transition state and that explicit inclusion of such a structure is probably necessary for a proper quantitative description of substituent effects on [3,3]-sigmatropic migrations or other pericyclic reactions.

Acknowledgment. Support of this work by the NIH (Grant GM 27022) is gratefully acknowledged.

Transition-Metal Binding Site of Bleomycin. A Synthetic Analogue Capable of Binding Fe(II) to Yield an Oxygen-Sensitive Complex

Masami Otsuka, Makoto Yoshida, Susumu Kobayashi, and Masaji Ohno*

Faculty of Pharmaceutical Sciences, University of Tokyo Hongo, Bunkyo-ku, Tokyo 113, Japan

Yukio Sugiura

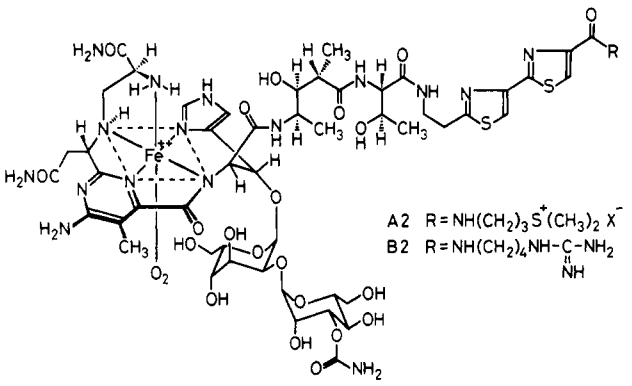
Faculty of Pharmaceutical Sciences, Kyoto University Kyoto 606, Japan

Tomohisa Takita and Hamao Umezawa

Institute of Microbial Chemistry, Kamiosaki Shinagawa-ku, Tokyo 141, Japan

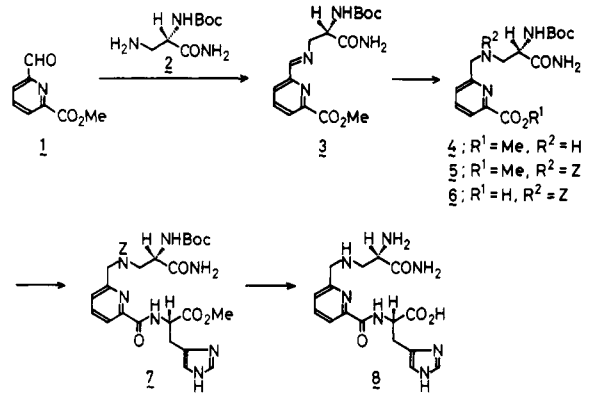
Received July 6, 1981

Bleomycin (BLM) is an antitumor antibiotic clinically used in the treatment of squamous cell carcinoma and malignant lymphoma.[1] In addition to its medicinal importance, BLM has attracted a great deal of structural[2] and synthetic[3-6] studies because of the unique structure and interesting biological activity. The mechanism by which the drug exhibits antitumor activity is currently under active investigation, and two important capabilities of BLM are considered essential. BLM is capable of producing strand breaks in DNA[1] and binding Fe(II) to yield an oxygen-sensitive complex, BLM–Fe(II).[7] A number of possible transition-metal binding sites of BLM were proposed on the basis of



The proposed structure for bleomycin-Fe(II) complex[8a]

Scheme I



theoretical and spectroscopic studies.[8] Among them, the X-ray structural analysis[9] of the Cu(II) complex of P-3A isolated from a culture broth of BLM demonstrated the most direct evidence for the metal binding site, and an acid hydrolysis product of BLM–Co(III) was recently shown to be analogous to the structure of P-3A–Cu(II).[10] However, these complexes are biologically inactive and do not activate molecular oxygen. We wish to report the first successful synthesis of an analogue of the pyrimidine moiety of BLM capable of binding Fe(II) to yield an oxygen-sensitive complex at physiological values of pH.

