Linearized bacteriophage λ DNA was labeled with ^{32}P individually at the left (L) and right (R) ends (3′ fill-in reaction) with AMV reverse transcriptase. An 18 base homopyrimidine oligonucleotide, 5′-T*$T_3CT_6CT_4$CT-3′, with thymidine–EDTA (T*)[13] at the 5′ end, was synthesized by automated methods. The oligonucleotide–EDTA (0.8 μM) was mixed with $Fe(NH_4)_2(SO_4)_2$ (1.0 μM) and spermine (1.0 mM). The oligonucleotide–EDTA·Fe(II)/spermine was added to a solution of L (or R) ^{32}P end-labeled λ DNA (approximately 4 μM in base pairs) in 100 mM NaCl and 25 mM tris–acetate at pH 7.0.[14] After 0.5 h incubation at 24 °C, 4 mM dithiothreitol was added to initiate strand cleavage. The reaction was allowed to continue for 6 h (24 °C). The double strand cleavage products were separated on a 0.4% vertical agarose gel which resolves DNA segments up to 25 kbp in size (Figure 3). Therefore, DNA uniquely labeled at the R and L ends allows cleavage site analysis of the entire 48.5 kbp of the λ genome.

No cleavage was observed for the first 25 kbp of the L end-labeled DNA (Figure 3A, lane 3). However, a single major cleavage site 9.4 kbp from the right (R) end of the 48.5 kbp DNA was visualized on the autoradiogram (Figure 3A, lane 5).[15] This was quantitated by scintillation counting of the individual bands. The oligonucleotide–EDTA·Fe probe afforded double strand cleavage of λ DNA at the target sequence, 5′-$A_4GA_6GA_4$GA-3′, with an efficiency of 25% (Figure 3).[16] Within the limits of our detection, overexposure of the autoradiogram revealed no secondary cleavage sites. We estimate that all secondary sites were cleaved at least 30-fold less efficiently than the primary sequence.[17]

In order to prevent random shearing of the DNA caused by pipetting or vortexing,[3,4] large DNA is routinely manipulated by embedding it in low melting point (LMP) agarose and diffusing reagents into the matrix. To test whether triple helix mediated site-specific cleavage can occur with oligonucleotide–EDTA·Fe *within an agarose matrix*, end-labeled λ DNA was embedded in 1% LMP agarose gel, and the cleavage reactions were performed as described above (24 °C, pH 7.0, 6 h, initial incubation time was extended to 1 h to allow for complete diffusion into the matrix) followed by electrophoresis. Primary site-specific double strand cleavage occurred in the agarose matrix with an efficiency of 25% (Figure 3B, lane 2).

In conclusion, this work has implications for human genetics. The way is now clear for the development of a triple helix strategy to isolate large segments of genomic DNA for mapping and sequencing.

Acknowledgment. This work was supported in part by the National Institutes of Health (GM 35724), the Caltech Consortium in Chemistry and Chemical Engineering (Founding members: E. I. duPont de Nemours and Company, Inc., Eastman Kodak Company, Minnesota Mining and Manufacturing Company, and Shell Oil Company Foundation), and a National Research Service Award (T32GM07616) from the NIH-GM to S.A.S.

(13) Dreyer, G. B.; Dervan, P. B. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 968.

(14) Concentrations given are final dilutions.

(15) The complementary fragment, 39.1 kbp from the L terminus, was not resolved from the intact λ DNA but can be visualized in underexposed autoradiograms as a shoulder with intensity equal to the 9.4 kbp fragment.

(16) 25% efficiency means that one-quarter of all the DNA underwent double strand cleavage at one site.

(17) Although the above conditions are optimal for single-site cleavage, reaction parameters can be modified for the purposes of searching large DNA for sequences of *partial* homology with the primary target site. For example, identical reaction conditions performed at the reduced temperature of 0 °C for 24 h resulted in three minor sites of cleavage (25-fold less efficient) which map to 22.4, 37.9, and 47.7 kbp from the L terminus. Sequences partially homologous with the primary site are found at each of these positions. One site, 5′-AA*CA*AAAAAAG-3′, contains two mismatches in the first 12 nucleotides from the 5′ end of the oligonucleotide–EDTA·Fe probe. Two other sites, 5′-AAAAGAAAAA*T*GAA-3′ and 5′-AAA*T*GAA*T*AAAGAA-3′, contain one and two mismatches in the first 14 base pairs, respectively. All other sequences in λ DNA with a similar degree of sequence homology (two mismatches in 12) were not cleaved within the limits of our detection and have in common the mismatch at or adjacent to the T* which contains the EDTA·Fe(II) cleaving moiety.

