by 75:24:1, 150 mL) yielded *N*-methylpelletierine (expt. 1, 16 mg; expt. 2, 13 mg) and *N*-methylallosedridine (expt. 1, 13 mg; expt. 2, 15 mg), respectively.

^{13}C NMR Spectra. Spectra were recorded on a Bruker AM 500 spectrometer under standard conditions, with TMS as internal reference (δ 0.0 ppm). The spectra are shown in Figure 1. Coupling constants are summarized in Table I.

Acknowledgment. This investigation was supported by a grant from the Natural Sciences and Engineering Research Council of Canada.

Registry No. 2, 40199-45-9; **3**, 41447-16-9; acetate, 71-50-1; acetoacetate, 541-50-4; ethyl [1,2,3,4-$^{13}C_4$]acetoacetate, 84508-55-4; sodium hydroxide, 1310-73-2.

Trapping of a Carbocationic Intermediate in the Spontaneous Hydrolysis Reaction of 7β,8α-Dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]-pyrene: Mechanism of the Spontaneous and General Acid Catalyzed Hydrolysis Reactions of Bay-Region Benzo[*a*]pyrene 7,8-Diol 9,10-Epoxides

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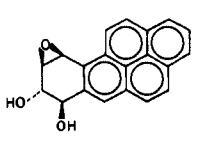
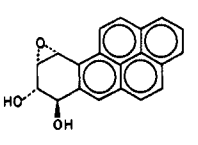
Abstract: The hydrolysis reactions of racemic 7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (**DE-1**) and racemic 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (**DE-2**) in 1:9 dioxane–water solutions are catalyzed by a series of general acids consisting of $Cl_2CHPO_3H^-$, $ClCH_2PO_3H^-$, $H_2PO_4^-$, and $C_2H_5PO_3H^-$. For the hydrolysis of **DE-1** catalyzed by H_3O^+, H_2O, and the above series of general acids, a plot of log k_{HA} vs pK_a gave a Brønsted α of 0.39. A similar Brønsted plot for the hydrolysis of **DE-2** catalyzed by H_3O^+, $Cl_2CHPO_3H^-$, $ClCH_2PO_3H^-$, $H_2PO_4^-$, and $C_2H_5PO_3H^-$ gave an α of 0.40. It is concluded that the mechanism of the hydrolyses of both **DE-1** and **DE-2** catalyzed by the above general acids with pK_a's < ca. 8, including H_3O^+, must occur by concerted proton transfer and benzyl C–O bond cleavage to yield carbocation intermediates. Dipolar intermediates are ruled out. An intermediate in the spontaneous reaction of **DE-1** was trapped, subsequent to its rate-limiting formation, by azide and *N*-acetylcysteine anions. It is proposed that the rate-limiting step for the spontaneous reaction of **DE-1** is formation of a benzylic carbocation intermediate, with a neutral water molecule acting as a proton donor. The rate constant for reaction of this carbocation with solvent is estimated to be 1.7×10^7 s^{-1}. Trapping of an intermediate by azide and *N*-acetylcysteine anions subsequent to a rate-limiting step in the spontaneous hydrolysis of **DE-2** was not detected. Possible explanations for the differences in the hydrolysis reactions of **DE-1** and **DE-2** are given.

Introduction

The hydrolysis reactions of the bay-region diol epoxide metabolites (**DE-1** and **DE-2**)[1] of the environmental carcinogen, benzo[*a*]pyrene, have received considerable attention.[2-4] The rate data between pH 4–10 accurately fit the equation $k_{obsd} = k_H[H^+] + k_0$, where k_H is the second-order rate constant for the acid-catalyzed process[2e,3a] and k_0 is the rate constant for the spontaneous reaction that predominates at higher pH (> ca. 5.5 for **DE-1** and 7.0 for **DE-2**).[3a] The hydrolyses of **DE-1** and **DE-2** are also reported to be catalyzed by general acids such as acetic acid, dihydrogen phosphate, and protonated amines.[3b,4]



DE-1



DE-2

The acid-catalyzed hydrolyses of simple epoxides have been extensively studied; mechanisms proposed for these reactions include either attack of water on protonated epoxide or cleavage of a C–O bond of protonated epoxide to give a carbocation intermediate.[5] Acid-catalyzed hydrolyses of aryl-substituted epoxides generally proceed with cleavage of the benzyl C–O bond.[6] (+)-(*R*)-Styrene oxide is converted to racemic styrene glycol in aqueous perchloric acid, which is compelling evidence for an intermediate benzyl carbocation in this case.[6b] **DE-1** and **DE-2**

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[‡] National Institutes of Health.

(1) Complete names for (−)-**DE-1** and (+)-**DE-2**, the stereoisomers shown, are (−)-7*R*,8*S*-dihydroxy-9*R*,10*S*-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene and (+)-7*R*,8*S*-dihydroxy-9*S*,10*R*-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, respectively.

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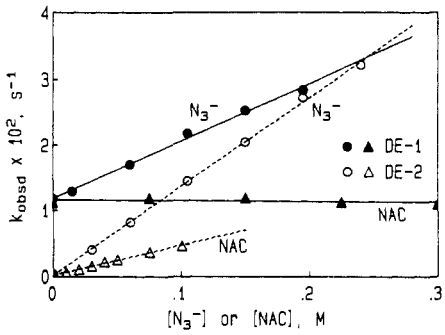


Figure 1. Plots of k_{obsd} for reaction of **DE-1** and **DE-2** vs the concentrations of sodium azide $[N_3^-]$ and total *N*-acetylcysteine [NAC] in 5:95 (v/v) dioxane–water solution, 25 °C. Reactions in *N*-acetylcysteine solutions (μ = 0.1 M, $NaClO_4$) were carried out at pH 7.00 for **DE-1** and pH 8.00 for **DE-2**. Reactions in sodium azide solutions were carried out at pH 8.00 (μ = 0.3 M, $NaClO_4$).

undergo benzyl C–O bond cleavage to yield mixtures of cis and trans hydration products from the acid-catalyzed reactions.[2e,3a,4] These results are best interpreted by mechanisms in which aryl-stabilized carbocations are intermediates. Very similar product ratios from both dihydrogen phosphate catalyzed and hydronium ion catalyzed hydrolyses of **DE-1** and **DE-2** suggest that benzylic carbocations are also intermediates in the general acid catalyzed reactions.[3b]

