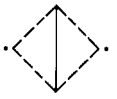
termediate. Furthermore, analysis of the ground state eigenvector indicates that the most significant structure is the singlet diradical

which is consistent with the cycloaddition reactions of 1,3-di-*tert*-butylcyclobutadiene.[9] The associated triplet diradical



is of course ruled out as the transition state in this reaction because there is little distortional stabilization in the triplet state.

Of course the inclusion of strain energy will appreciably modify the surface. It should be pointed out, however, that the π-electronic stabilization is quite large and it seems quite possible that the effects of strain energy will not completely negate the conclusions.

Acknowledgment. We wish to thank Professor R. Pettit for suggesting this problem to us. We also acknowledge the support of the Robert A. Welch Foundation, Houston, Texas.

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E. A. Halevi
Department of Chemistry, Technion Institute
Haifa, Israel

F. A. Matsen, T. L. Welsher*
Department of Chemistry, Department of Physics
University of Texas
Austin, Texas 78712

Received May 24, 1976

The Nuclear Overhauser Enhancement of the ^{31}P Magnetic Resonance Spectrum of Inorganic Orthophosphate in Aqueous and Nonaqueous Media

Sir:

The magnitude of the nuclear Overhauser enhancement[1,2] (NOE) of the ^{31}P resonance signals of phosphates resulting from strong irradiation of protons depends on several factors present in the solvating medium, namely, the pH, the nature of the countercation, the concentration of the phosphate, the ionic strength of aqueous solutions, and whether the solvent is water, deuterium oxide, or an anhydrous organic liquid. In this communication, some data are presented to show these dependencies and to explain some of the problems we have encountered in quantitating proton-decoupled ^{31}P spectra. These findings may also explain some quantitative differences between previously published[3] ^{31}P NOE data and similar quantitative ^{31}P data being gathered in a number of different laboratories where the ^{31}P NOE was apparently not present.[4,5]

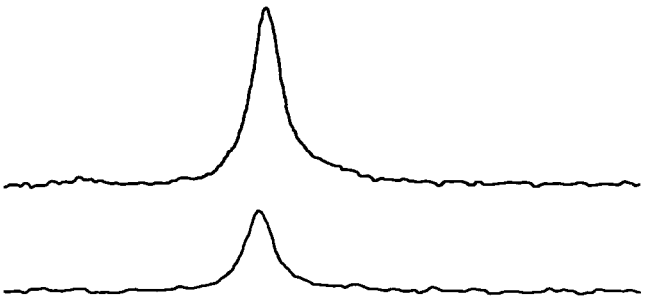


Figure 1. Comparable ^{31}P NMR spectra of tetra-*n*-hexylammonium orthophosphate (0.01 M) in 50% benzene–cyclohexane showing the nuclear Overhauser enhancement effect. The bottom spectrum is the normal ^{31}P signal obtained without irradiation of protons. The top spectrum shows the effect of strong proton irradiation; the NOE factor[2] is 2.21.

For this work, the ^{31}P spectra were obtained on a Bruker HFX-5 spectrometer[6] operating at 36.43 MHz for ^{31}P (90.00 MHz for ^{1}H) and equipped for continuous-wave and broadband, homo- and heteronuclear, ^{1}H and ^{31}P decoupling and Fourier transform[5] detection of resonances with signal averaging. Ten or thirteen millimeter spinning sample tubes were employed at a temperature of 31 °C, and the spectrometer was field-frequency stabilized through use of a 1-mm concentric capillary containing the stabilization reference, D_2O, and the phosphorus intensity reference, methylene diphosphonate (pD 9.5, sodium countercation), which shows no NOE in D_2O solution.[5] To ensure precision in the intensity measurements, 16K data point spectra were gathered employing a sweep width of 1250 Hz (400 μs per data point) and an 8-s cycling time. Signal widths at half height were almost always greater than 2 Hz,[7] and with triplicate measurements the NOE values could usually be determined to $\pm 1\%$ on the relatively concentrated (0.01 M in P) samples of this study.

