from daunomycin for which the half-life under similar conditions is 53 s.[13] Furthermore, the availability of reducing enzymes and the concentration of hydroquinones produced by them may have an important effect on the binding of the quinone methide from menogaril to biological macromolecules. The slow uncatalyzed tautomerization of the quinone methide and its reactivity with nucleophiles may explain the low recovery of menogaril and its metabolites in animal[14] and human[15] metabolic studies.

(13) Kleyer, D. L.; Koch, T. H. *J. Am. Chem. Soc.* **1984**, *106*, 2380.
(14) Dodion, P.; Egorin, M. J.; Engisch, K. L.; Bachur, N. R. *Cancer Res.* **1985**, *45*, 5352.
(15) Egorin, M. J.; Van Echo, D. A.; Whitacre, M. Y.; Forrest, A.; Sigman, L. M.; Engisch, K. L.; Aisner, J. *Cancer Res.* **1986**, *46*, 1513.

High-Resolution Electrophoretic NMR

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Here we report a high-resolution electrophoretic NMR experiment which is based on pulsed field gradient FT-NMR. The NMR system is described, and spectra in the presence of electric fields are shown for alkylammonium ions separately and in a mixture. The combination of electrophoresis with high-resolution NMR offers relatively painless determination of mobilities μ for ions in mixtures with internal calibration and with direct indication of complicating effects such as convection and electro-osmosis. It also opens new possibilities for spectral editing in NMR and for the measurement of rates of ionic reactions.

Pulsed field gradient NMR (PFGNMR) was developed by Tanner and Stejskal for the measurement of diffusion and flow.[1,2] The application of this technique to the study of ionic drift in electric fields was apparently first suggested by Packer.[3] Holz and Muller investigated magnetic fields resulting from current flow and found that undesirable gradients are minimized when the electric and magnetic fields are parallel.[4] They then used the parallel field geometry for a demonstration of electrophoretic NMR in a low field proton spin-echo NMR system which permitted no resolution of chemical shifts. In their experiment large concentrations of ions were apparently required to permit detection. The ionic strengths were large, and currents up to 250 mA were used to obtain the necessary electric fields. Accordingly, heating was a severe problem, and the samples required stabilization by a gelling agent.[5]

The experiment described here involves electrophoresis in free solution in a capillary tube. The total ionic concentrations are in the 10-mM range, and current densities and durations are kept below 0.5 A/cm^2 and 1 s, respectively, so that heating effects and convection can be avoided.[6] In the standard PFGNMR sequence, two magnetic field gradient pulses, of amplitude G and duration δ, are inserted into the spin echo sequence (90°-τ-180°). The first is applied between the 90 and 180° pulses, and the second is between the 180° pulse and the echo as shown in Figure 1. In our version of the electrophoretic experiment, an electric field pulse of amplitude E_{dc} is applied during the interval t_f between the two gradient pulses, and the polarity of this field is alternated in successive pulses. For convenience in dealing with the electrodes,

(1) Stejskal, E. O.; Tanner, J. E. *J. Chem. Phys.* **1965**, *42*, 288-292.
(2) Stejskal, E. O. *J. Chem. Phys.* **1965**, *43*, 3597-3603.
(3) Packer, K. J. *Mol. Phys.* **1969**, *4*, 355-368.
(4) Holz, H.; Muller, Ch. *Ber. Bunsenges. Phys. Chem.* **1982**, *86*, 141-147.
(5) Holz, M.; Lucas, O.; Muller, C. *J. Magn. Reson.* **1984**, *58*, 294-305.
(6) Jorgenson, J. W. In *New Directions in Electrophoretic Methods*; Jorgenson, J. E., Phillips, M., Eds.; American Chemical Society: Washington, DC, 1987; pp 182-198.

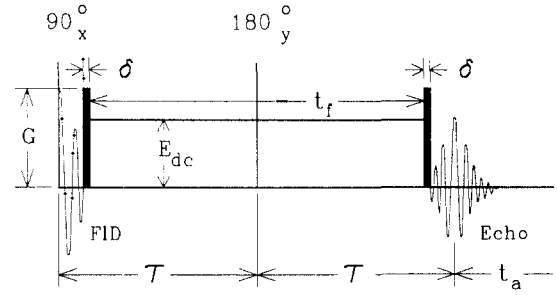

Figure 1. The pulse sequence for high resolution electrophoretic NMR. The acquisition time variable is t_a (see text).