Preparation of the 8,9-Epoxide of the Mycotoxin Aflatoxin B_1: The Ultimate Carcinogenic Species

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Aflatoxin B_1 (AFB_1, **1**) is formed by ubiquitous fungi and is one of the most potent environmental mutagens and carcinogens, causing worldwide concern about contamination of agricultural products.[1] It requires metabolic activation to a highly labile, as yet unisolated, electrophilic species which forms covalent adducts with DNA.[2] Metabolic activation can be mimicked by in situ chemical activation of AFB_1 by peroxy acids,[3] by photooxidation,[4] or by solvolysis of 8-acyloxy-9-hydroxy derivatives of AFB_1.[5] Indirect evidence derived from structures of adducts with nucleic acids and other nucleophiles points to the fugitive intermediate being the *exo*-8,9-epoxide **2**.[3-6] Numerous attempts to prepare this epoxide have failed,[7-10] which has raised questions as to its existence and role in carcinogenesis.[7] The epoxide could be expected to be highly labile by virtue of the fact that it is fused to a tetrahydrofuran ring and would be prone to acid-catalyzed solvolysis at C8.[11,12] We wish to report the preparation of **2** by a chemical oxidation of AFB_1 and the finding that the compound is stable enough to permit storage and manipulation.[13] Epoxide **2** has the properties associated with in vivo activation of AFB_1, supporting the hypothesis that it is the ultimate carcinogen. The availability of the epoxide will permit direct investigation of its chemistry and metabolism.[14]

Synthesis of epoxide **2** involved oxidation of AFB_1 with dimethyldioxirane (Scheme I), which had been prepared from potassium peroxysulfate as a distilled 0.05 M solution in acetone.[15] Approximately 1.5 equiv of the dioxirane was added to AFB_1 dissolved in either acetone or methylene chloride.[16] The reaction

(1) More than 5000 articles on aflatoxins have been abstracted by *Chemical Abstracts*. For a review, see: Busby, W. F., Jr.; Wogan, G. N. In *Chemical Carcinogens*, 2nd ed.; Searle, C., Ed.; American Chemical Society Series, 1984, Vol. 182, pp 945-1136.

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(12) Havel, J. J.; Chan, K. H. *J. Org. Chem.* **1976**, *41*, 513-516.

(13) **Caution**: The toxicological properties of the epoxide **2** are presently being investigated. On the basis of structure and the chemical properties reported herein it should be assumed that **2** is highly toxic and carcinogenic. Manipulations should be carried out in a well-ventilated hood using disposable latex gloves. Particular care should be taken when handling crystalline material due to electrostatic properties. Aflatoxins can be destroyed by treatment with commercial NaOCl solutions.

(14) The stability of **2** is sufficient that vendors of fine chemicals should be able to market it to investigators not wanting to prepare their own material.

(15) Murray, R. W.; Jeyaraman, R. *J. Org. Chem.* **1985**, *50*, 2847-2853. Adam, W.; Chan, Y.-T.; Cremer, D.; Gauss, J.; Scheutzow, D.; Schindler, M. *J. Org. Chem.* **1987**, *52*, 2800-2803.

(16) Although water has been shown to accelerate epoxidations with dimethyldioxirane,[17] the dimethyldioxirane solution and the reaction solvent were dried (molecular sieves or K_2CO_3) to minimize hydrolysis of the epoxide.

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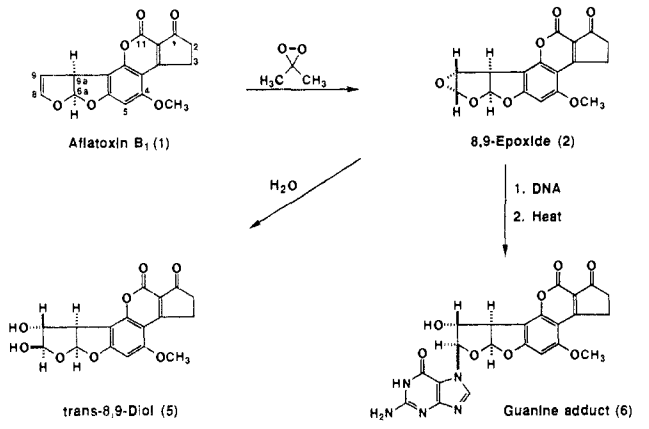
Scheme I