Large well-formed crystals of orthophosphoric acid[8] (Matheson, Coleman and Bell) were dissolved in deionized water and neutralized with the appropriate concentrated base (10 M NaOH or concentrated tetra-*n*-butyl- or tetra-*n*-hexylammonium hydroxides) to produce the samples of this study. The sodium chloride was recrystallized from deionized water; the free acid of EDTA (ethylenediamine-tetraacetic acid) and the D_2O were commercial preparations. For aqueous solution studies, the appropriate salt of EDTA (0.001–0.1 M) was added to the samples to ensure that diminished NOE factor values were not the result of paramagnetic contributions to the ^{31}P T_1 relaxation. Such contributions were not likely with the nonaqueous systems since the theoretical[1,2,9] NOE was detected, implying that the predominant T_1 relaxation mechanism operating was dipole–dipole relaxation.

The anhydrous samples were prepared by evaporation of aqueous tetra-*n*-hexylammonium phosphate solutions at 30 °C, drying each sample by successive evaporations from acetone and benzene followed by anhydrous benzene, and finally purging the sample of benzene by evaporations from neat cyclohexane.[10] Only very dry preparations of hexylammonium phosphates are soluble to any significant degree in cyclohexane.

Figure 1 shows a typical ^{31}P NOE experiment. The sample was an equimolar mixture of the mono- and dianionic forms of the tetra-*n*-hexylammonium orthophosphates in a 50% benzene in cyclohexane solution. The top trace was obtained while strongly irradiating a broad band of the ^{1}H NMR (nuclear magnetic resonance) spectrum; the bottom trace shows the standard "proton coupled" ^{31}P spectrum. There are no changes in the spectral features of this resonance upon irra-

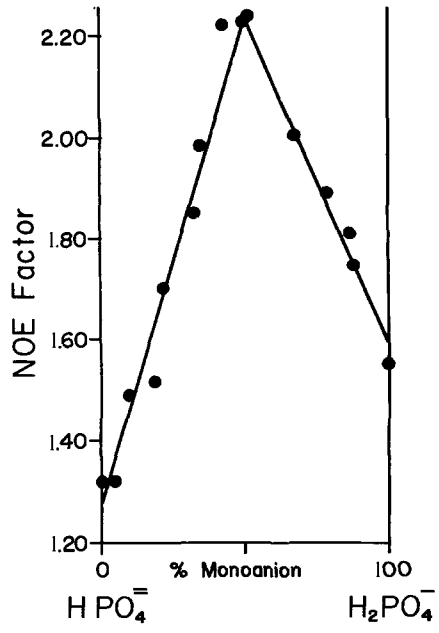


Figure 2. The NOE factor observed for a series of preparations of tetra-*n*-hexylammonium orthophosphate in cyclohexane as a function of the mole ratio of the mono- and dianionic salts. Substitution of deuterium for the exchangeable protons in all cases results in an NOE factor of 1.00; i.e., the enhancement is eliminated.

diation of protons except for a marked change in signal area; the chemical shift (−1.98 ppm) and the signal width at half height (6.1 Hz) remain constant; i.e., this is a case where the NOE is not accompanied by a "decoupling" of the spectrum. The NOE factor[2] is 2.21.

Figure 2 shows the relationship between the NOE factor and the relative proportions of the othophosphate mono- and dianions in anhydrous cyclohexane solutions. The maximum NOE of 2.23 is obtained when there is an equal proportion of mono- and dianions in solution. (For ax systems of the type ^{31}P-^{1}H, the maximum NOE factor is 2.235,[9] i.e., a 123.5% increase in the signal area.) On either side of this point the NOE falls off in a linear fashion with the mole ratio of the two anions. Observations involving ionic species other than those indicated in Figure 2 were not possible because of solubility limitations; however, what measurements could be made showed that the total relationship was oscillatory in nature since the NOE values progressively increased beyond the limits of Figure 2. This behavior is similar to that observed[11] for the pH dependence of the ^{31}P T_1 relaxation times of quaternary ammonium orthophosphates in water.