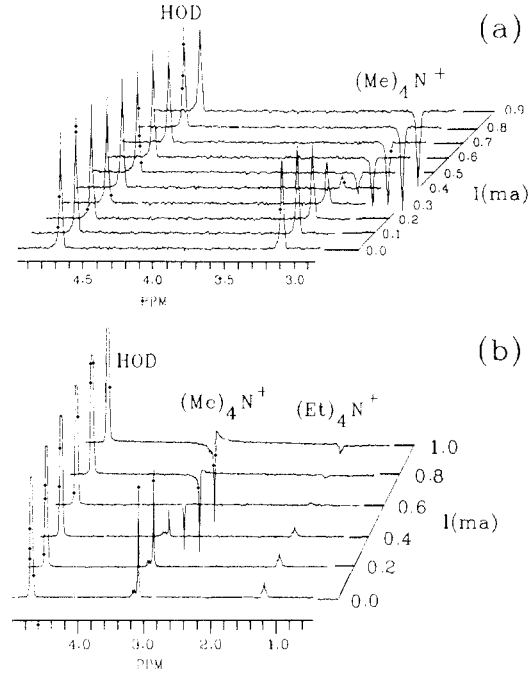

Figure 2. NMR spectra obtained with K = 310 cm^{-1}, δ = 0.75 ms, t_f = 0.75 s, and T = 298 K: (a) 10 mM Me_4NCl in D_2O and (b) 5 mM Me_4NCl and 5 mM Et_4NCl in D_2O. For an ion with mobility μ, the signal amplitudes are proportional to $\cos[K\mu t_f I/(\sigma\kappa)]$ (see text).

a vertical U-tube cell was adopted which gives a counterflow of ions.[4] With this arrangement the echo amplitude at 2τ is given by[2,4,7]

$$M(2\tau) = M(0) \cos(Kvt_f) \exp[-DK^2(t_f + 2\delta/3) - 2\tau/T_2] \quad (1)$$

where $K = \gamma G\delta$, $v = \mu E_{dc}$, D is the tracer diffusion coefficient, and T_2 is the nuclear spin–spin relaxation time. Also, $E_{dc} = I/(\sigma\kappa)$ where I is the current, σ is the cross-sectional area of the tube, and κ is the conductivity. In the FT experiment, data collection commences at the peak of the echo.[8] Fourier transformation then produces an NMR spectrum in which the peaks of the various species show attenuations as described by eq 1.

The experiments were performed on a Bruker WM 250 NMR spectrometer with a custom 10-mm ^{1}H probe built by Cryomagnet Systems. A computer-controlled gradient generator, built in-house for this experiment, produced 0–10 A current pulses for periods up to 12 ms with areas (A·s) reproducible to 1 ppm. Such accurate control is achieved by means of active regulation of pulse amplitudes.[9] This unit drives a pair of opposed Helmholz coils with a separation of 13 mm which produce a gradient of 23.3 G cm^{-1} A^{-1}.[6] A 550 V electric field generator capable of providing gateable currents from 0 to 100 mA was also constructed in-house.[9] The cell consisted of Pt electrodes in a glass U-tube with a cross-sectional area of σ = 0.0268 cm^2.

(7) Saarinen, T. R.; Johnson, C. S., Jr. *J. Magn. Reson.* **1988**, in press.
(8) James, T.; McDonald, G. *J. Magn. Reson.* **1973**, *11*, 58-61.
(9) Saarinen, T. R.; Woodward, W. S. *Rev. Sci. Instrum.* **1988**, in press.