Scheme II

3 n = 1

4 n = 2

was complete within 15 min at room temperature giving the epoxide and acetone as the only products. The present procedure avoids the presence of electrophiles or nucleophiles. Solvent and excess dimethyldioxirane were removed by evaporation in a stream of nitrogen to leave **2** as a solid which could be recrystallized from acetone and methylene chloride.[13] The crystalline material underwent a phase transition near 230 °C but did not melt below 300 °C. The compound is stable for at least 12 h at room temperature in the solid form or in solutions of acetone and methylene chloride and can be stored for long periods at −10 °C. The infrared spectrum (Nujol mull) of **2** showed major absorptions at 1750, 1625, 1597, 1505, 1310, 1232, 1136, 990, 936, 831 cm^{-1}.

Mass spectral analysis of **2** supported the epoxide formulation. The low resolution spectrum had a prominent molecular ion at m/z 328, the empirical formula of which was confirmed by exact mass measurement as $C_{17}H_{12}O_7$ (m/z 328.0590 observed, 328.0583 calculated). Major fragments included m/z 270 (loss of $C_2H_2O_2$ from the epoxyfuran ring), m/z 242 (a further loss of CO), and m/z 214 (loss of a second CO).

The ^{13}C NMR spectrum[18] of **2** was assigned by comparison with the spectrum of AFB_1.[19] The only significant differences lie in C8 (δ 82.63 vs 145.2) and C9 (δ 56.90 vs 102.7), reflecting the conversion of the olefin to the oxirane. The ^1H NMR spectrum is shown in Figure 1B; for comparison, the spectrum of AFB_1 is shown in Figure 1A. The signals for the epoxide protons H8 and H9 appear as doublets at δ 5.48 and 4.01, $J_{8,9}$ = 1.8 Hz, $J_{9,9a}$ = <0.5 Hz. Protons 6a and 9a appear as doublets at δ 6.19 and 4.53, $J_{6a,9a}$ = 5.9 Hz. The remaining signals were not significantly altered by epoxidation. Assignments were aided by a COSY spectrum (not shown) which established coupling between the pairs of signals assigned to positions 2 and 3, 8 and 9, and 6a and 9a; correlation was not observed between H9 and H9a. By comparison, the oxirane protons of 1,3-dioxabicyclo[2.1.0]hexane (**3**), which we have prepared by oxidation of 2,3-dihydrofuran with dimethyldioxirane, appeared at δ 5.17 and 3.61 ppm (J = 1.8 Hz), while those of 1,3-dioxobicyclo[3.1.0]heptane (**4**), derived from 2,3-dihydropyran, appeared at δ 4.67 and 3.13 ppm (J = 2.7 Hz).[21] Structural effects on chemical shifts of the epoxide protons are noteworthy. The epoxide of dihydropyran has been prepared previously by other procedures;[12,20] epoxides of dihydrofurans were