During our synthetic study[4] of BLM, a strategy was developed for the elaboration of the pyrimidine moiety of BLM, affording pyrimidoblamic acid as a key intermediate to the total synthesis of bleomycin aglycon.[5] The synthetic approach has been considered to provide the most reliable evidence for the controversial transition-metal binding sites of BLM and now extended to the synthesis of the analogue of the pyrimidine moiety of BLM. The key features of the approach include (1) simplification of the pyrimidine nucleus of BLM to pyridine nucleus, (2) use of a simplified side chain, [((*S*)-2-amino-2-carbamoylethyl)amino]-methyl group, (3) use of histidine for β-hydroxyhistidine of BLM, and (4) the assembly of such fragments to provide a simplified analogue which corresponds to the amine-pyrimidine–imidazole region of BLM.

Thus, methyl 6-formylpyridine-2-carboxylate (**1**)[11] was treated with (*S*)-3-amino-2-[(*tert*-butoxycarbonyl)amino]propionamide (**2**)[4] in an equal molar ratio in CH_3CN in the presence of an activated molecular sieve at 25 °C for 12 h (Scheme I). The resulting Schiff base **3** was hydrogenated in the presence of 5% Pd–C in MeOH, affording yellow foam **4** upon workup and chromatography on silica gel (eluted with 9:1 CH_2Cl_2–MeOH) (**4**: 60% yield from **1**, $[\alpha]^{20}_D$ +36.2° (*c* 1, $CHCl_3$); $M^+ + 1$, 353). Then, the secondary amino group of **4** was protected with the benzyloxycarbonyl group (*Z*), since it was found that the secondary amino group caused difficulty in the peptide formation between **4a** ($R^1 = R^2 = H$) and histidine methyl ester. Treatment of **4** with a little excess of benzyl chloroformate in the presence of 0.1 N NaOH in CH_2Cl_2 (3 h, 25 °C) afforded colorless foam **5** upon workup and chromatography on silica gel (eluted with 9:1 CH_2Cl_2–MeOH) in 88% yield (**5**: $[\alpha]^{20}_D$ +77.7° (*c* 1, $CHCl_3$); M^+, 486). Condensation of **5** with histidine methyl ester[12] was carried out smoothly. Methyl pyridine-2-carboxylate derivative **5** was hydrolyzed (0.1 N LiOH, 30 min at 0 °C and then 1 h at

(1) Umezawa, H. *Prog. Biochem. Pharmacol.* **1976**, *11*, 18.
(2) Takita, T.; Muraoka, Y.; Nakatani, T.; Fujii, A.; Umezawa Y.; Naganawa, H.; Umezawa, H. *J. Antibiot.* **1978**, *31*, 801.
(3) Umezawa, Y.; Morishima, H.; Yoshioka, T.; Otsuka, M.; Ohno, M. In "Bleomycin"; Hecht, S. M., Ed.; Springer-Verlag: New York, 1979; pp 63–74.
(4) Umezawa, Y.; Morishima, H.; Saito, S.; Takita, T.; Umezawa, H.; Kobayashi, S.; Otsuka, M.; Narita, M.; Ohno, M. *J. Am Chem. Soc.* **1980**, *102*, 6630.
(5) Takita, T.; Umezawa, Y., Saito, S.; Morishima, H.; Umezawa, H.; Muraoka, Y.; Suzuki, M.; Otsuka, M.; Kobayashi, S.; Ohno, M. *Tetrahedron Lett.* **1981**, *22*, 671.
(6) Tsuchiya, T.; Miyake, T.; Kageyama, S.; Umezawa, S.; Umezawa, H.; Takita, T. *Tetrahedron Lett.* **1981**, *22*, 1413.
(7) Sausville, E. A.; Peisach, J.; Horwitz, S. B. *Biochem. Biophys. Res. Commun.* **1976**, *73*, 814.
(8) (a) Takita, T.; Muraoka, Y.; Nakatani, T.; Fujii, A.; Iitaka, Y.; Umezawa, H. *J. Antibiot.* **1978**, *31*, 1073. (b) Dabrowiak, J. C.; Greenaway, F. T.; Longo, W. F.; Husen, M. V.; Crooke, S. T. *Biochim. Biophys. Acta* **1978** *517*, 517. (c) Murakami, H.; Mori, H.; Taira, S. *J. Theor. Biol.* **1976**, *59*, 1. (d) Vos, C. M.; Westra, G.; Schipper, D. J. *J. Inorg. Biochem.* **1980**, *13*, 165. (e) Oppenheimer, N. J.; Rodriguez, L. O.; Hecht, S. M. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 5616.
(9) Iitaka, Y.; Nakamura, H.; Nakatami, T.; Muraoka, Y.; Fujii, A.; Takita, T.; Umezawa, H. *J. Antibiot.* **1978**, *31*, 1070.
(10) Tsukayama, M.; Randall, C. R.; Santillo, F. S.; Dabrowiak, J. C. *J. Am. Chem. Soc.* **1981**, *103*, 458.
(11) Mathes, W.; Sauermilch, W.; Klein, T. *Chem. Ber.* **1953**, *86*, 584.
(12) Greenstein, J. P.; Winitz, M. "Chemistry of the Amino Acids"; Wiley: New York, 1961; Vol. 2, p 1061.

