Biosynthesis of *N*-Methylpelletierine: Vindication of a Classical Biogenetic Concept

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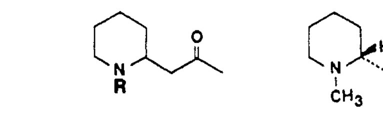
Abstract: The ^{13}C NMR spectrum of a sample of *N*-methylpelletierine (**2**), generated biosynthetically from sodium [1,2,3,4-$^{13}C_4$]acetoacetate in *Sedum sarmentosum* Bunge, shows that the C_3 side chain of the alkaloid is derived as an intact unit from the C_4 precursor. The ^{13}C NMR spectrum of a sample of the alkaloid, biosynthetically derived from sodium [1,2-$^{13}C_2$]acetate, shows that it is the $-COCH_3$ unit and not the $-CH_2CO-$ unit of the side chain that is derived from an intact acetate precursor. These results constitute evidence in support of classical biogenetic concepts and disprove three other possible routes of biosynthesis.

The skeleton of the alkaloids related to pelletierine (**1**) was shown 20 years ago to originate from lysine[1-3] and acetate.[3-5] A



1 R = H **3**

2 R = CH_3

great deal of tracer evidence[1-3,6,7] defines the mode and stereospecificity of incorporation of L-lysine (**4**) into the piperidine ring of the alkaloids, via bound cadaverine and Δ^1-piperideine (**5**) (Scheme I). Surprisingly, the route whereby the C_3 side chain is elaborated from acetate is not well understood, four different pathways being consistent with the observed incorporation pattern of acetate.

The major reason for this lack of precise knowledge probably lies in the dominating influence that early biogenetic ideas of Sir Robert Robinson[8] and Clemens Schöpf[9] continued to exert when tracer studies of alkaloid biosynthesis began. It was then generally assumed that the biochemical process whereby the propanone side chain is attached to the piperidine nucleus in the biosynthesis of pelletierine (**1**) would mimic the route whereby the compound had been synthesized (Scheme I, path A), via a Mannich reaction between Δ^1-piperideine and acetoacetic acid, accompanied by a spontaneous decarboxylation.[10-19] The putative intermediate, **6**, of the process has not been isolated. The synthesis was, in fact, originally devised[10] on the basis of "biogenetic" thinking.[8,9]

The observed distribution of label from sodium [1-^{14}C]acetate[3,4] and [2-^{14}C]acetate[5] within pelletierine[3] (**1**) and *N*-methylpelletierine[3-5] (**2**) was consistent with this route (Scheme I, path A). Attempts to demonstrate the direct derivation of the propanone side chain of **2** from β-hydroxybutyrate, a C_4 acid closely related to acetoacetate, were unsuccessful.[2] Nonetheless, it is generally accepted[20-22] on the basis of the above evidence that the biosynthetic route to pelletierine and its relatives is as shown in Scheme I, path A.

Yet the available tracer evidence is equally consistent with any one of three other routes (Scheme I, paths B, C, and D), each of which leads to one and the same distribution of label in the alkaloid, when ^{14}C-labeled or singly ^{13}C-labeled acetate serves as the precursor (Scheme II). This and the important recent finding by Leete and Kim[23] that the biosynthesis of the tropane moiety of cocaine does not take place by a route analogous to path A, as had hitherto been assumed, but by a route analogous either to path B or to path C, with the latter being favored, prompted us to investigate the mode of assembly of the pelletierine (**1**) side chain, as found in *N*-methylpelletierine (**2**) and in *N*-methylallosedridine (**3**), in *Sedum sarmentosum*.

The four routes, paths A–D, may be distinguished from one another by means of a pair of tracer experiments with bond-labeled substrates: An experiment with [1,2,3,4-$^{13}C_4$]acetoacetate discriminates between paths A and B on the one hand and paths C and D on the other (Scheme IIIb). An experiment with [1,2-$^{13}C_2$]acetate differentiates between paths A and D on the one hand and paths B and C on the other (Scheme IIIa). The predicted distribution of label in **1** from these experiments is shown in Scheme III.