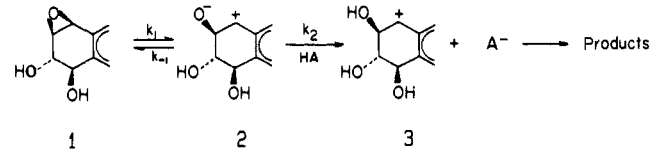
The spontaneous reactions of epoxides with water may occur by a number of kinetically equivalent mechanisms. One or more water molecules may be part of the rate-limiting transition state, acting as either a nucleophile, a proton donor, or both. The spontaneous reaction of 1,3-cyclohexadiene mono-oxide yields only trans 1,2-diol.[7] Water therefore appears to act as a nucleophile in this reaction, adding directly to neutral epoxide. For spontaneous reactions of other epoxides, intermediates are postulated. For example, the pH–rate profile for the hydrolysis of precocene I oxide exhibits two plateaus.[8] An intermediate in the hydrolysis reaction of precocene I oxide at pH 8.5, in the region of the first plateau, was trapped by nucleophilic reaction subsequent to the rate-determining step. Evidence supported a hydroxy carbocation intermediate in this case. In contrast, the spontaneous reactions of benzene oxide and naphthalene oxide are postulated to proceed via zwitterionic intermediates.[9,10]

Conclusive evidence demonstrating the presence of intermediates in the spontaneous reactions of diol epoxides has not been reported. A mixture of cis and trans hydration products, with the cis product predominating, is formed from the k_0 reaction of **DE-1**.[3a] This product distribution is consistent with the intermediacy of a carbocationic species, but does not prove it. In this paper we report definitive experimental data demonstrating that the spontaneous reaction of **DE-1** proceeds with rate-limiting formation of an intermediate. Evidence is provided that suggests that this intermediate is the hydroxy carbocation **3**.

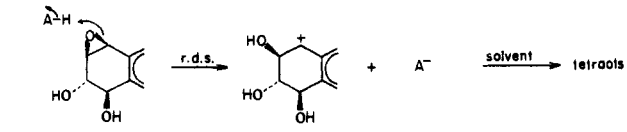
Results and Discussion

Reactions of DE-1. Provided in Figure 1 are plots of k_{obsd} for reaction of **DE-1** and **DE-2** vs the concentrations of the nucleophilic reagents azide ion (N_3^-) and *N*-acetyl-L-cysteine (AcNH-$CH(CH_2SH)CO_2Na$) at pH 7–8, where the spontaneous hydrolysis reactions predominate in the absence of nucleophilic reagent. The rate constants for reaction of **DE-1** in solutions of *N*-acetylcysteine at pH 7.0 show no detectable increase as the concentration of *N*-acetylcysteine is increased to 0.3 M. However, reverse phase C(18) HPLC analyses of the product mixture from reaction of (±)-**DE-1** in 0.3 M *N*-acetylcysteine solution showed

Scheme I



Scheme II



that the yields of tetraol hydrolysis products were reduced by >90% compared to their yields from reaction of **DE-1** in the absence of *N*-acetylcysteine. Four additional products with shorter retention times, formed in approximately equal amounts, eluted from HPLC. These products were assigned as the cis and trans adducts from reaction of *N*-acetyl-L-cysteine with both (+) and (−) enantiomers of **DE-1**.[11,12] *N*-Acetylcysteine must therefore be capturing an intermediate, subsequent to a rate-limiting step. *This rate-limiting step must be the formation of an intermediate in the spontaneous reaction of DE-1.* Concerted mechanisms for the spontaneous reaction of **DE-1** with solvent, leading to either cis hydration or trans hydration, are therefore ruled out.

The rate constant for reaction of **DE-1** in 0.015 M sodium azide solution is only ca. 10% greater than that in the absence of azide, yet >90% of the product is comprised of approximately equal amounts of two azide adducts. Therefore, these adducts must also result primarily from capture of an intermediate by the nucleophilic reagent subsequent to a rate-limiting step. As the concentration of N_3^- is increased, both the rate of reaction of **DE-1** and the relative yield of one of the adducts is increased. This observation is consistent with the reaction of azide ion with **DE-1** by a competing bimolecular process to yield only trans adduct.[13]

Several possible mechanisms for the spontaneous reactions of **DE-1** are outlined in Schemes I and II. In Scheme I, benzyl C–O bond cleavage in **1** leads to the dipolar intermediate **2**,[9] which may be trapped by nucleophiles or protonated by solvent to yield carbocation **3**. In carbocation **3**, an electron-deficient benzyl carbon is located α to the newly formed hydroxyl group. With the assumption that substitution of a positively charged carbon atom in place of a neutral carbon at the α-position of an alcohol has the same acid-strengthening effect as substitution of a positively charged nitrogen in place of neutral nitrogen,[14] then the pK_a of an α-hydroxy carbocation can be estimated to be ca. 12. Since much of the positive charge of **3** is delocalized into the aromatic rings, its pK_a should be somewhat higher than 12, but lower than that of water. If the pK_a of **3** is assumed to be 13–14, then protonation of **2** by a neutral water molecule at pH > ca. 6 is calculated to be much faster than diffusion-controlled protonation of **2** by hydronium ion.[15]

(7) Ross, A. M.; Pohl, T. M.; Piazza, K.; Thomas, M.; Fox, B.; Whalen, D. L. *J. Am. Chem. Soc.* **1982**, *104*, 1658.
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(11) (+)-**DE-1** and (−)-**DE-1**, when allowed to react separately with *N*-acetyl-L-cysteine at pH 7.0, each yielded two of the four adducts.
(12) Thiolate and azide anions have been shown to be very effective in capturing carbocations: (a) Ritchie, C. D.; Wright, D. J.; Huang, D.; Kamego, A. A. *J. Am. Chem. Soc.* **1975**, *97*, 1163. (b) Richard, J. P.; Rothenberg, M. E.; Jencks, W. P. *Ibid.* **1984**, *106*, 1361.
(13) Reactions of nucleophiles with **DE-1** and **DE-2** occur exclusively at the benzyl C(10) carbon, ref 2d.
(14) (a) Hine, J.; Kokesh, F. C. *J. Am. Chem. Soc.* **1970**, *92*, 4383. (b) Fox, J. P.; Jencks, W. P. *Ibid.* **1974**, *96*, 1436.
(15) The reverse of the protonation of **2** by a neutral water molecule is deprotonation of **3** by hydroxide ion, a favorable proton transfer reaction that should proceed with a diffusion-limited rate constant (k_{-2}) of ca. 5×10^9 M^{-1} s^{-1} *if 2 were to exist.* From the equilibrium expression ($K_2 = k_2/k_{-2}$) for the reaction of **2** with H_2O to yield **3** and hydroxide ion, the pK_a for **3** and K_w, a value for $k_2 = k_{-2}K_w/K_a$ can be estimated to be 10^8–10^9 s^{-1}. For protonation of **2** by H^+ at pH > 6 at the diffusion-controlled limit, the value of the rate constant $k_{diff}[H^+]$ is estimated to be $< 5 \times 10^3$ s^{-1}. This argument has been presented previously, ref 8.