0002-7863/88/1510-3332$01.50/0

Typical results are illustrated in Figure 2 for D_2O solutions. The proton spectra in Figure 2a for a 10 mM solution of Me_4NCl show the HOD peak and the methyl proton peak at various currents. The constancy of the HOD peak amplitude for currents up to 0.8 mA indicates that electro-osmosis is negligible in a properly coated cell.[10] However, above 0.9 mA with $t_f = 0.75$ s additional attenuation, probably resulting from heat induced convention, becomes evident. Figure 2b shows proton spectra for a solution 5 mM in both Me_4NCl and Et_4NCl. Spin splitting reduces the intensity of the Et_4N^+ signals, and J modulation complicates the interpretation of the methylene intensities. However, the methyl signal for Me_4N^+ and the center line of the methyl signal for Et_4N^+ provide sufficient information for the determination of corresponding mobilities. A fit of methyl intensities versus I (Figure 2a) to a cosinusoidal function yields $\mu = 3.6 \times 10^{-4}$ cm^2 V^{-1} s^{-1} which can be compared with the zero concentration result, 3.79×10^{-4} cm^2 V^{-1} s^{-1}, from conductivity measurements.[11] For the mixture we obtain 3.9×10^{-4} cm^2 V^{-1} s^{-1} and 2.5×10^{-4} cm^2 V^{-1} s^{-1} for the methyl and ethyl ammonium ions, respectively.

The potential of this method is obvious from Figure 2. The sensitivity can be improved by the use of multiple capillary tubes and higher frequency spectrometers; however, the necessity of long T_2's remains a limitation. The technique is especially promising for small ions and for microemulsions. A two-dimensional version of the experiment, based on Fourier transformations with respect to both t_f and t_a, has also been developed for the study of velocity distributions. Applications will be published elsewhere.

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(10) Herren, B. J.; Shafer, S. G.; Alstine, J. V.; Harris, J. M.; Snyder, R. S. *J. Colloid Interface Sci.* **1987**, *115*, 46-55.
(11) Kay, R. L.; Evans, D. F. *J. Phys. Chem.* **1965**, *69*, 4216-4221.

Phenoxazinone Synthase: Enzymatic Catalysis of an Aminophenol Oxidative Cascade

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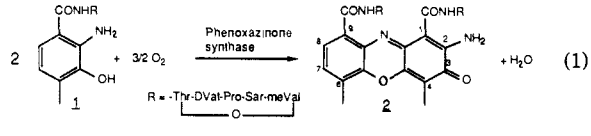
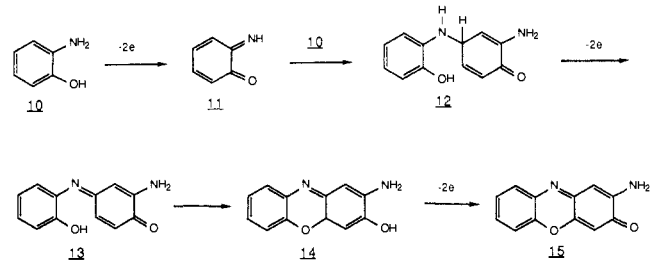
Phenoxazinone synthase catalyzes the oxidative coupling of two molecules of an aminophenol to form the phenoxazinone chromophore.[1] This reaction constitutes the final step in the biosynthesis of actinomycin **2**[2] and is a complex six-electron oxidative condensation (eq 1). The mechanism of the enzymatic reaction has not been elucidated and was of interest to us because of the novelty of the chemistry involved.



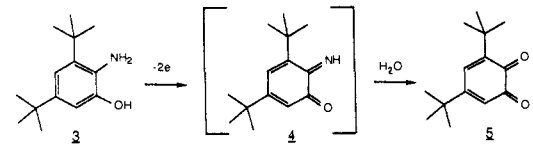
(1)

Phenoxazinone synthase has been cloned and overproduced in *Streptomyces lividans*[3] and can be readily isolated in 100-mg quantities.[4] The subunit molecular weight is 88 000 daltons. In its active form the enyzme consists primarily of a mixture of dimers and hexamers.[4] The enzyme is a copper-containing protein[5] and catalyzes the oxidation of a wide variety of aminophenols to phenoxazinones.[2]

