The Active Site of Staphylococcal Nuclease: Paramagnetic Relaxation of Bound Nucleotide Inhibitor Nuclei by Lanthanide Ions

(nuclear magnetic resonance/3',5'-thymidine diphosphate/gadolinium/
calcium-binding proteins/substrate geometry)

BRUCE FURIE*, JOHN H. GRIFFIN*, RICHARD J. FELDMANN†, EDWARD A. SOKOLOSKI‡, AND
ALAN N. SCHECHTER*

* Laboratory of Chemical Biology, National Institute of Arthritis, Metabolism, and Digestive Diseases; † Computer Center Branch, Division of Computer Research and Technology; and ‡ Laboratory of Chemistry, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

Communicated by C. B. Anfinsen, April 12, 1974

ABSTRACT The structure of 3',5'-thymidine diphosphate bound in the active site of staphylococcal nuclease (EC 3.1.4.7) was studied by measuring the relaxation rate enhancement of substrate analog nuclei by a paramagnetic metal ion. The lanthanide ion, Gd(III), was substituted for Ca(II) in the formation of the ternary complex of nuclease-Gd(III)-3',5'-thymidine diphosphate. Measurements were made of the transverse relaxation rates of protons and the longitudinal and transverse relaxation rates of the phosphorus nuclei of the bound nucleotide. Internuclear distances between the metal ion and atoms of the 3',5'-thymidine diphosphate nucleotide were determined from these data by the Solomon-Bloembergen equation. In general, these distances corresponded closely to those determined by previous x-ray crystallography of the thymidine diphosphate complex.

These internuclear distances were also used with a computer program and graphics display to solve for metal-nucleotide geometries, which were consistent with the experimental data. A geometry similar to the structure of the metal-nucleotide complex bound to nuclease determined by x-ray analysis was one of the solutions to this computer modeling process. For staphylococcal nuclease, the nuclear magnetic resonance and x-ray methods yield compatible high resolution information about the structure of the active site. However, differences of uncertain significance exist between the two structures.

The absence of suitable electronic and magnetic properties of calcium ions has limited spectroscopic investigations of the interaction of these ions with calcium-binding proteins. Recently, the use of lanthanide ions as substitutes for calcium and other metal ions has facilitated the study of metal ion interaction with metal-binding proteins (1-5), in particular, staphylococcal nuclease (6-9).

Staphylococcal nuclease has a single polypeptide chain of 16,900 molecular weight (10); its three-dimensional structure is known from x-ray crystallographic studies (11). Nuclease activity has an absolute requirement for calcium (10). Trivalent lanthanide ions bind tightly to nuclease, compete for a single Ca(II)-binding site, competitively inhibit enzymatic activity, and substitute for Ca(II) in the formation of

a ternary complex composed of nuclease-metal ion-nucleotide inhibitor (9).

A substantial theoretical and experimental foundation exists for the determination of absolute distances between protein-bound paramagnetic metal ions and nuclei of the protein, substrate, or bound water molecules by the use of nuclear magnetic resonance (NMR) relaxation techniques (12). This approach has been extended to the use of paramagnetic lanthanide ions in biological systems (13-15). We report here the application of this method to the examination of the solution structure of a nucleotide inhibitor bound to nuclease in the presence of gadolinium(III). A comparison of the solution structure of this nucleotide substrate analog with that determined by x-ray crystallography is discussed.

MATERIALS AND METHODS

Staphylococcal nuclease (EC 3.1.4.7; nucleate 3'-oligonucleotidohydrolase) was prepared and purified as described (16, 17). Exchangeable protons in nuclease were substituted with deuterium by successive lyophilization of a nuclease solution from 99.7% D₂O (Diaprep).

Lanthanide ions were obtained as the anhydrous chloride salts from K & K Laboratories, dissolved in D₂O, and kept acidic to prevent hydrolysis. 3',5'-dTDP (CalBiochem) was prepared in D₂O. Solutions for ³¹P studies were passed through a 1 × 7-cm column of Chelex-100 (BioRad) equilibrated with 99.7% D₂O. All solutions were adjusted to pH 6.9 by titration with DCl or NaOD.