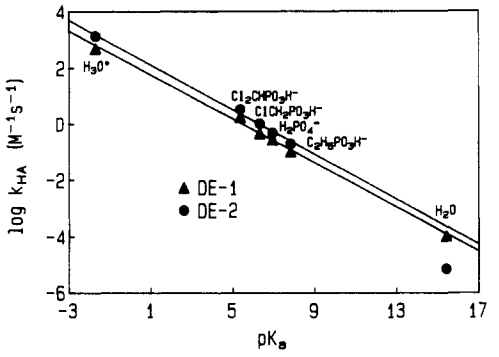

Figure 2. Brønsted plots of log k_{HA} for general acid catalyzed hydrolysis of **DE-1** and **DE-2** in 1:9 dioxane–water solution, 25 °C, vs pK_a of the general acid. Data for H_3O^+ are from solutions at ionic strength 0.1 M ($NaClO_4$);[3a] all other data are from solutions at ionic strength 0.2 M ($NaClO_4$). The solid regression line for **DE-1** is calculated from all **DE-1** data (α = 0.39), and that for **DE-2** is calculated from all **DE-2** data except that for H_2O (α = 0.40). If only the data points for phosphate and phosphonate anions are used in Brønsted correlations, α for **DE-1** is calculated to be 0.49 and α for **DE-2** is calculated to be 0.51.

In Scheme II, proton transfer from water (HA) is concerted with C–O bond breaking and leads directly to the carbocation **3**. Two candidates for the structure of the trapable intermediate in the spontaneous reaction of **DE-1** are therefore the dipolar species **2** and its protonated form, the hydroxy carbocation **3**. In both Schemes I and II, a neutral water molecule acts as a proton donor, and thus may be regarded as a general acid. It is therefore instructive to discuss the hydrolyses of **DE-1** and **DE-2** catalyzed by other general acids, and then consider the special case of the spontaneous (water-catalyzed) reaction.

General Acid Catalysis in the Hydrolysis of DE-1 and DE-2. We have previously reported the hydrolyses of **DE-1** and **DE-2** catalyzed by acetic acid, dihydrogen phosphate, protonated amines, and phenol.[3b] In this paper we also report the bimolecular rate constants for the hydrolyses of **DE-1** and **DE-2** catalyzed by a series of monohydrogen phosphonates. In Figure 2 are Brønsted plots for the reactions of both **DE-1** and **DE-2**, catalyzed by H_3O^+, H_2O, $H_2PO_4^-$, and this series of phosphonates.[16]

General acid catalysis in the hydrolyses of **DE-1** and **DE-2** can also be rationalized by the mechanisms of either Scheme I or Scheme II. If the general acid catalyzed reactions of **DE-1** were to follow the mechanism outlined in Scheme I, then protonation of the dipolar intermediates **2** by buffer acids HA must occur in rate-limiting steps for general acid catalysis to be observed. Proton transfer to **2** from general acids with pK_a's lower than that of **3** would be thermodynamically favorable, and should occur at the diffusion-controlled rate. Therefore, a Brønsted α = 0 should be observed for those acids given in Figure 2 with pK_a's < ca. 13. This is clearly not the case. Instead, a Brønsted correlation for the general acid catalyzed hydrolysis of **DE-1** that includes all general acids of Figure 2, including H_3O^+ and H_2O, gives an α = 0.39. If only the data points for dihydrogen phosphate and the phosphonate anions are used, a Brønsted α = 0.49 is calculated. *These data show that the zwitterion **2** cannot be an intermediate in the hydrolysis of **DE-1** catalyzed by these general acids and support the concerted mechanism provided in Scheme II.* This same type of argument can also be given to support a concerted mechanism for the general acid catalyzed hydrolysis of **DE-2**.

Requirements for concerted general acid catalysis in aqueous solutions have been proposed by Jencks.[17] These requirements are that the site of catalysis must undergo a large change in pK in the course of the reaction and that the pK_a of the catalyst must be intermediate between those of the reactant and product sites. Thus, proton transfer from the catalyst to the reactant site should be unfavorable, but proton transfer from the catalyst to the product site should be favorable. These criteria are met in the general acid catalyzed reactions of **DE-1** and **DE-2** when the pK_a's of the general acids are lower than those estimated for the carbocation intermediates. Dihydrogen phosphate and the phosphonate anions given in Figure 2 have lower pK_a's than that estimated for **3**, for example, but higher pK_a's than protonated epoxide. Therefore, proton transfer from one of these general acids to **1** would be unfavorable, but proton transfer from this same general acid to **2** would be favorable.

Mechanism of the Hydronium Ion Catalyzed Hydrolysis of DE-1 and DE-2. The observation that the H_3O^+ data points fit the Brønsted correlations for the general acid catalyzed hydrolysis of **DE-1** and **DE-2** is suggestive that H_3O^+ also acts as a general acid. We have determined the solvent deuterium isotope effect $k(H_3O^+)/k(D_3O^+)$ for the hydronium ion catalyzed hydrolysis of **DE-1** and **DE-2** to be 0.67 and 0.70, respectively. These inverse isotope effects are rather close to the value of 0.75 observed for the hydronium ion catalyzed hydrolysis of 2-(*p*-nitrophenoxy)-tetrahydropyran, an acetal that is thought to undergo hydrolysis by a mechanism in which H_3O^+ acts as a general acid.[18] They are much larger than the value of ca. 0.37 for the isotope effect on the hydronium ion catalyzed hydrolysis of acetals that are thought to hydrolyze with specific acid catalysis.[18] Acid-catalyzed acetal hydrolyses are mechanistically analogous to acid-catalyzed epoxide hydrolyses and should serve as good models for purposes of estimating isotope effects for epoxide reactions. Inverse isotope effects for the hydronium ion catalyzed hydrolyses of several simple epoxides that most likely occur with specific acid catalysis have been determined to be in the range 0.45–0.53,[5c] and that, for the acid-catalyzed hydrolysis of tetramethylethylene oxide, is reported to be 0.34.[5d] Thus, the Brønsted correlation and solvent isotope effects support mechanisms in which H_3O^+ acts as a general acid catalyst in the hydrolysis of both **DE-1** and **DE-2**.

Mechanism of the Spontaneous Reaction of DE-1. In the possible mechanisms outlined in Schemes I and II for the spontaneous reaction of **DE-1**, water acts as a proton donor either during the rate-limiting step or subsequent to it. In this sense, water may be regarded as another general acid. This reaction is a special case, however, not only because water is the solvent but also because its pK_a is substantially higher than those of the other general acids given in Figure 2.

