Fig. 5 indicated that the racemization progressed with time at 37°C. A plot of enantiomeric excess [i.e. the difference between the percentages of (*R*)-EtOX and (*S*)-EtOX] vs time yielded a straight line with a $t_{1/2}$ of 25.1 min (Fig. 6). Results of temperature-dependent experiments (Fig. 6) indicated that the nucleophilic substitution reaction underwent an apparent first-order reaction. Racemization $t_{1/2}$ of repeated experiments were reproducible with standard deviations within 5% of the mean values shown in Fig. 4. Retention times of EtOX enantiomers were shorter than those of MeOX, indicating that heteronucleophilic substitution of enantiomeric EtOX in methanol and enantiomeric MeOX in ethanol may be studied by the method described herein.

Arrhenius plots ($\log t_{1/2}$ vs $1000/T$) of data shown in Fig. 6 and the data obtained using enantiomerically pure (*S*)-EtOX are shown in Fig. 4. It was apparent that experiments conducted using either (*R*)-EtOX or (*S*)-EtOX gave essentially the same results. These results further confirmed an earlier finding that enantiomers of EtOX had the same racemization $t_{1/2}$ in acidic ethanol [4] although enantiomers differ in conformational preferences [14–17]. The thermodynamic parameters involved in the formation of activated complex are shown in Table 1.

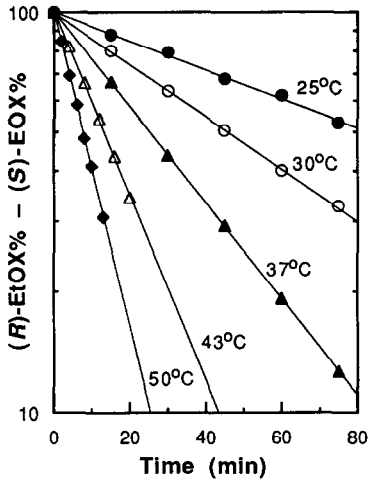

Figure 6
Temperature-dependent racemization of (*R*)-EtOX (0.125 mM) in ethanol containing 0.57 M H_2SO_4. Each data point represents an enantiomeric excess [difference between the percentage of (*R*)-EtOX and the percentage of (*S*)-EtOX] determined at the indicated time and temperature. Experiments were performed and samples were processed and analysed as described in 'Materials and Methods'.

It is interesting to note the difference in slopes in the $\log t_{1/2}$ vs $1000/T$ plots (Fig. 4). The rates of ethanolysis of enantiomeric EtOX were consistently faster than the methanolysis of enantiomeric MeOX (Fig. 4). The enthalpies of activation in the ethanolysis of enantiomeric EtOX, determined by CSP-HPLC and

spectropolarimetric methods, were consistently lower than those in the methanolysis of enantiomeric MeOX (Table 1). Methanol is a stronger attacking nucleophile than ethanol [18]. Consequently, CH_3OH is a better leaving group than C_2H_5OH. In addition to the relative nucleophilicity of attacking and leaving groups, the stereoselectivity in the nucleophilic attack also plays an important role in determining the relative rates in the homonucleophilic substitution and the resulting racemization reactions described in this study. A higher stereoselectivity in the homonucleophilic substitution of an enantiomer leads to a lower rate of racemization. Since the exact stereoselectivity cannot be determined in this study, it is not possible to assess the relative contributions of nucleophilicity of the leaving and attacking groups to the rates of homonucleophilic substitution reactions observed in this study.

Reaction mechanism

The results of acid-catalysed racemization reactions of MeOX enantiomers in methanol and EtOX enantiomers in ethanol are consistent with those obtained by a spectropolarimetric method [4, 10] (see also Table 1). The proposed mechanism of acid-catalysed stereoselective homonucleophilic substitution reactions of MeOX enantiomers in methanol and EtOX enantiomers in ethanol is essentially the same as that described earlier [4, 10] and is summarized below.

1,4-Benzodiazepines, with or without an asymmetric centre, exist as conformational racemates [14–17]. For example, diazepam and nordiazepam each exists in both P (plus) and M (minus) conformations, which are mirror images of each other [14–17]. The M conformation binds preferentially to the benzodiazepine receptors [17]. Because the 3-substituents are preferentially in quasi-equatorial position [14–17], the *R*- and *S*-enantiomers of 3-substituted 1,4-benzodiazepines are predominantly in P and M conformation, respectively. According to Decorte *et al.* [16], (*S*)-MeOX is expected to exist predominantly (>97%) in M conformation.

In Fig. 7, an N4-protonated and highly solvated (*S*)-MeOX predominantly in M conformation is depicted as intermediate I. The (*S*)-MeOX is shown with bifurcated hydrogen bonds between the N4 hydrogen and the oxygens from attacking nucleophiles. A hydrogen bond is formed between the hydrogen of an attacking nucleophile (R_2OH) and the oxygen of a leaving group (R_1OH). Due to hydrogen bonding, the attacking nucleophiles are ready to replace the leaving nucleophile (R_1OH). The actual nucleophilic substitution takes place following transient bonding of an attacking nucleophile to C3 (intermediate II). Thus the positive charge illustrated in intermediate II is not a pure carbocation. Due to steric factor, the formation of (*S*)-R_2OX is expected to be favoured over the formation of (*R*)-R_2OX, the latter existing predominantly in P conformation. This chain of events results in a stereoselective nucleophilic substitution. The resulting (*R*)-R_2OX also undergoes stereoselective (*R*-selective) nucleophilic substitution by a mechanism similar to that depicted for (*S*)-R_1OX. The subsequent stereoselective nucleophilic substitutions of both (*R*)-R_2OX and (*S*)-R_2OX eventually lead to racemization of the starting (*S*)-R_1OX. In intermediate II, the leaving nucleophile (R_1OH) may also serve as an attacking nucleophile, forming the starting substrate (*S*)-R_1OX. The proposed mechanism (Fig. 7) applies to both MeOX and EtOX enantiomers.