Proton NMR spectra were recorded at 220 MHz at a probe temperature of 22°C ± 0.5° (unless stated otherwise), with a Varian Associates HR 220 NMR Spectrometer equipped with the Varian Fourier transform accessory. The solutions, contained in a 5-mm NMR tube, were given 25 or 100 transients, each of which corresponded to a 90° pulse. All chemical shift values were measured relative to external tetramethylsilane in CCl₄. Unless stated otherwise, the solutions studied (500 μl) contained 20 mM nucleotide and 0.1 M NaCl in D₂O at pH 6.9. Aliquots of lanthanide solutions (1 mM) were added to the nucleotide-protein solutions in increments of 4-10 μl. The temperature dependence of the transverse relaxation rate (1/T₂) was determined with a Varian temperature regulator to change the probe temperature.

Abbreviations: NMR, nuclear magnetic resonance; 3',5'-dTDP, 3',5'-thymidine diphosphate; (1/T₂)_M and (1/T₁)_M, paramagnetic contribution to the transverse and longitudinal relaxation rates, respectively.

^{31}P NMR spectra with proton decoupling were recorded at a probe temperature of $28^\circ\text{C} \pm 1^\circ$ on a Varian XL-100 NMR Spectrometer operating at 40.5 MHz and equipped with a Digilab computer for Fourier transform analysis. Data were collected for 20 min per spectrum, using 900 90° pulses. A D_2O external lock was used. Measurements of longitudinal relaxation rate (T_1) were obtained at 22°C by the $180^\circ\text{-}\tau\text{-}90^\circ$ pulse technique (18).

The value of $1/T_2$ was determined from the linewidths at half-height of assignable resonances and were plotted as a function of the ratio of Gd(III) concentration to nucleotide concentration. The values of the paramagnetic contribution to the transverse and longitudinal relaxation rates [$(1/T_2)_\text{M}$ and $(1/T_1)_\text{M}$, respectively] were determined by linear extrapolation of these data to a 1:1 complex of Gd(III):nucleotide and subtraction of the diamagnetic contribution from the $(1/T_2)_\text{observed}$ and $(1/T_1)_\text{observed}$.

Equilibrium dialysis was performed with Visking 20 dialysis tubing (Union Carbide) prepared in 1% NaHCO_3 at 100° for 10 min in a cell with a 2-ml compartment divided by the dialysis membrane. Experiments used 40 mM $3',5'\text{-dTDP}$ in D_2O (pH 6.9), GdCl_3 in D_2O , and nuclease. After equilibration for 8 hr, samples of each solution were removed, and the proton NMR spectra were obtained.

The structure of the bound nucleotide inhibitor was examined with a DEC PDP-10 computer equipped with an Adage AGT-30 graphics display computer and a DEC-34D graphics terminal (19). The structure of $3',5'\text{-dTDP}$, from the coordinates taken from x-ray crystallographic data (11), was modified by addition of protons using known bond angles and bond lengths. Within the constraints of the atomic Van der Waal radii, the covalent bond lengths, and the distances from Gd(III) to atoms of the nucleotide inhibitor determined experimentally, a series of possible conformations was generated. This was accomplished by rotation of the N1-C1' , C4'-C5' , C5'-O5' , and C3'-O3' bonds in increments of 10° or less and the use of a least mean squares analysis for determining the position of Gd(III) §.

RESULTS

The proton magnetic resonance spectrum of $3',5'\text{-dTDP}$ at 220 MHz showed a singlet at 7.63 ppm (C6-H), a triplet at 6.18 ppm (C1'-H), a singlet at 4.11 ppm (C4'-H), a singlet at 3.81 ppm (C5',5''-H), a broad multiplet centered approximately at 2.5 ppm (C2',2''-H), and a large singlet at 1.74 ppm (CH_3). These assignments are based on the analysis by Ts'o *et al.* (20) of related compounds. The ^{31}P spectrum of $3',5'\text{-dTDP}$ at 40.5 MHz showed two overlapping resonances at 110.08 ppm upfield of P_4O_6 .

The effect of temperature on the paramagnetic and diamagnetic transverse relaxation rate of the nucleotide protons was determined in order to establish whether $3',5'\text{-dTDP}$ was in rapid or slow exchange with the nuclease-metal ion complex. A decrease in the observed transverse relaxation rate with increasing temperatures was observed for the C6-H proton and C5',5''-H protons of 20 mM $3',5'\text{-dTDP}$ in the presence of nuclease and Gd(III) or La(III), suggesting conditions of fast chemical exchange compared to the paramagnetic or diamag-

netic relaxation rate. These results imply that the rate of exchange of the nucleotide between the bound and free states is greater than any of the relaxation rates of the nucleotide nuclei in the ternary complex and that the observed proton resonances represent a weighted average for the nuclei resonances of the bound and unbound nucleotide.