The kinetic solvent deuterium isotope effect $k_0(H_2O)/k_0(D_2O)$ on the spontaneous reaction of **DE-1** is 2.62 ± 0.02. For reactions in which rate-limiting formation of dipolar intermediates have been proposed, much lower solvent kinetic deuterium isotope effects of ca. 1.3 are reported.[9] A somewhat larger isotope effect on the rate of formation of a dipolar intermediate such as **2** might be rationalized if the transition state structure is very "late" and the oxyanion group of **2** is tightly solvated by water molecules. If **2** is solvated with three water molecules with fractionation factors of 0.7 for the coordinating L–O hydrogenic atoms,[19] a solvent isotope effect ($k(H_2O)/k(D_2O)$) of 2.9 is calculated on the equilibrium constant for **1** → **2**. Since the C–O bond would not be completely broken at the transition state for the reaction **1** → **2**, coordination of the developing oxyanion of the transition state with two water molecules instead of three would be expected. The fractionation factor for the coordinating L–O bonds should also be >0.7 because the oxyanion is only partially formed. If only two water molecules solvate the transition state, the isotope effect should therefore be less than ca. 2.0. The observed isotope effect is substantially greater than this estimated value, and therefore we conclude that the spontaneous reaction of **DE-1** most likely proceeds by either a different rate-limiting step or a different mechanism.

Simple proton transfer from water to **2** should be thermodynamically unfavorable on the basis of pK_a considerations. For an unfavorable proton transfer reaction, diffusion of the conjugate

(16) Only data for general acids of a common charge type, except for those of H_3O^+ and H_2O, are used in these Brønsted plots. Rate data for the hydrolysis of **DE-1** and **DE-2**, catalyzed by general acids of different charge types, do not form good Brønsted correlations. In particular, the hydrolysis of **DE-1** and **DE-2** catalyzed by protonated amines may involve more complicated mechanisms.

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(18) Fife, T. H. *Acc. Chem. Res.* **1972**, *5*, 264.

(19) Gold, V.; Grist, S. *J. Chem. Soc. Perkins Trans. 2* **1972**, 89.

base of the proton donor away from the species protonated is the rate-limiting step,[20] and therefore the primary kinetic solvent deuterium isotope effect for the reaction should be close to unity. In the protonation of **2** by water, hydroxide ion is generated. From the fractionation factor of 0.4–0.5 for LO^-,[19,21] a secondary isotope effect of ca. 2–2.5 can be estimated for the overall reaction of **1** → **3** by the mechanism of Scheme I in which the second step is rate-limiting. The calculated isotope effect for this mechanism is slightly less than the observed kinetic solvent deuterium isotope effect for the spontaneous reaction of **DE-1** (2.62). Hydroxide ion is also formed in the concerted mechanism of Scheme II, and it can be argued that the magnitude of the kinetic isotope effect for this mechanism should be similar to that expected for the mechanism of Scheme I with the second step rate-limiting. The observed solvent deuterium isotope effect alone therefore cannot be used to distinguish unambiguously between them.

If the mechanism for the reaction of **DE-1** with water is the same as that for the reaction of **DE-1** with other general acids, then the bimolecular rate constant for the reaction of **DE-1** with water might be expected to fall on or near a Brønsted line formed by the other general acids. On the Brønsted plot for **DE-1** (Figure 2), the data points for H_3O^+, H_2O, $H_2PO_4^-$, and the series of phosphonates fit reasonably well to a regression line ($\alpha = 0.39$). One might conclude therefore that water is also acting as a general acid and that the reaction of **DE-1** with water occurs by the same concerted mechanism as the reaction of **DE-1** with other general acids of lower pK_a's. This conclusion presents a dilemma because the pK_a of water is higher than that estimated for the product carbocation **3**, and **3** should therefore become immediately deprotonated by the newly formed hydroxide ion. The criterion of Jencks' rule that the pK_a of the catalyst must be intermediate between the initial and final pK_a values of the substrate site would therefore be violated.

We have already concluded that **2** cannot be an intermediate in the hydrolysis of **DE-1** catalyzed by general acids with sufficiently low pK_a's. A concerted mechanism for proton transfer from these general acids to **DE-1** would be presumably "enforced"[22] because of the instability of the zwitterion **2** in the presence of the general acid. The dilemma of a concerted mechanism for proton transfer from water to the epoxide oxygen in the spontaneous reaction of **DE-1** is alleviated somewhat if the dipolar species **2** *is too unstable to exist*; i.e., there is no energy barrier for its collapse to epoxide **1**. The epoxide oxygen must be then be partially protonated in the transition state leading to the carbocationic intermediate, which ultimately reacts to form stable products. A concerted mechanism for this step would be "enforced"[22] because of the instability of the dipolar species even in the presence of water. If there is no energy barrier for collapse of the zwitterion **2** to epoxide **1**, and proton transfer from **3** to hydroxide ion is thermodynamically favorable, then the encounter complex for deprotonation of **3** by HO^- will not have a significant lifetime. The rate-limiting step for the spontaneous reaction of **DE-1** is therefore most likely either diffusion of hydroxide ion away from the carbocation, or conformational isomerization of the carbocation.[23] The actual proton-transfer step would therefore preceed the rate-limiting physical process, and could thus be either protonation of the zwitterion **2** by water or the concerted process (Scheme II), depending on whether **2** is sufficiently stable to exist in the presence of water. The relatively good fit of the water point to the Brønsted correlation for the other general acids with lower pK_a's may therefore be fortuitous. Since the rate-limiting step for the water reaction would be different from that of the other general acids, the water point for the spontaneous reaction of **DE-1** should actually fall below the Brønsted line for the other data points.

Scheme III

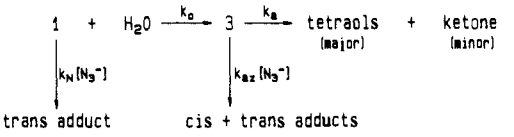

Table I. Biomolecular Rate Constants for General Acid Catalyzed Hydrolysis (k_{HA})[a] and Nucleophilic (k_N) Reactions of **DE-1** and **DE-2**, 25 °C

HA	k_{HA}(DE-1), M^{-1} s^{-1}	k_{HA}(DE-2), M^{-1} s^{-1}
$Cl_2CHPO_3H^-$	1.79 ± 0.07	3.42 ± 0.05
$ClCH_2PO_3H^-$	0.47 ± 0.04	1.04 ± 0.04
$H_2PO_4^-$	0.28 ± 0.02	0.50 ± 0.03
$C_2H_5PO_3H^-$	0.104 ± 0.002	0.193 ± 0.002
N	k_N(DE-1), M^{-1} s^{-1}	k_N(DE-2), M^{-1} s^{-1}
N_3^-	0.088 ± 0.003[b]	0.13 ± 0.01[b]
N-acetyl-L-cysteine[c]	1.23 ± 0.03[d]	2.3 ± 0.4[e]

[a] 1:9 dioxane–water, μ = 0.2 M ($NaClO_4$). [b] 5:95 dioxane–water, μ = 0.3 M ($NaClO_4$). [c] N refers to thiolate anion. [d] 5:95 dioxane–water, μ = 0.3 (KCl). [e] 5:95 dioxane–water, μ = 0.1 M ($NaClO_4$), average of values obtained at pH 8.0, 8.5, and 9.0.