The orthophosphate NOE observed with aqueous solutions also varies with the anionic form. At pH 0.5, which corresponds to the free acid, I have observed an NOE factor of 2.05 using a 0.01 M solution (see ref 3 where a value of 1.30 was obtained, presumably with the neat acid). At the same concentration of P, the tetra-*n*-butylammonium salt at pH 6.8 (pK_a 2) shows no enhancement (NOE factor 1). However, at pH 6.8 and 0.01 molar concentrations the corresponding sodium salt shows an NOE factor of 1.31 whereas the potassium salt shows only 1.15, thus demonstrating that the NOE factor also varies with the nature of the countercation.[12] Disolution of the samples in D_2O, or replacement of the exchangeable protons with deuterium eradicates the enhancement.

Moreover, the NOE factor may also vary with concentraton as shown in Figure 3 where the reciprocal of the NOE factor is plotted as a function of the concentration of tetra-*n*-butylammonium phosphate at pH 6.8 (pK_a 2). No enhancement is observed until the phosphate concentration is about 1.3 M. Thereafter, the reciprocal of the NOE factor decreases in a

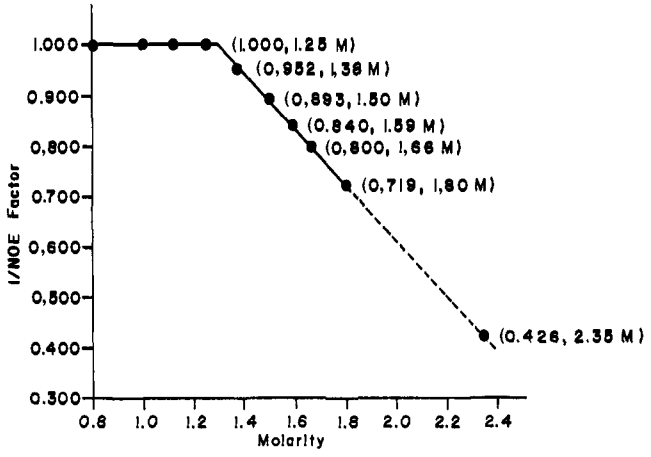
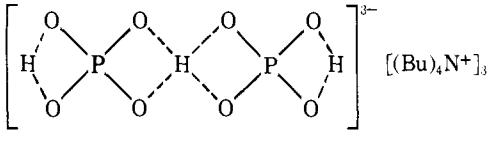


Figure 3. The reciprocal of the ^{31}P NOE factor of tetra-*n*-butylammonium orthophosphate vs. the molarity of the phosphate in simple aqueous solution at pH 6. The 1/NOE values and their molarities are indicated in the figure. The extrapolated point corresponds to an NOE of 2.235 at the molarity of the neat salt.

relatively linear fashion.[13] When this curve is extrapolated to the value corresponding to the maximum theoretical enhancement for a ^{31}P-^{1}H ax system, the phosphorus concentration indicated corresponds to that in the neat salt, $[(Bu)_4N^+]_{1.5}H_{1.5}PO_4^{-1.5}$, or the corresponding mixed salt existing at the pK_2 of phosphoric acid.

The effect indicated in Figure 3 may reflect the progressive depletion of water in the sample or perhaps the progressive ordering of that amount of water remaining, since a similar phenomonon was also observed with the sodium phosphates upon the addition of molar quantities of sodium chloride. The NOE may simply reflect the ionic strength of the solution.[12]

On the molecular level, the data of Figure 2 may be interpreted to indicate that in anhydrous media a new species, perhaps a simpler dimer, is formed at a 1:1 mole ratio of the mono- to the dianion. Such a structure could involve a tetrahedrally coordinated proton and two nested protons such as are observed in crystalline orthophosphoric acid.[14]



It is clear, however, that the ^{31}P NOE depends on a large number of factors and that the contribution of each of these factors to the total phenomenon will have to be evaluated before a cogent interpretation of orthophosphate NOE data can be made. Further, other experiments[15] indicate that similar data will be obtained with any phosphate so that the entire question of ^{31}P NOE values and their meaning depends upon the accumulation of considerably more data than are currently available.