In order to determine the contribution of the paramagnetic lanthanide ion to the enhanced relaxation rates of the nuclei of the substrate analog bound in the ternary complex [nuclease-Gd(III)-nucleotide], we determined the bulk paramagnetic contribution of free Gd(III), the contribution of the binary complex [nucleotide-Gd(III)], and the diamagnetic contribution to the observed transverse relaxation rates. In solutions of 20 mM $3',5'\text{-dTDP}$ and 0.1 mM GdCl_3 in the absence of nuclease, extensive broadening was observed for all of the assigned resonances. For example, a linewidth of 13.6 and 4.0 Hz was observed for the C6-H resonance in the presence and absence of Gd(III), respectively. The metal-induced broadening of the C6-H resonance in the presence of Gd(III) could be due to the complexing of Gd(III) and $3',5'\text{-dTDP}$ or to the bulk paramagnetic effect of unbound Gd(III) on the nucleotide protons. No metal-induced broadening of the C6-H linewidth (4.0 Hz) was observed in thymidine (20 mM) with the addition of Gd(III) to 0.1 mM concentration. Therefore, the bulk paramagnetic effect is negligible at the Gd(III) ion concentrations (<0.1 mM) used in these experiments.

However, Gd(III) binds to $3',5'\text{-dTDP}$ and induces line-broadening of the nucleotide resonances in the absence of nuclease. Consequently, in a solution containing nuclease, $3',5'\text{-dTDP}$, and Gd(III), the relaxation rate enhancement is due to the Gd(III)- $3',5'\text{-dTDP}$ or the ternary complex of nuclease-Gd(III)- $3',5'\text{-dTDP}$. Equilibrium dialysis was used to determine the relative fractions of the binary and ternary complexes in a solution of nuclease, Gd(III), and $3',5'\text{-dTDP}$. Nuclease (0.3 mM) and Gd(III) (0.2 mM) were introduced into the compartment of an equilibrium dialysis chamber. $3',5'\text{-dTDP}$ (40 mM) was added to the other compartment. After equilibration, the proton NMR spectrum of the contents of each compartment was examined. The proton resonances of $3',5'\text{-dTDP}$ were markedly broadened in the sample taken from the compartment containing nuclease. This compartment could potentially contain binary and ternary complexes. However, the $3',5'\text{-dTDP}$ resonances obtained from

FIG. 1. Effect of Gd(III) on the transverse relaxation rate of protons of $3',5'\text{-dTDP}$ complexes with nuclease. The NMR tube contained 20 mM $3',5'\text{-dTDP}$ (pH 6.9), 10 mg/ml of nuclease, and varying concentrations of GdCl_3 . $(1/T_2)_\text{M}$, for each of the assigned protons, was obtained by subtraction of the diamagnetic component of $(1/T_2)$ from the observed value of $(1/T_2)$. Error bars indicate the range of uncertainty.

§ The principles of the computer program, developed by R. Feldmann and B. Furie with assistance from G. Knott, will be presented in more detailed form in a subsequent communication describing the geometries of various bound nucleotides.

FIG. 2. Effect of Gd(III) on the longitudinal and transverse relaxation rate of the phosphorus nuclei of 3',5'-dTDP complexes with nuclease. The NMR tube contained 20 mM 3',5'-dTDP (pH 6.9), 10 mg/ml of nuclease, and varying concentrations of GdCl₃. The $(1/T_2)_M$ (●—●) or $(1/T_1)_M$ (○—○) for the phosphorus nuclei, obtained by subtraction of the diamagnetic component from the observed $1/T_2$ or $1/T_1$, is plotted against the ratio of the Gd(III) concentration to the nucleotide concentration.

the other compartment, containing potentially only binary complexes, showed no measurable linebroadening. These results show that at the concentrations of nuclease, Gd(III), and 3',5'-dTDP used, most of the Gd(III) ions are bound in the ternary complex and negligible quantities of 3',5'-dTDP exists in the binary form.