The mechanism depicted in Fig. 7 can be classified as nucleophilically assisted and as a mixed S_N1 and S_N2 reaction. The proposed mechanism is consistent with the observed apparent first order kinetics, thermodynamic parameters involved in the formation of activated complex, and kinetic isotope effects described in this and earlier reports [4, 10].

Conclusions

A relatively simple CSP-HPLC method was developed to study acid-catalysed stereoselective homonucleophilic substitution and the resutling racemization of enantiomeric MeOX and EtOX. This represents an alternative method and does not require more specialized instrumentation such as those used in earlier studies [4, 6–10]. The described method may be adopted to study stereoselective heteronucleophilic substitution and other similar types of reactions. Enantiomeric MeOX and EtOX undergo solvent-assisted homonucleophilic substitution reaction in acidic methanol and ethanol, respectively. The reaction is initiated by protonation at the N4 position, followed by transient formation of a highly solvated C3 carbocation and subsequent stereoselective nucleophilic attack from one

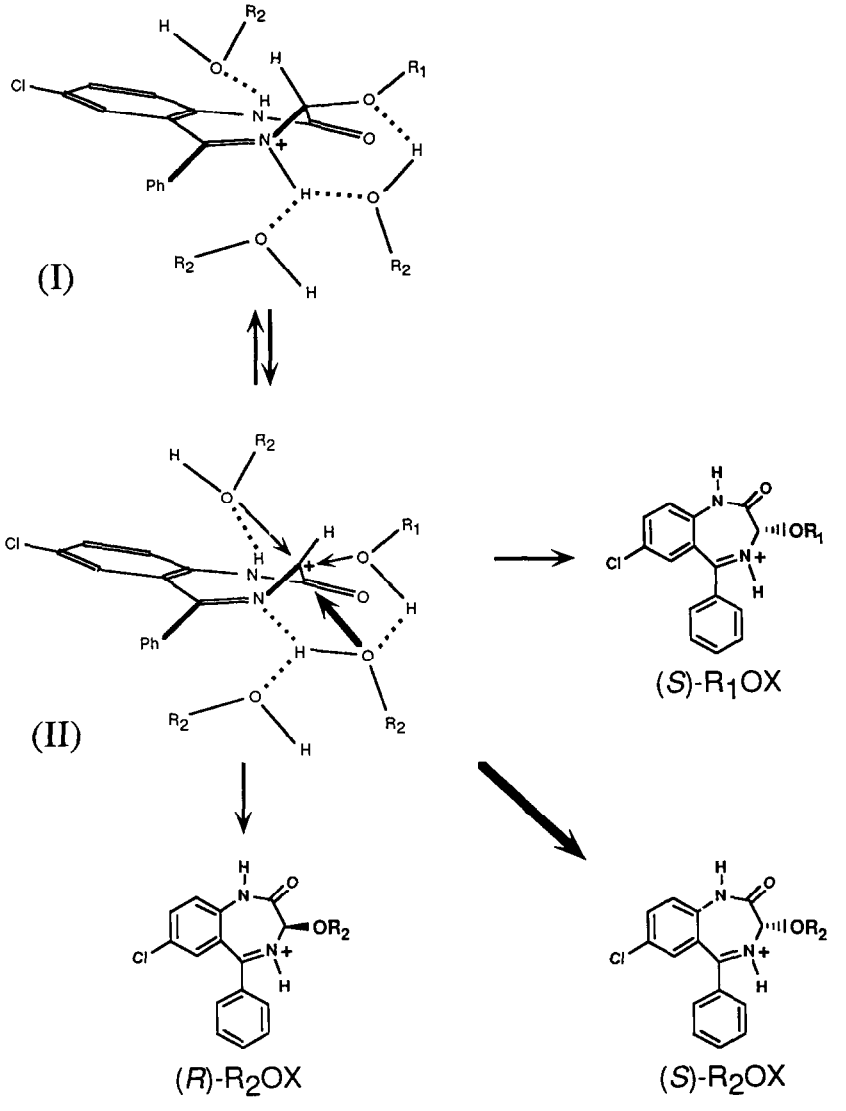

Figure 7
Proposed mechanism in acid-catalysed stereoselective homonucleophilic substitution and the resulting racemization of (*S*)-MeOX in methanol and (*S*)-EtOX in ethanol (in M conformation). Similar mechanism is proposed for the *R*-enantiomer in P conformation. R_1OH and R_2OH ($R_1 = R_2$ = methyl or ethyl) indicate the leaving and attacking nucleophile, respectively. Arrows indicate transient bonding to C3 and origin of nucleophilic attack at C3. For simplicity, only three of many possible attacking nucleophiles are shown.

stereoheterotopic face of the C3 carbon. The highly favoured orientation for stereoselective nucleophilic attack results from the preferred conformation of an enantiomer (M conformation for *S*-enantiomer and P conformation for *R*-enantiomer). Racemization is then observed as a consequence of the stereoselective nucleophilic substitution. The proposed mechanism satisfactorily explains the stereoselective homonucleophilic substitution of enantiomeric MeOX and EtOX, and their subsequent racemization. Hetereonucleophilic substitutions of other 3-alkoxy-1,4-benzodiazepines are predicted to follow similar mechanism.

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