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Thomas Glonek
Research Resources Center
University of Illinois at the Medical Center
Chicago, Illinois 60612
Received June 1, 1976

Baccharin, a Novel Potent Antileukemic Trichothecene Triepoxide from *Baccharis megapotamica*[1,2]

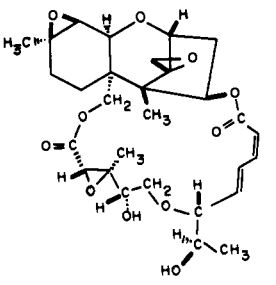
Sir:

In the course of a continuing search for tumor inhibitors from plant sources, we found that an ethanol extract of *Baccharis megapotamica* Spreng (Asteraceae)[3] showed significant inhibitory activity in vitro against cells derived from human carcinoma of the nasopharynx (KB) and in vivo against P-388 leukemia in mice.[4] We report herein the isolation and structural elucidation of one of the novel and very potent antileukemic principles, baccharin (**1**), which appears to be the first reported trichothecene triepoxide isolated from natural sources.

Fractionation of the alcohol extract, guided by a combination of P-388 in vivo assay in mice and KB testing in vitro showed that the inhibitory activity was concentrated, successively, in the ethyl acetate layer of an ethyl acetate–water partition and in the methanol layer of a 90% aqueous methanol–petroleum ether partition. Column chromatography on alumina (activity II, III) gave several active fractions; chromatography on Silica Gel 60 of one of these fractions followed by preparative TLC on Silica Gel 60 afforded baccharin (**1**, 0.004%): mp 200–230 °C (dichloromethane–methanol); $[\alpha]^{24}D$ +41.5° (*c* 2.2, $CHCl_3$); uv_{max} (EtOH) 259 nm (ϵ 18 700); ir ($CHCl_3$) 2.72, 2.9 (broad), 5.68, 5.80, 6.08, and 6.22 μ; mass spectrum (chemical ionization: methane gas reagent) *m/e* 563.2476 ($M^+ + H$, calcd 563.2492), 409, 275, 257, 137; NMR ($CDCl_3$) δ 0.75 (3 H, s, 14-H), 1.20 (3 H, d, J = 5.6 Hz, 14′-H), 1.37 (3 H, s, 16-H), 1.65 (3 H, s, 12′-H), 2.48 (1 H, d of d, $J_{3\alpha,4\alpha}$ = 8.8; $J_{3\alpha,3\beta}$ = 16 Hz, 3α-H), 2.75, 3.16 (each 1 H, d, J = 4.0 Hz, 13-H), 3.11 (1 H, d, $J_{10,11}$ = 5.8 Hz, 10-H), 3.37 (1 H, s, 2′-H), 4.24, 4.42 (each 1 H, d, J = 12.2 Hz, 15-H), ≈5.80 (1 H, 4-H), 5.82 (1 H, d, $J_{9',10'}$ = 11 Hz, 10′-H), 5.98 (1 H, d of d, $J_{6',7'}$ = 2; $J_{7',8'}$ = 15.5 Hz, 7′-H), 6.60 (1 H, d of d, $J_{8',9'} = J_{9',10'}$ = 11 Hz, 9′-H), 7.48 (1 H, d of d, $J_{7',8'}$ = 15.5; $J_{8',9'}$ = 11 Hz, 8′-H).