The concentration dependence of Gd(III)-induced linebroadening of the inhibitor resonances was evaluated at Gd(III) concentrations between 10 and 50 μ M. The observed value of $(1/T_2)_M$ was the same at 0.5 and 1.2 mM nuclease. Furthermore, the effect of temperature on the relaxation rates of the inhibitor nuclei indicate that rapid chemical exchange of the inhibitor with the protein-metal complex also exists with respect to the NMR time scale. It therefore is possible to study the paramagnetic effect of bound Gd(III) on the nuclei of the inhibitor in the ternary complex at low ratios of Gd(III) to nucleotide. By linear extrapolation of the molar ratio of Gd(III) to 3',5'-dTDP to a 1:1 complex, the paramagnetic contribution to the transverse and longitudinal relaxation rates can be determined for the ternary complex (21).

The effect of Gd(III) concentration on the metal-induced relaxation rate of the proton resonances of 3',5'-dTDP bound to nuclease is presented in Fig. 1. Error in the addition of microliter quantities of metal ions was estimated to be 0.5 μ l (1 μ M). Similarly, the effect of Gd(III) concentration on the longitudinal and transverse relaxation rate enhancement of the phosphorus resonances of 3',5'-dTDP complexed to nuclease is shown in Fig. 2.

The extrapolated values of $(1/T_1)_M$ and $(1/T_2)_M$ to a [Gd(III)]/[nucleotide] ratio of 1:1 for the nuclei studied are given in Table 1. From these data it is possible to calculate an absolute distance from the paramagnetic Gd(III) ion to different inhibitor nuclei by the Solomon-Bloembergen equation (22, 23) relating $(1/T_2)_M$ or $(1/T_1)_M$ (in sec⁻¹) and r (in cm), the metal-nucleus distance. The value of the correlation time, τ_c , was determined from measurements of $(1/T_1)_M$ and $(1/T_2)_M$ of the C-2 protons of the four histidines of nuclease in the nuclease-Gd(III) binary complex (E. Nieboer, D. East, J. S. Cohen, and A. N. Schechter, unpublished observations). This method yielded a τ_c of 2.6×10^{-9} sec. If the scalar term of the Solomon-Bloembergen equation is neglected, then the

TABLE 1. Internuclear distances from Gd(III) to proton and phosphorus nuclei of 3',5'-dTDP

	$(1/T_1)_M^a$ sec ⁻¹	$(1/T_2)_M^a$ sec ⁻¹	NMR analysis (Å)	Computer fitted NMR analysis (Å)	X-ray analysis (Å)
C6-H	—	1,400	7.8 ± 0.3	8.1	7.7
C4'-H	—	15,300	7.7 ± 0.5	7.6	8.4
C5',5'-H	—	42,100	$6.5^b \pm 0.3$	6.2, 6.9	6.4, 6.7
CH ₂	—	4,000	$9.6^b \pm 0.3$	7.6, 9.5, 10.3 ^a	7.1, 7.4, 8.4 ^a
C3'-P	{7,600}	{23,000}	$\{6.2^b \pm 0.1\}$	6.6	10.0
C5'-P			$\{5.7^b \pm 0.3\}$	5.5	4.5

^a Relaxation rates of the nuclei of 3',5'-dTDP in the ternary complex at a 1:1 ratio of Gd(III):3',5'-dTDP.

^b Observed as the root mean sixth distance from Gd(III) to equivalent nuclei. Distances of Gd(III) to nuclei used in the computer modeling process were chosen arbitrarily from a set of pairs that are compatible with the experimentally determined root mean sixth distance.

^c Positions of protons arbitrarily fixed at indicated positions.

^d Determined from the longitudinal relaxation rate.

^e Determined from the transverse relaxation rate.

internuclear distance, r , from Gd(III) to either proton or phosphorus nuclei in the ternary complex of nuclease-Gd(III)-nucleotide may be calculated from the experimental values of $(1/T_1)_M$ or $(1/T_2)_M$. The calculated distances are presented for 3',5'-dTDP in Table 1. These distances may be compared to the internuclear distances obtained from the x-ray data.

The root mean sixth phosphorus-Gd(III) distance determined from the transverse relaxation rate is 5.7 Å. This value is, at best, a lower limit since relaxation mechanisms through hyperfine interactions between Gd(III) and phosphorus were presumed negligible but may, in fact, be operative. Therefore, the root mean sixth phosphorus-Gd(III) distance of 6.2 Å determined from the longitudinal relaxation rate, where hyperfine contributions are negligible, represents a more accurate estimation of the average distance.