0002-7863/81/1503-6986$01.25/0

Table I. ESR Parameters for Divalent Metal Complexes of Pyridine-Model Ligand and Bleomycin

complex	$g_{\parallel}$ (g_z)	$g_{\perp}$ (g_x, g_y)	$A_{\parallel}$, G	A_N, G	N-hfs (line)
PYML-Cu(II)	2.206	2.048	179.4		
PYML-Co(II)	2.022	2.255	92.5	13	3
PYML-Co(II)-O_2	2.093	2.005	22.5		
PYML-Fe(II)	no ESR signals				
PYML-Fe(II)-^{14}NO	2.009	2.036 1.972		25.6	3
PYML-Fe(II)-^{15}NO	2.009	2.036 1.972		35.6	2
BLM-Cu(II)	2.211	2.055	183.0		
BLM-Co(II)	2.025	2.272	92.5	13	3
BLM-Co(II)-O_2	2.098	2.007	20.2		
BLM-Fe(II)	no ESR signals				
BLM-Fe(II)-^{14}NO	2.008	2.041 1.976		23.6	3
BLM-Fe(II)-^{15}NO	2.008	2.040 1.976		31.6	2

Table II. Visible Absorption Characteristics for Fe(II) Complexes of Pyridine-Model Ligand and Bleomycin with Dioxygen Analogues

complex	λ_{max}, nm (ϵ)
PYML-Fe(II)	465 (300)
PYML-Fe(II)-CO	390 (2000)
PYML-Fe(II)-C_2H_5NC	490 (1800)
PYML-Fe(II)-NO	470 (1650)
BLM-Fe(II)	476 (380)
BLM-Fe(II)-CO	380 (3000)
BLM-Fe(II)-C_2H_5NC	495 (2700)
BLM-Fe(II)-NO	470 (2300)