Treatment of baccharin (**1**) at room temperature with acetic anhydride in pyridine gave baccharin diacetate, mp 254–256 °C; uv_{max} (EtOH) 262 nm (21 000); ir ($CHCl_3$) 5.75 (broad with shoulders), 6.10 and 6.25 μ; mass spectrum (chemical ionization: methane gas reagent) *m/e* 647.2692 ($M^+ + H$, calcd 647.2704); NMR ($CDCl_3$) δ 0.75 (3 H, s, 14-H), 1.24 (3 H, d, J = 6.6 Hz, 14′-H), 1.36 (3 H, s, 16-H), 1.74 (3 H, s, 12′-H), 2.03, 2.16 (each 3 H, s, $-COCH_3$), 2.46 (1 H, d of d, $J_{3\alpha,4\alpha}$ = 8.0; $J_{3\alpha,3\beta}$ = 16 Hz, 3α-H), 2.76, 3.16 (each 1 H, d, J = 3.9 Hz, 13-H), 3.07 (1 H, d, $J_{10,11}$ = 5.3 Hz, 10-H), 3.37 (1 H, s, 2′-H), 4.23, 4.49 (each 1 H, d, J = 12.5 Hz, 15-H), ≈4.30 (1 H, 4′-H), 5.06 (1 H, d of q, $J_{12',13'}$ = 6.6, $J_{12',6'}$ = 4 Hz, 13′-H), ≈5.75 (1 H, 4-H), 5.80 (1 H, d, $J_{9',10'}$ = 11 Hz, 10′-H), ≈5.90 (1 H, 7′-H), 6.60 (1 H, d of d, $J_{8',9'} = J_{9',10'}$ = 11 Hz, 9′-H), 7.47 (1 H, d of d, $J_{7',8'}$ = 15.5; $J_{8',9'}$ = 11 Hz, 8′-H).

Inspection of the above spectral data for baccharin (**1**) and its diacetate suggested that these compounds were related to the roridins[5] whose structures are distinguished by a 12,13-epoxytrichothecene central ring system which is spanned by a dienic macrolide ester side chain. However, careful comparison of the spectral data for **1** and its diacetate (as well as the spectral data for a number of structurally similar compounds that have been isolated from *B. megapotamica*) with the data published for the roridins[5,6] clearly showed that **1** (and other so far isolated active principles) is more highly oxygenated than the known roridins.[7]



1

The chemical structure and molecular stereochemistry of **1** were determined by a direct single-crystal x-ray analysis using a crystal obtained by slow evaporation of solvent from a solution of **1** in dichloromethane–methanol. Crystals of **1** conform to space group $P2_12_12_1$ with a = 10.389 (1) Å, b = 30.160 (2) Å, c = 10.172 (1) Å, and Z = 4. Intensity measurements were made by diffractometry with Cu Kα radiation made monochromatic by Bragg reflection from a highly oriented graphite crystal. Within a single octant of reciprocal space, surveyed to sin θ/λ 0.562, scattered intensity significantly above background [$I > 3\sigma(I)$] was measured by scintillation counting at 2471 of 2726 locations.

The structure was solved by application of the program MULTAN[9] and refined by difference Fourier and least-squares method to R = 0.07 for the significant reflections. C and O atoms were refined using anisotropic thermal parameters, and all hydrogen atoms in the molecule, with the exception of those associated with the hydroxy groups, were clearly identifiable from different maps, and fixed contributions for them were included in the least-squares calculations. Loosely bound water of solvation is found at four sites in the asymmetric unit with one site fully and the others partially occupied.

A view of the molecular structure of **1** as found in the crystal is shown in Figure 1. Although the absolute configuration of **1** has not been established by the analysis, the figure is drawn to conform to the absolute stereochemistry derived from the x-ray analysis of the *p*-iodobenzenesulfonate of verrucarin A.[10] The 12,13-epoxytricothecene structure is confirmed with epoxide functionalities found also at C(9)–C(10) and C(2′)–C(3′), a hydroxy group at C(4′), and a hydroxyethyl group at C(6′). In the central nucleus, the six-membered oxa-ring B adopts a chair conformation, the five-membered ring C an envelope form with the flap at C(12). The presence of the 9,10 epoxide unit, which acts stereochemically as a double bond, leads to a 1,2-diplanar conformation for the six-membered A ring typical of substituted cyclohexenes.

Note should be taken that isolation of **1** and related compounds from *B. megapotamica* constitutes the first known case of the appearance of 12,13-epoxytrichothecenes in higher plants; all previous isolations of such compounds have been from various species of fungi. At the moment, we cannot exclude the possibility that these compounds result from fungal