On the basis of above discussions, the most probable mechanisms for the spontaneous reaction of **DE-1** are either the rate-limiting formation of a dipolar intermediate **2** (Scheme I), or a rate-limiting physical process involving carbocation–hydroxide diffusional separation or carbocation isomerization. On the basis of the large kinetic solvent deuterium isotope effect, we favor one of the latter mechanisms.

Lifetime of the Intermediate from the Spontaneous Reaction of DE-1. The reactions of carbocations with nucleohiles have been extensively studied.[12,24-26] Highly stabilized carbocations have sufficient lifetimes such that their rates of reaction with water and nucleophiles can be measured directly.[24] The ratios of the rate constants for their reactions with azide ion and with water (k_{az}/k_s) are relatively constant, and are on the order of 10^6–10^7 M^{-1}. Other somewhat less stable carbocations have reduced k_{az}/k_s ratios. These reduced ratios have been interpreted to indicate that the reactions of these carbocations with azide ion occur at the diffusion-controlled limit,[12b,25,26] whereas k_{az} for reaction of the more highly stabilized carbocations is activation limited.

A mechanism that accounts for the reaction of **DE-1** with azide ion is given in Scheme III. At lower concentrations of azide, both cis and trans azide adducts are formed from trapping of the intermediate carbocation **3** by azide, subsequent to the rate-limiting step. There is also a second-order reaction of **DE-1** with azide that yields only trans adduct. Thus, the rate data for reaction of **DE-1** in 1:9 dioxane–water solutions fit the equation $k_{obsd} = k_N[N_3^-] + k_0$, where k_N is the bimolecular rate constant for reaction of **DE-1** with N_3^- (Table I). At very low concentrations of azide ion, the trans:cis ratio of azide adducts is ca. 1.2:1. As the concentration of azide ion is increased, the ratio of these adducts from **DE-1** increases as predicted by the mechanism of Scheme III.

In 7.5 mM sodium azide solution, the yield of tetraols from **DE-1** was determined to be ca. 17%. If the remainder of the product is assumed to be azide adducts and the amount of adduct resulting from bimolecular reaction of azide ion with **DE-1** corrected for, then the ratio of adducts:tetraols resulting from reaction of **3** with water and azide under these conditions can be calculated to be 4.6:1.0. This ratio is equal to $k_{az}[N_3^-]/k_s$, from which k_{az}/k_s can be calculated to be 6.1 × 10^2 M^{-1}. This value of k_{az}/k_s is

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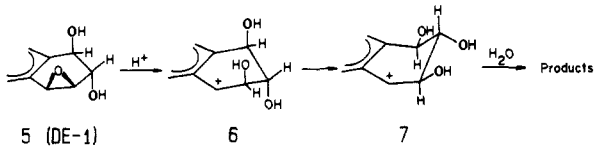
(21) Schowen, R. L. *Prog. Phys. Org. Chem.* **1972**, *9*, 275.
(22) Jencks, W. P. *Acc. Chem. Res.* **1980**, *13*, 161.
(23) (a) Sayer, J. M.; Yagi, H.; Silverton, J. U.; Friedman, S. L.; Whalen, D. L.; Jerina, D. M. *J. Am. Chem. Soc.* **1982**, *104*, 1972. (b) Gillilan, R. E.; Pohl, T. M.; Whalen, D. L. *Ibid.* **1982**, *104*, 4481.
(24) (a) Ritchie, C. D.; Virtanen, P. O. I. *J. Am. Chem. Soc.* **1972**, *94*, 4966. (b) Ritchie, C. D. *Acc. Chem. Res.* **1972**, *5*, 348.
(25) Richard, J. P.; Jencks, W. P. *J. Am. Chem. Soc.* **1982**, *104*, 4689.
(26) For a review and discussion of literature data for reactions of carbocations with N_3^- and water, see: Ta-Shma, R.; Rappoport, Z. *J. Am. Chem. Soc.* **1983**, *105*, 6082.

Scheme IV



5 (DE-1) 6 7

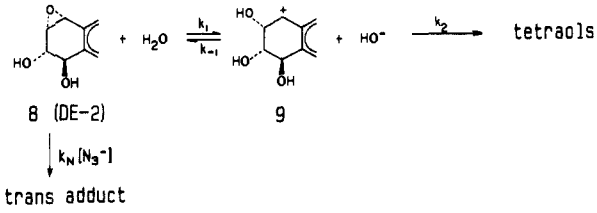
close to values of k_{az}/k_s observed for reactions of a series of 1-phenylethyl carbocations, for which it is argued convincingly that the reactions of the carbocations with azide ion occur at the diffusion-controlled rate.[12b] If it is therefore assumed that the rate constant for reaction of the intermediate **3** with azide ion is also at the diffusion-controlled limit, with a value of 5×10^9 M^{-1} s^{-1},[12b] then the rate constant k_s for reaction of **3** with water can be calculated to be 1.6×10^7 s^{-1}.

An interesting aspect of the spontaneous reaction of **DE-1** is that for the carbocation **3** to be the trapable intermediate, as we have postulated, hydroxide ion must be formed along with the carbocation when it is generated. In order for the carbocation **3** to be trapped by external nucleophiles, the highly basic hydroxide ion must diffuse away from the carbocation faster than it collapses with it to form diols. For this mechanism to be reasonable, hydroxide ion must not be formed in a location favorable for collapse with the electron-deficient benzyl carbon. It has been previously postulated that the acid-catalyzed reaction of **DE-1** occurs with axial opening of the epoxide ring (Scheme IV), and that the initially formed carbocation **6** undergoes conformational isomerization to **7** at a rate that exceeds that of tetraol formation.[23] The predominant cis hydration product then results from axial attack of solvent on **7**. This proposal was based on the observations of Goering and Josephson,[27] who showed that "axial" attack of water on cyclohexenyl cations is the most favorable route to product formation. If hydroxide ion is generated in the spontaneous reaction of **DE-1**, it is most likely positioned on the same side of the ring as the newly formed axial hydroxyl group. It will therefore not be in a location favorable for axial attack on the carbocation. For axial attack of this hydroxide ion, either ring inversion or carbocation rotation must first occur. It is also known that the hydroxide ion is a much poorer nucleophile toward carbocations than either azide or thiolate ions,[12a] so perhaps it is not reasonable for the rate of diffusion of hydroxide away from the carbocation to exceed the rate of diol formation.