Because of the similarity of the chemical shift of the C5'-P and C3'-P resonances, we were unable to determine the transverse or longitudinal relaxation time directly. However, it might be expected that if the Gd(III)-phosphorus distances were considerably different for C5'-P and C3'-P, differential linebroadening or a complex decay in the T_1 determination representing the summation of the first-order rate processes of C5'-P and C3'-P would have been evident. Neither was observed. These data, therefore, suggest that the Gd(III)-phosphorus distances to C5'-P and C3'-P may be similar. The Gd(III)-C5'-P distance may be limited to 5.5–6.2 Å on the basis of experimental uncertainty. The pair of metal-phosphorus distances used for modeling are arbitrarily chosen within the limits of these data.

Computer analysis of the Gd(III)-nucleotide geometry determined a set of possible conformations within the tolerances of the experimental NMR data. A Ramachandran plot of the N1-C1' and C4'-C5' bond angles of the allowable structures indicated that all of the solutions were clustered near the position of the structure derived from x-ray data. The computer-generated solution, which resembles the structure of the 3',5'-dTDP inferred from crystallographic studies, is shown in Fig. 3. Although similar, these structures differ in the orientation of the thymidine ring, the position of the metal ion, and the extension of the phosphate groups into space.

FIG. 3. CALCOMP plots of the structure of 3',5'-dTDP bound to the nuclease-metal ion complex determined by NMR relaxation enhancement studies and x-ray crystallographic analysis. (Right) X-ray crystal structure of nuclease-bound 3',5'-dTDP as extracted from the X-ray model of nuclease-Ca(II)-3',5'-dTDP. (Left) One of the possible conformations of nuclease-bound 3',5'-dTDP, derived from the computer modeling process based on the data of the NMR relaxation studies. (A) Both structures, centered about the ribose ring, demonstrate the similarity of the ribose and thymidine ring orientation. In this view, the metals differ in their position along a locus perpendicular to the plane of the page. (B) Both structures, centered about the metal ion, are shown to occupy the same space relative to the metal. The differences are manifestations of variations of the orientation of the nucleotide to the metal in the two structures.

The internuclear distances between Gd(III) and the C6-H, C4'-H, C5',5''-H, CH₃, C3'-P, and C5'-P nuclei, determined from the computer solution to the NMR data, are presented in Table 1 for comparison with the experimental NMR data and the internuclear distances obtained from the x-ray model.

DISCUSSION

Knowledge of the features of the active site of staphylococcal nuclease has been derived from various solution studies (10) and from the x-ray analysis at 2.5-Å resolution of crystals of the complex of nuclease-Ca(II)-thymidine diphosphate (24). In this communication we have tested the NMR paramagnetic relaxation rate enhancement method for distance determination against the crystallographic data for the binding of the nucleotide inhibitor, 3',5'-dTDP. In parallel work, these methods have been used to measure metal-histidine residue distances in nuclease (ref. 8; and E. Nieboer, D. East, J. S. Cohen, and A. N. Schechter, unpublished observations).

These studies are based on the NMR relaxation approach, which permits the estimation of the distances between paramagnetic metal ions and other nuclei by measuring the enhanced relaxation rates of a nucleus due to the effects of the magnetic field of the metal ion (13-15, 25). The demonstration that lanthanide ions in general, and Gd(III) in particular, compete with Ca(II) for the single metal-binding site of nuclease (10) and bind with a 1:1 stoichiometry (9) indicates that the space occupied by Ca(II) and Gd(III) overlap. The

similarity of the ionic radius of Gd(III) (0.938 Å) and Ca(II) (0.990 Å), as well as recent experimental evidence (26), may justify our assumption in the interpretation of these experiments that both metal ion spheres are centered in roughly the same position in the metal-ion binding site of nuclease.

The interpretation of the Gd(III)-induced relaxation rate enhancement of the 3',5'-dTDP nuclei in the presence of nuclease was simplified by the demonstration that, under the conditions used, the relaxation rate enhancements of the 3',5'-dTDP resonances by Gd(III) arise entirely from the ternary complex of nuclease-Gd(III)-3',5'-dTDP and that 3',5'-dTDP undergoes rapid chemical exchange with the nuclease-Gd(III) complex.