25 °C) and neutralization with 0.1 N HCl and usual workup afforded almost pure free acid **6**. Treatment of **6** with *N,N'*-carbonyldiimidazole (THF, 0 °C, 1 h) followed by condensation with histidine methyl ester (THF, 25 °C, 12 h) afforded colorless oil **7** upon workup and chromatography on silica gel (eluted with 9:1 CH_2Cl_2–MeOH) (**7**: 83% yield based on **5**; mp 168–170 °C; $[\alpha]^{20}_D$ +60.1° (*c* 0.1, $CHCl_3$); M^+, 623). The protective groups of **7** were removed with 30% HBr–AcOH (2.5 h at 25 °C), affording crude methyl ester of **8** as a solid residue after workup. The methyl ester was treated with 1 N NaOH at pH 9–10, and the solution was neutralized with 1 N HCl. After removal of the solvent, the residue was purified by Amberlite CG-50 (H^+ form, eluted with 1% aqueous NH_3). Thus, the yellow solid *N*-[6-[[((*S*)-2-amino-2-carbamoylethyl)amino]methyl]pyridine-2-carbonyl]-L-histidine (**8**) was obtained in 87% yield upon usual workup (mp 120–122 °C, $[\alpha]^{20}_D$ +2.85° (*c* 1, H_2O); $M^+ + 1$ (FD), 376).[13] Next, the metal-binding property of the pyridine-model ligand **8** (PYML) was investigated. The 1:1 divalent metal complexes (PYML–Cu(II), PYML–Co(II), and PYML–Fe(II)) and dioxygen analogue adducts (PYML–Fe(II)–O_2, PYML–Fe(II)–CO, PYML–Fe(II)–CNEt, and PYML–Co(II)–O_2) were prepared according to the procedure previously described for the corresponding BLM complexes.[14,15] Figure 1 shows the ESR spectra for these divalent metal complexes of PYML at pH 7.2 and 77 K. The obtained ESR parameters of these metal complexes are remarkably close to those of the corresponding BLM–metal complexes (see Table I). The 1:1 PYML–Cu(II) complex has an absorption maximum at 597 nm (ϵ 140), circular dichroism (CD) extrema at 545 ($\Delta\epsilon$ +0.88) and 655 nm (–0.10), and Cu(II)/Cu(I) reduction potential of $E_{1/2}$ = –0.319 V vs. NHE, which well correspond to the characteristics [λ_{max} = 595 nm (ϵ 120), CD extrema = 555 ($\Delta\epsilon$ +1.21) and 665 nm (–0.60) and $E_{1/2}$ = –0.327 V] of the BLM–Cu(II) complex.[16]

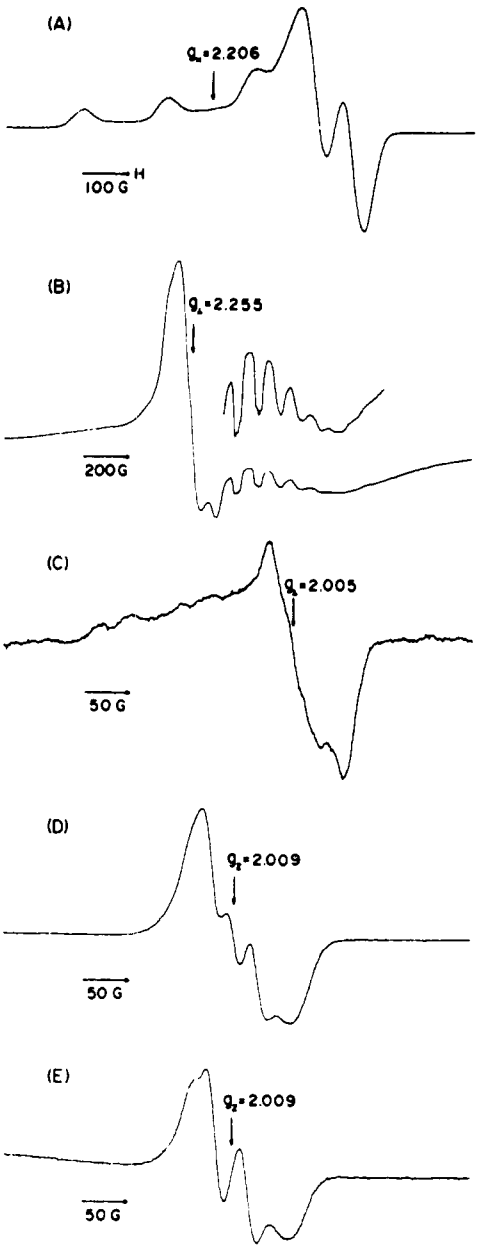


Figure 1. ESR spectra for (A) Cu(II), (B) Co(II), (C) CO(II)–O_2, (D) Fe(II)–^{14}NO, and (E) Fe(II)–^{15}NO complexes of PYML at pH 7.2 and 77 K.

The visible spectral constants of the PYML–Fe(II) complexes with dioxygen analogues also resemble those of the corresponding BLM

(13) Satisfactory combustion analyses were obtained for **7** and **8**, and all material described here were chromatographically homogeneous and gave MS, IR, and NMR (^{13}C and ^{1}H) spectra consistent with their structures.

(14) (a) Sugiura, Y. *J. Am. Chem. Soc.* **1980**, *102*, 5216. (b) *Ibid.* **1980**, *102*, 5208.