Reactions of DE-2. The slopes of plots of k_{obsd} vs $[N_3^-]$ for **DE-2** are the same within experimental error at pH 8.0 and 8.5. However, the slopes of plots of k_{obsd} vs the total concentration of *N*-acetylcysteine increased with increasing pH. Thus, the kinetic terms due to these nucleophilic reagents are consistent with mechanisms in which N_3^- and the thiolate anion (RS^-) of *N*-acetylcysteine react with neutral epoxide by concerted, bimolecular addition reactions. Yields of azide adduct from reaction of **DE-2** in dilute solutions of sodium azide at pH 8.0–8.5 were generally found to be ca. 10–20% greater than yields calculated from the rate data if it is assumed that azide adduct results only from bimolecular addition of azide to neutral epoxide. However, the calculated and observed adduct yields were sufficiently close that we hesitate to conclude that any of the adduct results from capture of an intermediate subsequent to a rate-limiting step. These data do not rule out the intermediacy of a carbocation in the spontaneous reaction of **DE-2**, but do show that if an intermediate exists, its capture by azide cannot be readily detected because of the favorable concerted, bimolecular addition of nucleophilic reagents to **DE-2**. This condition is made possible by the fact that the spontaneous reaction rate of **DE-2** is ca. 50 times slower than that of **DE-1**, yet the bimolecular rate constant for reaction of azide with **DE-2** exceeds the bimolecular rate constant for reaction of azide with **DE-1** by ca. 50%.

The kinetic solvent deuterium isotope effect ($k(H_2O)/k(D_2O)$) for the spontaneous reaction of **DE-2** (1.69 ± 0.13) is still rather large, but is significantly lower than that for the spontaneous reaction of **DE-1**. If the spontaneous reaction of **DE-2** proceeds by a mechanism similar to that of **DE-1** (Scheme II), then the isotope effect for **DE-2** would be expected to be closer to that of **DE-1**. A kinetically equivalent mechanism that would give a lower isotope effect is given in Scheme V, in which the second step, attack of HO^- on carbocation **9**, is partially or significantly rate-limiting. The transition state for this second step contains hydroxide ion partially bonded to carbocation **9**, and therefore only the secondary effect of a partially formed hydroxide ion contributes to the overall kinetic isotope effect if this step is rate-limiting. However, if external hydroxide ion reacts with the carbocation **9** in the rate-limiting step, then azide ion should also react with **9**. Azide is a better nucleophile than hydroxide toward carbocations, and should react with **9** at or near the diffusion-controlled limit. In solutions of 10^{-4}–10^{-3} M azide at pH 8–9, the concentrations of azide greatly exceed those of hydroxide ion (10^{-6}–10^{-5} M). In these dilute azide solutions, therefore, the rate of reaction of azide with the carbocation **9** ($k_{diff}[N_3^-]$) should be much greater than the rate of reaction of HO^- with **9** ($k_{OH}[OH^-]$). The two predominant pathways leading to product would thus be trapping of the intermediate **9** with azide and nucleophilic addition of azide to neutral epoxide. Consequently, the only products from reaction of **DE-2** in solutions of 10^{-4}–10^{-3} M azide at pH 8–9 should be azide adducts. However, in 0.5 mM sodium azide solution at pH 8.5, tetraols constitute ca. 60% of the product mixture from the reaction of **DE-2**. Therefore, the mechanism for the spontaneous reaction cannot involve attack of external HO^- on carbocation **9**.

Scheme V



8 (DE-2) 9

trans adduct

A possible mechanism for the spontaneous reaction of **DE-2** is one similar to that proposed for **DE-1**, except that in the reaction of **DE-2**, the newly formed hydroxide ion reacts at the electron-deficient benzyl carbon of **9** faster than it diffuses away. Such mechanism might account for the fact that **DE-2** gives ca. 60% cis hydration in its spontaneous reaction, but only ca. 10% cis hydration in the hydronium ion catalyzed hydrolysis.[3a]

It is not clear at this point why the spontaneous reaction of **DE-2** differs from that of **DE-1**.[28] Work is continuing in order to better understand the mechanisms of the spontaneous and nucleophilic addition reactions of these diol epoxides.

Summary

The hydrolysis reactions of **DE-1** and **DE-2** are catalyzed by H_3O^+ and a series of general acids with pK_a's 5–8. It is concluded that the mechanisms of these reactions involve proton transfer from the general acid to the epoxide oxygen, concerted with benzyl C–O bond cleavage of the epoxide in the rate-limiting step. Dipolar intermediates are ruled out.

On the basis of the large solvent kinetic deuterium isotope effect for the spontaneous reaction of **DE-1** and the observation that an intermediate of this reaction is trapped by nucleophilic reagents subsequent to a rate-limiting step, it is concluded that the spontaneous reaction of **DE-1** proceeds via a carbocation intermediate. On the basis of pK_a considerations, it is suggested that the rate-limiting step of this reaction is either diffusion of hydroxide ion away from the carbocation, or conformational isomerization of the carbocation. The actual proton-transfer step would thus preceed the rate-limiting process and could be either protonation of a zwitterion by water or concerted proton transfer from water

(27) Goering, H. L.; Josephson, R. R. *J. Am. Chem. Soc.* **1962**, *84*, 2779.

(28) The mechanism for the spontaneous reaction of **DE-1** may also differ from those of other cis diol epoxides of this series such as that derived from phenanthrene, which give greater yields of ketone, Whalen, D. L.; Ross, A. M.; Yagi, H.; Karle, J. M.; Jerina, D. M. *J. Am. Chem. Soc.* **1978**, *100*, 5212.

to the epoxide oxygen coupled with benzyl C–O bond cleavage, depending on whether the zwitterion has sufficient stability to exist in water. Although it is commonly accepted that the acid-catalyzed hydrolysis of aryl epoxides often proceeds via carbocation intermediates and such intermediates have been captured by nucleophiles,[8,29] this is the first example where such intermediate has been trapped in the spontaneous reaction of an epoxide in which neutral water acts as the proton donor. In the reaction of **DE-1** with nucleophilic reagents in the pH range where the spontaneous reaction predominates, adducts are formed both from reaction of the nucleophile with the carbocation intermediate generated in the spontaneous reaction of **DE-1** with water and from bimolecular addition of the nucleophile to neutral epoxide.

We were not able to detect the trapping of an intermediate in the spontaneous reaction of **DE-2** from product rate data for the reactions of **DE-2** in solutions containing nucleophilic reagents because of the relatively favorable nucleophile addition to neutral epoxide. The kinetic solvent deuterium isotope effect for the spontaneous reaction of **DE-2** is considerably lower than that for **DE-1**, which suggests a different mechanism or a different rate-limiting step for **DE-2**. Mechanisms that might account for the differences in the spontaneous reaction and nucleophilic addition reactions of **DE-1** and **DE-2** were suggested, but not proved.