In two separate experiments, cuttings of *Sedum sarmentosum* Bunge were kept in contact with sodium [1,2-$^{13}C_2$]acetate (experiment 1) and sodium [1,2,3,4-$^{13}C_4$]acetoacetate (experiment 2). In each instance the bond-labeled tracer was administered in admixture with an equimolar quantity of unenriched substrate, in order to reduce the probability that fully enriched acetoacetate would be generated, by dimerization of the bond-labeled acetate (experiment 1) or by regeneration, after possible breakdown to [1,2-$^{13}C_2$]acetate, of the administered fully labeled acetoacetate (experiment 2), if insufficient endogenous natural abundance material were present within the plant. After the plants had been kept in contact with tracer for 5 days, *N*-methylpelletierine[2,24] (**2**) and *N*-methylallosedridine[24,25] (**3**) were extracted, separated, and purified. Distribution of ^{13}C within the products was determined by ^{13}C NMR spectrometry at 125.8 MHz. Assignment of the signal due to the side chain CH_2 group in the spectrum of **2** was

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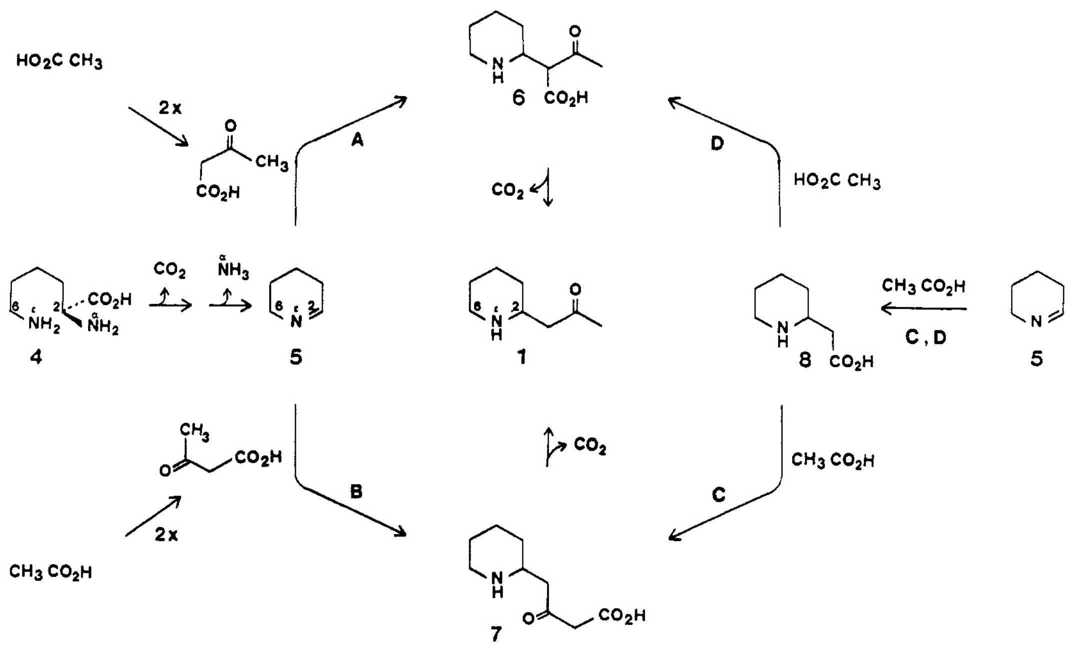
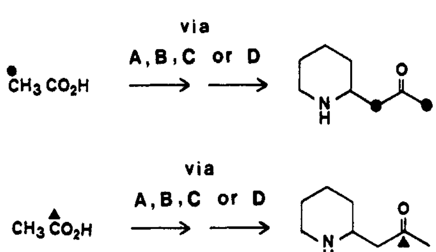
Table I