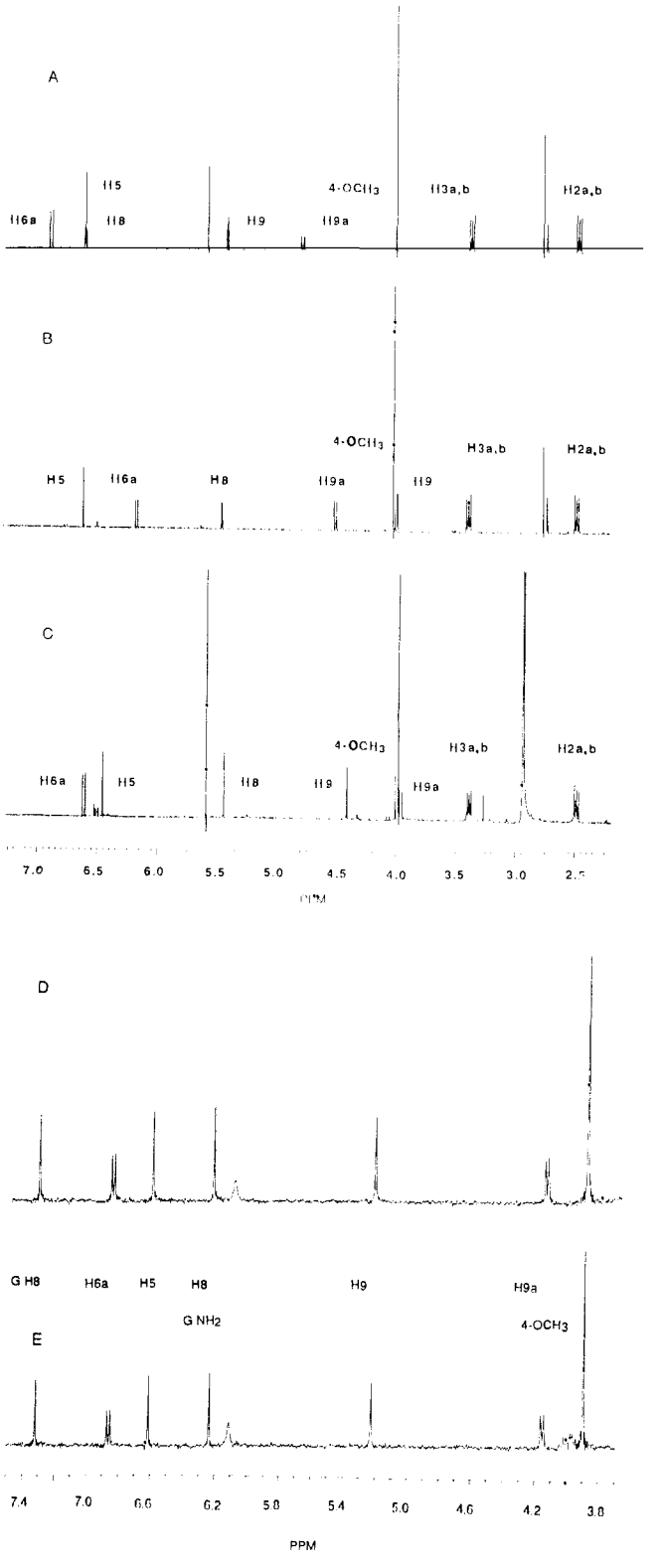


Figure 1. NMR (300.13 MHz) spectral evidence for the structure of **2** and the reaction of **2** with water and calf thymus DNA. AFB_1 (spectrum A) is epoxidized with dimethyldioxirane to yield epoxide **2** (spectrum B). The epoxide is converted to diol **5** (spectrum C) upon addition of water and a trace of acid. AFB_1-8,9-epoxide (a concentrated stock solution in acetone) was added directly to aqueous calf thymus DNA; hydrolysis and reverse-phase purification of the DNA adduct gave adduct **6** (spectrum D). This spectrum is identical with that of 8,9-dihydro-8-(N^7-guanyl)-9-hydroxyaflatoxin B_1 obtained by in situ oxidation of AFB_1 with 3-chloroperoxybenzoic acid/CH_2Cl_2 in the presence of calf thymus DNA (spectrum E). Spectra A–C were obtained in acetone-d_6; D and E were obtained in dimethylsulfoxide-d_6. The 9-hydroxy signal in spectra D and E is not observed due to rapid exchange caused by moisture in the NMR sample.

(17) Baumstark, A. L.; Vasquez, P. C. *J. Org. Chem.* **1988**, *53*, 3437–3439.

(18) ^{13}C NMR (acetone-d_6) δ 29.27 (C3), 35.29 (C2), 46.75 (C9a), 55.62 (C9), 56.90 (4-OCH_3), 82.63 (C8), 91.36 (C5), 104.42 (C3b), 102.25 (C9b), 112.10 (C6a), 117.80 (C11a), 153.89 (C9c), 154.92 (C11), 162.80 (C4), 166.92 (C5a), 177.59 (C3a), 201.48 (C1).

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previously unknown. The monoepoxides of furans are unstable, isomerizing to 1,4-dicarbonyl compounds;[22] in selected cases the epoxides can be observed at low temperature.[23] The bis-epoxide of methyl 3-furoate has been prepared.[24] It should be noted that the chemical shifts (δ 4.27 and 5.61) for H4 and H5 and the coupling between them (J = 1.5 Hz) in the bis-epoxide compare favorably with the values for H9 and H8 in **2**.