Two types of evidence suggest that H_3O^+ acts as a general acid catalyst in the hydronium ion catalyzed hydrolyses of **DE-1** and **DE-2**. The **DE-1** and **DE-2** data points for H_3O^+ fall near the Brønsted lines for the general acid catalyzed hydrolyses of **DE-1** and **DE-2** by other general acids, respectively, and the kinetic solvent deuterium isotope effects ($k(H_3O^+)/k(D_3O^+)$) for the hydronium ion catalyzed hydrolyses of **DE-1** and **DE-2** are much closer to the isotope effects expected for general acid catalyzed hydrolysis than they are to those expected for specific acid catalyzed hydrolysis.

The results of this study raise important questions about the mechanisms of reaction of **DE-1** and **DE-2** in biological systems. Our results suggest that in water solutions at pH ca. 7 containing dilute concentrations of certain biochemical nucleophiles such as glutathione, the mechanism of non-enzyme-catalyzed reaction of **DE-1** with the nucleophile will be capture of the carbocation subsequent to its rate-limiting formation. However, **DE-2** should react mainly by a second-order addition of nucleophile to neutral epoxide. **DE-1** and **DE-2** complexed with DNA also react via spontaneous reactions.[30] Although the mechanisms of these spontaneous reactions may not be similar to those of the free diol epoxides, different mechanisms for reaction of **DE-1** and **DE-2** might account for some of the differences in the amounts and types of covalent binding that occur.

Experimental Section

Solvents and Materials. Racemic samples of **DE-1** and **DE-2**,[31] (+)-**DE-1**[32] and (−)-**DE-1**,[32] were synthesized by published procedures. Sodium dichloromethylphosphonate was prepared from dichloromethylphosphonyl dichloride by a modification of a published procedure.[33] Sodium chloromethylphosphonate and sodium ethylphosphonate solutions were prepared by neutralizing solutions of chloromethylphosphonic acid and ethylphosphonic acid, purchased from Morton Thiokol, Inc. (Alpha Products) and Aldrich Chemical Co., respectively. Sodium azide (Aldrich Chemical Co.) was recrystallized from aqueous ethanol solution, and dioxane was distilled from sodium metal prior to use. Water for kinetic studies was deionized and glass-distilled. All other chemicals were of reagent grade and used without further purification. The L form of *N*-acetylcysteine was used in this work.

Kinetic Procedures. For each kinetic run, approximately 5–15 μL of a stock solution of **DE-1** or **DE-2** in dioxane was added to 2.0 mL of reaction solution in the thermostated cell compartment (25.0 ± 0.2 °C) of a Gilford Response spectrophotometer. Solutions of sodium azide and *N*-acetylcysteine also contained 2×10^{-4} M *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer to ensure constant pH and 10^{-4} M EDTA. Reactions were monitored at 348 nm, and pseudo-first-order rate constants were calculated by nonlinear regression analysis of the time vs absorbance data.

Kinetic solvent deuterium isotope effects ($k(H_2O)/k(D_2O)$) for the spontaneous reactions of **DE-1** and **DE-2** have been reported to be 2.4 ± 0.1 and 1.7 ± 0.2, respectively.[3b] These isotope effects have been redetermined by additional kinetic experiments and were found to be 2.62 ± 0.02 and 1.69 ± 0.13, respectively. The value of the isotope effect for **DE-1** is the average of six determinations in water (0.1 M $NaClO_4$, pH (pD) 8.0–8.6), in the absence of buffers. The isotope effect for **DE-2** is the average of nine determinations in water, pH (pD) 8.0–10.6, in the absence of buffers. The difference in pH of a kinetic solution measured before and after a given kinetic run varied from as little as 0.02 to as much as 0.7 pH units. Since the rates of the spontaneous reactions are pH independent, each kinetic run followed excellent first-order kinetics. For each run, generally 50 data points spaced throughout ca. 10 half-lives were used to calculate a rate constant, and the internal error in each rate constant ranged from 0.2–0.5%. It is important that buffer reagents of any appreciable concentrations not be present. Some buffers, e.g. 10^{-3} M Tris, contribute significant kinetic terms which, if not corrected for, lead to erroneous isotope effects.

The isotope effects for the spontaneous reactions of **DE-1** in water–dioxane solvents were found to be lower than that for its reaction in water. For example, we have measured the kinetic solvent deuterium isotope effects for the reactions of **DE-1** in 10% dioxane–90% water and 20% dioxane–80% water to be 1.92 and 1.80, respectively. It is reported that greater amounts of ketone product are formed from this reaction as the dioxane concentration of the solvent was increased,[3a] and presumably the mechanism and rate-limiting steps are changing.

Second-Order Rate Constants. Values of k_{HA} for $H_2PO_4^-$ and the phosphonate acids of Figure 2 were obtained from the slopes of least-squares plots of k_{obsd} vs $[RPO_3H^-]$, the concentration of the mono-anion form of each reagent. The bimolecular rate constants for the reaction of the thiolate form (RS^-) of *N*-acetylcysteine to **DE-1** and **DE-2** were determined from plots of k_{obsd} vs $[N\text{-acetylcysteine}]_{TOTAL}$. The slopes of these plots were set equal to $k_N(K_a/(K_a + [H^+]))$, where k_N is the bimolecular rate constant for the thiolate form of *N*-acetylcysteine and K_a is the apparent ionization constant for *N*-acetylcysteine. A plot of k_{obsd} for reaction of **DE-1** vs the concentration of *N*-acetylcysteine thiolate at pH 9.65 (ionic strength 0.3 M, KCl) provided a value of k_N equal to 1.23 ± 0.03 M^{-1} s^{-1}. From this plot and kinetic data at pH 7, it could be concluded that the thiolate form of *N*-acetylcysteine was responsible for the kinetic term.

The values of k_{HA} for the reactions of **DE-1** and **DE-2** with water were calculated by dividing their spontaneous reaction rate constants (0.0054 s^{-1} and 0.00035 s^{-1}, respectively) by 50 M, the approximate concentration of water in 1:9 dioxane–water mixture.

pK_a Values. The apparent pK_a values for the second ionization of phosphoric acid, chlorophosphonic acid, dichlorophosphonic acid, and ethylphosphonic acid in 1:9 dioxane–water solutions (μ = 0.2 M, $NaClO_4$) were determined by titration with a Radiometer automatic titration assembly and are as follows: $H_2PO_4^-$, 6.95; $ClCH_2PO_3H^-$, 6.32; $Cl_2CHPO_3H^-$, 5.36; $C_2H_5PO_3H^-$, 7.83. The apparent pK_a of *N*-acetylcysteine (RSH) in 5:95 dioxane–water solution (v/v, μ = 0.3 M, KCl) was determined by titration to be 9.65. A combination glass electrode, calibrated with standard buffer solutions in water, was used for these titrations. The apparent pK_a values thus determined are uncorrected for change of solvent. Solutions of 0.2 M $NaClO_4$ in water and in 1:9 dioxane–water solution having pH values of 2.5–3.5, prepared from diluting identical volumes of standard mineral acid, gave pH readings that agreed within 0.02 pH units.