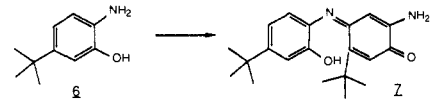
Scheme I



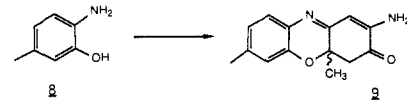
By using a variety of substituted aminophenols we have been able to block the synthesis of phenoxazinone at various intermediate stages.[6] Enzyme-catalyzed oxidation of 3,5-di-*tert*-butyl-2-aminophenol (**3**) generated the *o*-quinone **5**[7] (eq 2). This



(2)



(3)



(4)

product presumably arose from hydrolysis of the intermediate *o*-quinoneimine **4** which was too sterically hindered to undergo subsequent conjugate addition. Enzyme-catalyzed oxidation of 5-*tert*-butyl-2-aminophenol (**6**)[8] resulted in the formation of quinone-anil **7** in which the bulky *tert*-butyl group blocked the second conjugate addition (eq 3). Enzyme-catalyzed oxidation of 5-methyl-2-aminophenol (**8**) produced the dihydrophenoxazinone **9** in which the methyl group at C4a blocked the final dehydrogenation (eq 4). These results are incorporated into the mechanistic proposal shown in Scheme I.

Quinoneimine **11** reacts rapidly with aminophenol to form phenoxazinone.[9] This observation prompted us to determine if intermediates are released from the enzyme prior to phenoxazinone formation. Dihydrophenoxazinone **16**[10] was prepared and found to be instantaneously oxidized to phenoxazinone by air. To determine if the second conjugate addition occurred at the active site, the chirality at C4a of **9** was examined. Racemic **9** was

(1) Katz, E.; Weissbach, H. *J. Biol. Chem.* **1962**, *237*, 882.
(2) (a) Katz, E. *Antibiotics*; Gottlieb, D., Shaw, P. D., Eds.; Springer Verlag: New York, 1967; Vol. 2, p 276. (b) Hollstein, U. *Chem. Rev.* **1974**, *74*, 625.
(3) Jones, G.; Hopwood, D. *J. Biol. Chem.* **1984**, *259*, 14151.
(4) Choy, H.; Jones, G. *Arch. Biochem. Biophys.* **1981**, *211*, 55. Due to the high level of enzyme in the overproducing strain, we have been able to simplify the isolation procedure. Hydroxyapatite chromatography of the protein fraction precipitated by 35-45% ammonium sulfate resulted in pure enzyme.
(5) Samples were analyzed for iron, manganese, copper, molybdenum, cobalt, chromium, and nickel by inductively coupled plasma emission spectroscopy. The enzyme as isolated contained 0.5-2.0 coppers/subunit. However, addition of exogenous copper increased the activity of the preparation. The copper content of the reconstituted system is 5-7 coppers/subunit.
(6) Enzyme assays were run in sodium acetate buffer (3 mL, 100 mM, pH 5) containing 2-5 mM substrate (added from a freshly prepared stock solution in DMSO) and 5 μg of enzyme. Product formation was monitored spectrophotometrically. For product isolation, 5 mL of the assay mixture was treated with 100 μg of enzyme at 37 °C for 20 min. The reaction mixture was then extracted, and the product was purified by chromatography.
(7) This product was identified by NMR and MS comparison with literature spectra. Harmalker, S. P.; Sawyer, D. T. *J. Org. Chem.* **1984**, *49*, 3579.
(8) This was prepared by nitration followed by reduction of the corresponding phenol. (a) Kagan, H. B. *Tetrahedron Lett.* **1982**, *23*, 4315. (b) Yembrick, C. *The Oxidative Condensation of o-Aminophenols To Form Aminophenoxazinones*; Ph.D. Thesis, Ohio State University, 1961, University Microfilms International no. 62-2173.
(9) Nogami, T.; Hishida, T.; Yamada, M.; Mikawa, H.; Shirota, Y.; *Bull. Chem. Soc. Jpn* **1975**, *48*, 3709.
(10) Brockmann, H.; Seela, F. *Chem. Ber.* **1971**, *104*, 2751.

0002-7863/88/1510-3333$01.50/0