^{13}C Chemical shift, ppm		coupling constants, Hz: expt. 1 sodium [1,2-$^{13}C_2$]acetate		coupling constants, Hz: expt. 2 sodium [1,2,3,4-$^{13}C_4$]acetoacetate	
			N-Methylpelletierine (2)		
$-CH_3$	31.0	d	$^{1}J_{CH_3-CO} = 40.4 \pm 1.8$	dd	$^{1}J_{CH_3-CO} = 39.8 \pm 1.8$ $^{2}J_{CH_3-CH_2} = 14.4 \pm 1.8$
$-CH_2-$	47.1	s		dd	$^{1}J_{CH_2-CO} = 39.4 \pm 1.8$ $^{2}J_{CH_2-CH_3} = 13.7 \pm 1.8$
$-CO-$	207.8	d	$^{1}J_{CO-CH_3} = 39.5 \pm 1.8$	dd	$^{1}J_{CO-CH_3} = 39.4 \pm 1.8$ $^{1}J_{CO-CH_2} = 39.5 \pm 1.8$
			N-Methylallosedridine (3)		
$-CH_3$	24.3	d	$^{1}J_{CH_3-CHOH} = 40.4 \pm 1.8$	d	$^{1}J_{CH_3-CHOH} = 39.8 \pm 1.8$
$-CH_2-$	39.6	s		d	$^{1}J_{CH_2-CHOH} = 38.6 \pm 1.8$
$-CH(OH)-$	68.1	d	$^{1}J_{CH(OH)-CH_3} = 39.5 \pm 1.8$	dd	$^{1}J_{CH(OH)-CH_3} = 38.6 \pm 1.8$ $^{1}J_{CH(OH)-CH_2} = 38.6 \pm 1.8$

Scheme I. Four Possible Routes for the Derivation of the Skeleton of Pelletierine (**1**) from Δ^1-Piperideine (**5**) and Acetate



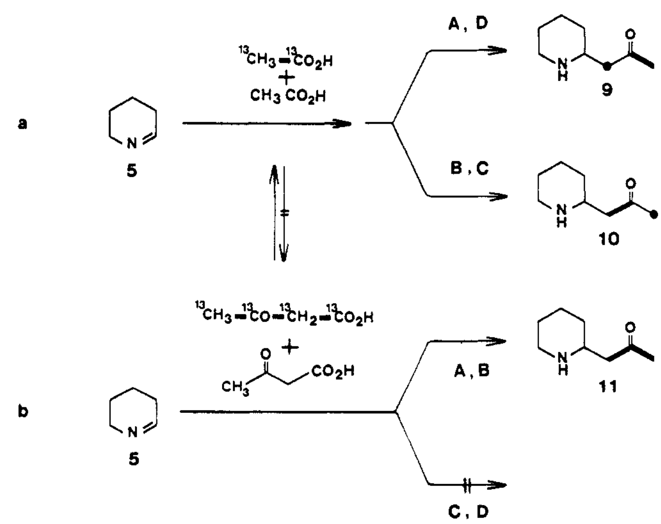
Scheme II. Derivation of the Propanone Side Chain of Pelletierine from Methyl-Labeled (●) and from Carboxyl-Labeled (▲) Acetate via Any One of the Four Possible Routes A, B, C or D, Shown in Scheme I



made after base-catalyzed protium–deuterium exchange at the carbon atoms α to the carbonyl group. Assignment of the ^{13}C NMR spectrum of **3** rests on homonuclear ^{1}H–^{1}H and heteronuclear ^{1}H–^{13}C shift correlation spectra.

The ^{13}C NMR spectra of the samples of *N*-methylpelletierine (**2**) (Figure 1A) and *N*-methylallosedridine (**3**), isolated from the feeding experiment with acetate (experiment 1), indicated enrichment (specific incorporation:[26a] **2**, 0.3%; **3**, 0.1%): In each

Scheme III. Derivation of the Propanone Side Chain of Pelletierine from [1,2-$^{13}C_2$]Acetate (Sequence a)[a] and [1,2,3,4-$^{13}C_4$]Acetoacetate (Sequence b)[b]



[a] The heavy bar denotes incorporation of the intact C_2 unit of acetate. [b] The heavy bars denote incorporation of an intact C_3 unit derived by decarboxylation of acetoacetate.

case satellites were observed in the signals due to the C-methyl carbon and the carbonyl or carbinol carbon (Table I). The signal

(26) (a) Specific incorporation = % ^{13}C above natural abundance within the product/% $^{13}C_2$ above natural abundance within the precursor (i.e., 49.7) × 100. (b) Specific incorporation = % ^{13}C above natural abundance within the product/% $^{13}C_4$ above natural abundance within the precursor (i.e., 48.0) × 100.