The lack of detectable coupling between H9 and H9a in epoxide **2** requires[25] a torsional angle of ~90° establishing that the epoxide has the exo configuration, i.e., dimethyldioxirane attacks the 8,9-double bond in AFB_1 from the less hindered exo face. Confirmatory evidence for the stereochemistry of epoxidation was obtained from nuclear Overhauser difference spectra; strong nuclear Overhauser effects were observed between protons H8 and H9 and between H6a and H9a, but only a weak effect was seen between anti protons H9 and H9a.

Introduction of ^2H_2O and a trace of ^2HCl into the acetone-d_6 NMR sample of epoxide **2** caused hydrolysis to give *trans*-8,9-diol **5**[26] in essentially quantitative yield. The NMR signals for protons H6a, H8, H9, and H9a of **3** (Figure 1C) appeared at δ 6.65, 5.45, 4.43, and 3.97 with $J_{6a,9a}$ = 5.9 Hz, $J_{8,9}$ = <0.5 Hz, and $J_{9,9a}$ = <0.5 Hz.

Epoxide **2** reacts with DNA with regio- and stereospecificity at the N^7 position of deoxyguanosine. Treatment of calf thymus DNA with **2** (12 h at pH 6.5 and 5 °C) produced extensive covalent reaction. Acid hydrolysis of purine–deoxyribose linkages followed by reverse-phase HPLC purification yielded an adduct identified as 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-AFB_1 (**6**) by ^1H NMR (Figure 1D); the spectrum was identical with spectra reported for the adduct produced from AFB_1 by microsomal activation and by chemical activation with 3-chloroperoxybenzoic acid.[5,6,27,28] The spectrum shown in Figure 1E was obtained from adduct formed from calf thymus DNA to which AFB_1 had been bound using in situ activation[6] with 3-chloroperoxybenzoic acid.

The preparation of DNA adducts by in situ chemical activation has serious problems. Activation of AFB_1 with peroxy acids requires a two-phase system with the oxidant and aflatoxin in methylene chloride and the DNA in aqueous buffer.[3] A single-phase system is unacceptable due to oxidative modifications of the DNA by the peroxy acid. The two-phase system is limited to nonbiological systems because of the destructive effect of organic solvents on cells. Photooxidative activation[4] requires intense UV light which is mutagenic in its own right. In addition, it is possible that psoralen-type nonoxidative DNA photoadducts are being formed.[29,30] Some research groups have used 8,9-dihalides as surrogates for the epoxide for in vivo studies.[8] Although the dihalides are highly mutagenic, their relevance to the molecular mechanism of action of **2** is questionable since the adducts derived from the dihalides have different structures. Certainly, the availability of AFB_1 epoxide will now facilitate investigations ranging from chemical activation of oncogenes to the total metabolism of this human carcinogen.

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Stereoselective 3 + 2 and Stereospecific 2 + 2 Cycloaddition Reactions of Alkenes and Quinones

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In search of new cycloaddition reactions for the stereoselective preparation of carbocyclic and heterocyclic ring systems,[1] we have found that highly substituted dihydrobenzofurans and bicyclo[4.2.0]octenediones can be obtained stereoselectively via titanium(IV)-catalyzed addition of unactivated alkenes to quinones.[2,3] More importantly, the bicyclo[4.2.0]octenediones are produced stereospecifically. The nature of the product formed is dependent upon substituents present on the alkene and the quinone and on the catalyst.

Thus, *trans*- or *cis*-β-methylstyrenes, **1** or **2**, bearing strong electron-donating groups on the aromatic ring (X = 2- or 4-OMe) stereoselectively produce *trans*-dihydrobenzofurans **6**[4,5] in good yield upon reaction with 2-alkoxy-1,4-benzoquinones **3a–c** or benzoquinone **3d** and $TiCl_4$ or $TiCl_4/Ti(OiPr)_4$ mixtures (Scheme

(1) For a compilation of many examples, see: (a) Desimoni, G.; Tacconi, G.; Barco, A.; Piero Pollini, G. *Natural Product Synthesis through Pericyclic Reactions*; ACS Monograph 1980; American Chemical Society: Washington, DC, 1983. See, also: (b) Inouye, Y.; Kakisawa, H. *Bull. Chem. Soc. Jpn.* **1971**, *44*, 563. (c) Lora-Tamayo, M. *Tetrahedron* **1958**, *4*, 17.