The ion product of water is found to be slightly greater in 20 wt % dioxane–80 wt % water solution (2.4×10^{-14} M^2) compared to that in pure water (1.0×10^{-14} M^2).[34] At ionic strength 0.1 M, the ion product of water is 1.59×10^{-14} M^2.[35] We therefore estimate the ion product of water in 1:9 dioxane–water solution (ionic strength 0.1 M) to be ca. 2.0×10^{-14} M^2. For Figure 2, we therefore estimate the pK_a's of H_3O^+ and H_2O to be −1.7 and 15.4, respectively.

Product Studies. Solutions from kinetic studies were also utilized for product studies. At the end of a kinetic run an aliquot of either 2-

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(31) (a) Yagi, H.; Hernandez, O.; Jerina, D. M. *J. Am. Chem. Soc.* **1975**, *97*, 6881. (b) Yagi, H.; Thakker, D. R.; Hernandez, O.; Koreeda, M.; Jerina, D. M. *Ibid.* **1977**, *99*, 1604.

(32) Yagi, H.; Akagi, H.; Thakker, D. R.; Mah, H. D.; Koreeda, M.; Jerina, D. M. *J. Am. Chem. Soc.* **1977**, *99*, 2358.

(33) Sayer, J. M.; Jencks, W. P. *J. Am. Chem. Soc.* **1973**, *95*, 5637.

(34) Harned, H. S.; Owen, B. B. *The Physical Chemistry of Electrolytic Solutions*, 3rd ed.; Reinhold Publishing Corp.: New York, 1958; p 756.

(35) Chiang, Y.; Kresge, A. J.; Tang, Y. S.; Wirz, J. *J. Am. Chem. Soc.* **1984**, *106*, 640.

naphthaleneethanol or cinnamyl alcohol was added to serve as a standard. The pH of the solution was adjusted to ca. 7, and it was then analyzed directly by HPLC on a Waters C18 Resolve Radial Pak column. The products were eluted with 55% methanol-45% water, 1.5 mL/min, and monitored by UV detection at 254 nm. The yields of tetraols were determined by comparing the areas of their HPLC peaks with the area of the peak due to the standard compound. It was assumed that the acid-catalyzed reactions of **DE-1** and **DE-2** at pH 4 yielded 100% tetraols, and the yields of tetraols from **DE-1** and **DE-2** under other conditions were determined relative to their yields at pH 4.

The azide adducts were analyzed with the same HPLC conditions used for analyzing tetraols, except for the solvent flow rate. The retention times (min) for the products from **DE-1**, with HPLC flow rate 3 mL/min, were as follows: tetraol from trans hydration, 5.4; tetraol from cis hydration, 10.1; azide adduct from cis addition, 20.9; azide adduct from trans addition, 26.1; 2-naphthaleneethanol (standard), 8.1. The retention times (min) for the products from **DE-2**, with HPLC flow rate 2.5 mL/min, were as follows: tetraol from trans hydration, 6.6; tetraol from cis hydration, 8.8; 2-naphthaleneethanol, 10.9; azide adduct(s), 16.2.

The *N*-acetylcysteine adducts from **DE-1** were analyzed by HPLC as described above, with 20% methanol-80% water as the eluent and a flow rate of 1.0 mL/min. The retention times (min) of the products were as follows: trans adduct from (-)-**DE-1**, 6.4; trans adduct from (+)-**DE-1**, 7.3; cis adduct from (-)-**DE-1**, 9.4; cis adduct from (+)-**DE-1**, 10.1.

Only two *N*-acetylcysteine adducts formed in equal amounts were detected from the reaction of racemic **DE-2** in solutions containing *N*-acetyl-L-cysteine at pH 8.5, and the HPLC retention times (min) of these adducts (20% methanol-80% water as eluent, 0.5 mL/min) were 13.1 and 15.5. The diastereomeric adducts presumably are formed from trans addition of the thiolate group to the (+) and (-) enantiomers of **DE-2**.

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Reagents and Methods for the Solid-Phase Synthesis of Protein-EDTA for Use in Affinity Cleaving

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Abstract: Synthetic procedures for the introduction of the metal chelator ethylenediaminetetraacetic acid (EDTA) at unique amino acid positions of proteins by solid-phase methods are described. Two protected derivatives of EDTA compatible with Merrifield solid-phase protein synthesis employing *N-tert*-butyloxycarbonyl- (Boc-) protected amino acids were developed. The first reagent is a dipeptide with three of four carboxyl groups of EDTA protected as benzyl esters and the fourth coupled to a γ-aminobutanoic acid linker, referred to as tribenzyl-EDTA-GABA (BEG). A second reagent is the tricyclohexyl ester of EDTA, TCE. BEG and TCE allow the modification of the NH_2 terminus and/or lysine side chains of resin-bound peptides and proteins. Upon deprotection and cleavage from the resin, a protein is produced with EDTA at a defined amino acid position. The availability of protein-EDTA conjugates extends the affinity cleaving method to the study of protein-DNA complexes in solution.

Introduction

High-resolution crystallographic views of DNA binding proteins and protein-DNA complexes reveal the structural complexity of protein-DNA interactions.[1-4] The combination of direct protein-DNA contacts mediated by multiple hydrogen bonds and sequence-dependent DNA conformational effects limits our ability to make detailed structural predictions, even if a new DNA binding protein can be assigned to a structural class such as helix-turn-helix,[1-3] double-barreled helix,[4] zinc binding finger,[5] or scissor grip leucine zipper.[6] In the absence of high-resolution crystallographic and nuclear magnetic resonance data, solution methods, such as affinity cleaving, are needed to determine the topology of protein-DNA complexes and correlate sequence similarities with known structural classes.[7-8]

Affinity Cleaving. Attachment of EDTA·Fe to a DNA binding moiety creates a DNA cleaving molecule that functions under physiologically relevant pH, temperature, and salt conditions.[9] The cleavage reaction can be initiated by addition of a reducing agent such as dithiothreitol or sodium ascorbate.[9] If the DNA binding molecule is sequence specific, the EDTA·Fe cleaves at highly localized sites on DNA restriction fragments and plasmids.[10-16] Because the EDTA·Fe cleaving moiety is not sequence

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