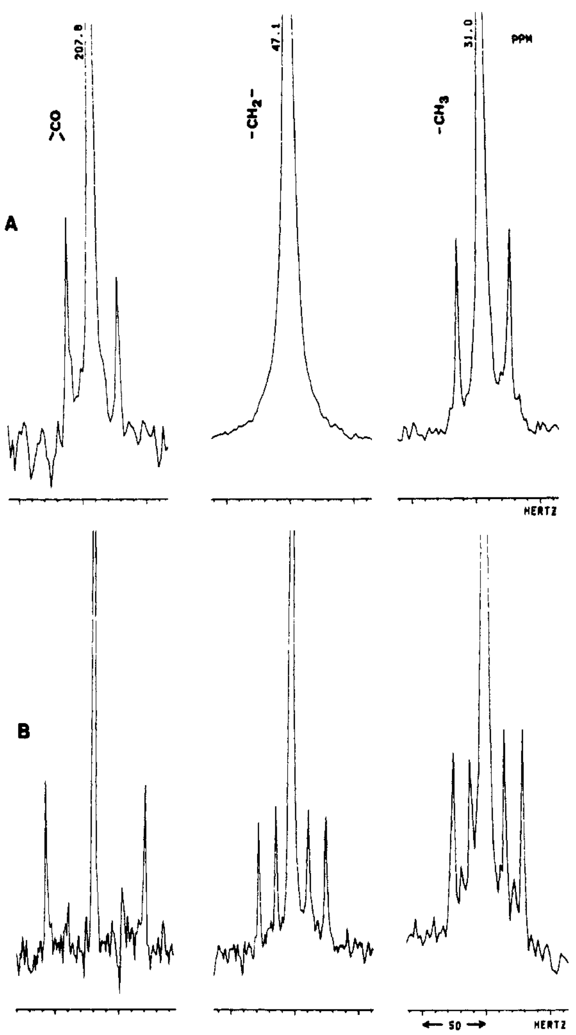


Figure 1. Signals due to the side chain carbon atoms in the proton noise decoupled ^{13}C NMR spectra (125.8 MHz) of the samples of *N*-methylpelletierine (**2**) obtained from cuttings of *Sedum sarmentosum* after administration of sodium [1,2-$^{13}C_2$]acetate (A) (16 mg of **2** in 0.6 mL of $DCCl_3$) and sodium [1,2,3,4-$^{13}C_4$]acetoacetate (B) (13 mg of **2** in 0.6 mL of $DCCl_3$), with TMS as internal reference (∂ 0.0 ppm).

due to the methylene carbon atom of the side chain appears as a single line. Furthermore, a variant of the 1D-INADEQUATE sequence[27,28] confirmed coupling between the satellites of the signals due to the methyl and the carbonyl and carbinol carbons, respectively. Thus, an intact acetate unit had entered the $-COCH_3$ and the $-CHOHCH_3$ units, respectively, of the side chain of *N*-methylpelletierine (**2**) and of *N*-methylallosedridine (**3**). Only paths A or D, but neither path B nor path C, are consistent with this distribution of label.

The second tracer experiment (experiment 2), with [1,2,3,4-$^{13}C_4$]acetoacetate, distinguished between paths A and D. The ^{13}C NMR spectrum of the sample of *N*-methylpelletierine obtained from this feeding experiment (specific incorporation 0.8%)[26b] (Figure 1B) showed satellites in the signals of all three side chain carbon atoms, each of which appears as a doublet of doublets (Table I).

Particularly noteworthy is the two-bond long-range coupling between the C-methyl carbon and the methylene carbon of the side chain. This coupling pattern provides clear evidence that the C_3 side chain was derived intact from acetoacetate, since cleavage of the $^{13}C_4$ precursor to [$^{13}C_2$]acetate before incorporation into the alkaloid would have led to dilution with natural abundance material and resulted in a labeling pattern identical with that observed in experiment 1. Indeed, close inspection of the spectrum (Figure 1B) reveals the presence of low-intensity satellites, ^1J = 40 Hz, in the signal due to the C-methyl carbon and in that due to the carbonyl carbon atom. The latter could not be observed with satisfactory signal-to-noise ratio in a normal ^{13}C spectrum, even after a prolonged run, but was clearly observable with use of an INEPT pulse sequence[29] optimized for a long-range coupling, $^2J_{C,H}$ = 7 Hz.

The spectrum (Figure 1B) thus indicates that an intact C_3 unit, derived from [1,2,3,4-$^{13}C_4$]acetoacetate, has served as the precursor of the side chain of the alkaloid. Path A, which predicts entry of an intact acetoacetate chain, is consistent with this observation whereas path D, which proceeds via piperidineacetic acid (**8**) and involves stepwise introduction of two individual acetate units, is not.