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(3) Enamines, enols, and allylsilanes are known to give benzofuran and dihydrobenzofuran products (3 + 2 cycloadducts) upon reaction with quinones, for examples, see: (a) Allen, G. R. In *Organic Reactions*; Dauben, W. G., Ed.; Wiley: New York, 1973; Vol. 20, p 358. (b) Brimble, M. A.; Gibson, J. J.; Baker, R.; Brimble, M. T.; Kee, A. A.; O'Mahony, M. J. *Tetrahedron Lett.* **1987**, *28*, 4891. (c) Eugster, C. H.; Kuser, P. *Chimia* **1964**, *18*, 358. (d) Eugster, C. H.; Fumagalli, S. E. *Helv. Chim. Acta* **1971**, *54*, 959. (e) Maruyama, K.; Tai, S. *Chem. Lett.* **1985**, 681. (f) Banville, J.; Grandmaison, J. L.; Lang, G.; Brassard, P. *Can. J. Chem.* **1974**, *52*, 80. (g) Brannock, K. C.; Burpitt, R. D. U.S. Patent 3285937; *Chem. Abstr.* **1967**, *66*, 37762p. (h) Bernatek, E. *Acta Chem. Scand.* **1953**, *7*, 677. (i) Giza, C. A.; Hinman, R. L. *J. Org. Chem.* **1964**, *29*, 1453. (j) Grinev, A. N.; Pan, B.-K.; Terent'ev, A. P. *Zh. Obshch. Khim.* **1957**, *27*, 1087; *Chem. Abstr.* **1958**, *52*, 3761e. (k) Maruyama, K.; Naruta, Y.; Uno, H. *Tetrahedron Lett.* **1981**, 5221. For a theoretical discussion on the regioselectivity of these reactions, see: Rozeboom, M. D.; Tegmo-Larsson, I.-M.; Houk, K. N. *J. Org. Chem.* **1981**, *46*, 2338.

(4) All new compounds gave satisfactory spectra (^1H and ^{13}C NMR, IR, mass), elemental analysis, and/or exact mass. Full spectral and crystallographic data will be provided in the full paper.

(5) The methyl signal of the *trans*-2-aryl-3-methyl-2,3-dihydrobenzofurans appears at 1.3–1.4 ppm, whereas the methyl signal of the corresponding *cis*-dihydrobenzofuran isomers appears at ~0.7 ppm, for examples, see: (a) Gregson, M.; Ollis, W. D.; Redman, B. T.; Sutherland, I. O.; Dietrichs, H. H.; Gottlieb, O. R. *Phytochemistry* **1978**, *17*, 1395. (b) Gottlieb, O. R.; Mourao, J. C.; Yoshida, M.; Mascarenhas, Y. P.; Rodrigues, M.; Rosenstein, R. D.; Tomita, K. *Ibid.* **1977**, *16*, 1003. (c) Gottlieb, O. R.; DaSilva, M. L.; Ferreira, Z. S. *Ibid.* **1975**, *14*, 1825. (d) Aiba, C. J.; Fernandes, J. B.; Gottlieb, O. R.; Maia, J. G. S. *Ibid.* **1975**, *14*, 1597. (e) Schmid, E.; Frater, G.; Hansen, H.-J.; Schmid, H. *Helv. Chim. Acta* **1972**, *55*, 1625.

(21) Oxirane **3**: ^1H NMR (acetone-d_6) δ 1.88 (m, H4), 2.17 (m, H4′), 3.58 (m, H5), 3.61 (m, H3), 3.98 (m, H5′), 5.17 (d, J = 1.8 Hz, H2); ^{13}C NMR (acetone-d_6) δ 27.51 (C4), 56.92 (C3), 65.49 (C5), 82.24 (C2). Oxirane **4**: ^1H NMR (acetone-d_6) δ 1.35–1.48 (m, H5), 1.54–1.65 (m, H5′), 1.87–2.13 (H4 and H4′), 3.13 (m, H3), 3.47 (m, H6), 3.61 (m, H6′), 4.67 (d, J = 2.7 Hz, H2); ^{13}C NMR (acetone-d_6) δ 20.74 (C4 or C5), 22.56 (C5 or C4), 51.58 (C3), 62.95 (C6), 77.20 (C2).

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