The calculation of absolute distances from the values of $(1/T_2)_M$ presumes that the relaxation is dominated by dipole-dipole interactions, and that the scalar term of the Solomon-Bloembergen equation approaches zero. For the proton data, this assumption is probably valid. On the other hand, relaxation of the phosphate groups appears to have contact contributions, suggesting that the scalar term for transverse relaxation may not be insignificant. As in enolase (27), the absolute distance is best determined from the longitudinal relaxation rate since the distance obtained from transverse relaxation rates yields only a lower limit of the distance.

The accuracy of the NMR method for distance determination between nuclei and paramagnetic ions by measurement of relaxation rates has been estimated to be ± 0.1 Å (12). In a study that measures $(1/T_2)_M$, the measurement of the line-widths at half-height of various resonances may be limiting. The uncertainty of the distance measurements in this study was estimated to be between 0.3 Å and 0.5 Å (Table 1). Furthermore, relaxation of the coupled C1'-H triplet may yield only lower limits of the metal-nuclei distances because of chemical exchange spin decoupling (28).

The correlation time, τ_c , may be measured by determining the frequency dependence of $1/T_1$, by comparison of $1/T_1$ and $1/T_2$ or directly by electron paramagnetic resonance techniques (12, 27, 29). The determination of τ_c for the nuclease-Gd(III) complex by the second method probably represents a close approximation of the τ_c of the nuclease-Gd(III)-3',5'-dTDP complex. Since the value of τ_c , 2.6×10^{-9} sec, used in this study must be considered tentative, the absolute distance determinations, by necessity, are similarly tentative. However, an incorrect τ_c would not affect the relative distances and an error of a factor of 10 in τ_c changes the absolute distances by a factor of 1.46.

Despite these limitations, there is general agreement between the NMR and the x-ray determined distances (Table 1). The NMR data have been further evaluated with a computer program and a graphics display to generate possible conformations of nuclease-bound 3',5'-dTDP. There exists at least one solution for the conformation of 3',5'-dTDP and for its distance from the bound metal that is similar to the structure of nuclease-Ca(II)-3',5'-dTDP defined by x-ray crystallography. Thus, NMR and x-ray analysis of the nucleotide inhibitor yield a solution and crystal structure of the metal ion, ribose, and phosphate groups that are generally within the experimental uncertainty of the NMR data presented in Table 1 and electron density maps (with an estimated uncertainty of ± 0.5 Å).

In particular, the relationship of the thymidine and the ribose ring are nearly identical (Fig. 3A). The position of the

5'-phosphorus is different in the two structures; however, as described above, its position is somewhat arbitrary. The orientation of the nucleotide to the metal ion is also different for the NMR and x-ray-derived conformations. These differences are caused primarily by the experimentally significant discrepancy in the CH₂-Gd(III) distance determined by the two methods. The nucleotides basically occupy the same space relative to the metal, but the orientation of the plane of the ribose and thymidine rings differ. At present the basis of these differences is unclear. The NMR techniques have many limitations, but the x-ray method is also dependent upon the degree of isomorphism and interpretation of complex electron densities. The examination of tertiary structure of small molecules by relaxation enhancement methods has yielded solution structures for AMP (14) and ligands in several protein-ligand complexes (27, 30-32). Current relaxation studies attempting to determine the three-dimensional structure of lysozyme will be directly comparable to the structure derived from x-ray crystal analysis (33). Our results indicate that NMR relaxation approaches yield structural information that is in general compatible with the x-ray crystallography of the protein-ligand complex, and that the solution and crystal structure of nuclease-bound 3',5'-dTDP may be similar. However, experimentally significant differences in the results of the two methods exist. It is possible that with further developments the NMR approach may be useful in refining the geometry determined by x-ray crystal analysis or in independently determining three-dimensional structure at high resolution.

We thank Dr. A. S. Mildvan for his critical comments and suggestions, which were applicable in all phases of this work. We also thank Drs. J. S. Cohen, E. Nieboer, P. Schiller, and C. B. Anfinsen for their critical review of the manuscript and Mr. R. Bradley for his expert maintenance of the HR 220 NMR spectrometer and accessories. J.H.G. was supported by an NIH Special Postdoctoral Fellowship.

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