Together, the two tracer experiments disprove three of the four possible modes of entry of acetate into the C_3 side chain of the pelletierine skeleton. They provide long-overdue experimental proof that the biosynthetic route was indeed correctly predicted on the basis of biogenetic thinking,[8,9] and that it parallels the regiochemistry of biosynthesis of another Sedum alkaloid, sedamine, which arises from Δ^1-piperideine and phenylalanine, presumably via cinnamate and benzoylacetate.[30] Contrary to prediction, the biosynthetic route from acetate into the pelletierine side chain (route A, Figure 1), for which evidence is here presented, bears no similarity to the route into the acetate-derived moiety of cocaine (analogous to route C, Figure 1) that is supported by recent tracer evidence.[23]

Experimental Section

Labeled Compounds. Sodium [1,2-$^{13}C_2$]acetate (99.4% $^{13}C_2$, MSD Isotopes, Pte. Claire, Quebec, Canada) and ethyl [1,2,3,4-$^{13}C_4$]acetoacetate (96.0% $^{13}C_4$, Isotech Inc, Miamisburg, Ohio) were commercial products.

Tracer Experiments. Cuttings of *Sedum sarmentosum* Bunge were immersed in glass-distilled water (50 mL) in 100-mL beakers (ca. 40 g of plant material per beaker). A slow stream of oxygen (ca. 1 mL per min) was bubbled through the liquid. Solutions of labeled material were added to each beaker once a day on five successive days. The plant material was kept in contact with the tracer solution for an additional 5 days. The volume of the solution in each beaker was maintained by daily addition of glass-distilled water.

Experiment 1: Sodium [1,2-$^{13}C_2$]acetate (600 mg) was mixed with an equal quantity of unenriched sodium acetate in 50 mL of glass-distilled water. The solution was applied to the cuttings in five equal portions over 5 days.

Experiment 2: Ethyl [1,2,3,4-$^{13}C_4$]acetoacetate (500 mg) was mixed with an equal quantity of unenriched material and suspended in a solution of sodium hydroxide (700 mg) in water (40 mL). The mixture was kept at room temperature overnight and was then stored at 4 °C. Each day, on five consecutive days, immediately before feeding, 8 mL of the solution were withdrawn and neutralized with ice cold hydrochloric acid (6 M), with methyl orange as indicator. The volume of the mixture was made up to 12.5 mL before addition to the beakers containing the plant cuttings.

Isolation of the Alkaloids. The plant material from each of the two experiments (ca. 200 g of fresh weight) was homogenized in distilled water (150 mL). The pH of the resulting homogenate was adjusted to pH 10 with concentrated ammonia. The basified mixture was filled into a chromatography column and percolated with chloroform (2 L). The phases of the eluate were separated, the aqueous layer extracted with more chloroform (3 × 100 mL), and the combined chloroform solutions evaporated in vacuo.

The residue was dissolved in hydrochloric acid (10 mL, 1 M), the solution washed with ether (4 × 10 mL), and the combined ether extract back-extracted with hydrochloric acid (3 mL, 1 M). The aqueous solution was neutralized (solid sodium bicarbonate) and basified with potassium hydroxide solution (50% w/v, 2 mL). The basic solution was extracted with dichloromethane (3 × 10 mL), the organic extract was dried (anhydrous sodium sulfate), filtered and evaporated, and the residue was applied to a silica column (Merck 7734, 1.0 × 25 cm). Elution with chloroform/methanol/0.880 ammonia (85:14:1, 150 mL, followed

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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 (∂ 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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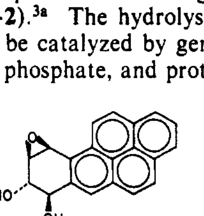
Received January 13, 1989

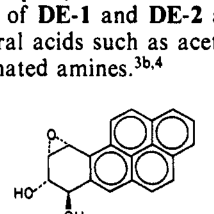
Abstract: The hydrolysis reactions of racemic 7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (**DE-1**) and racemic 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene (**DE-2**) in 1:9 dioxane–water solutions are catalyzed by a series of general acids consisting of $Cl_2CHPO_3H^-$, $ClCH_2PO_3H^-$, $H_2PO_4^-$, 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 $1.7 \times 10^7\ s^{-1}$. 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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(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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