(15) The PYML–Cu(II) complex was obtained as a solid, and it was recrystallized from water–ethanol. Other divalent metal complexes of PYML were confirmed to be single species spectroscopically. Full characterization of these complexes as well as the tervalent complexes will be reported in a separate paper.

(16) Sugiura, Y.; Ishizu, K.; Miyoshi, K. *J. Antibiot.* **1979**, *32*, 453. The redox potentials were measured at pH 7.2 and μ = 0.1 ($NaClO_4$) by cyclic voltammetry with a three-electrode system including a dropping mercury electrode.

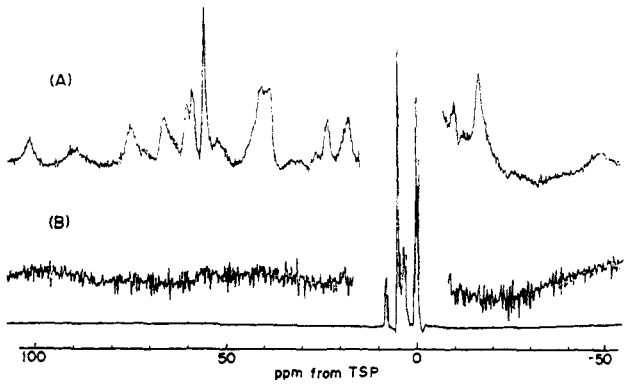


Figure 2. 220-MHz FT ^{1}H NMR spectra of (A) PYML-Fe(II) complex and (B) PYML-Fe(II)-CO complex at 20 °C and pD 7.6.

adduct complexes[14,17] (see Table II). The ESR spectra of PYML-Co(II) (Figure 1 and Table I) strongly support the presence of a nitrogen atom in the axial position. Similar to the BLM-Fe(II) complex, the PYML-Fe(II) complex showed large proton paramagnetic shifts which are due to the contact and pseudocontact effect of the central Fe(II) ion (see Figure 2). The magnitude of the proton chemical shifts suggests the presence of a high-spin Fe(II) ion ($S = 2$) for the PYML-Fe(II) complex. Upon CO binding to the PYML-Fe(II) complex, the pronounced proton paramagnetic shifts completely disappeared, indicating the presence of a diamagnetic Fe(II) ion ($S = 0$) in the CO adduct.[8e] The spin-trapping experiments[14] using *N-tert*-butyl-α-phenylnitron (BPN) or 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) at pH 6.9 clearly revealed that hydroxyl radicals are generated in the PYML-Fe(II)-O_2 complex system.[19] The ESR pattern and parameters were as follows: BPN spin adduct (triplet of doublet, $g = 2.0057$, and $a^N = 15.3$ G) and DMPO spin adduct (quartet, $g = 2.0058$, and $a^N = a_\beta^H = 15.2$ G). In contrast with the corresponding PYML-Fe(II)-O_2 complex system, the CO introduction strongly interfered with the O_2 activation by the PYML-Fe(II) complex. Carbon monoxide is in competition with dioxygen for interaction with the PYML-Fe(II) complex and is a typical O_2 antagonist, just as with the BLM-Fe(II) complex.

Of special significance is the fact that even such a simple oligopeptide like the synthetic model compound **8** is able to mimick the metal binding and dioxygen reduction by BLM ligand. In addition, the present results strongly support the proposed metal-binding site in which (1) the β-aminoalanine-pyrimidine-β-hydroxyhistidine region of the BLM molecule is substantially important for the Fe(II) and dioxygen interaction and (2) the gulose-mannose and methylvalerate moieties in BLM are not necessarily participating as direct ligands toward the Fe(II) binding.

On the basis of the results described here, we believe that there is good hope for the design of simple synthetic systems that will approach the mechanism of the reductive activation of molecular oxygen and explore the nature of chemical and biochemical oxidation with the PYML-Fe(II)-O_2 complex. Work is continuing in this effort.

Acknowledgment. This work was financially supported in part by Grants-in-Aid for Special Project Research from the Ministry of Education, Science and Culture of Japan.

(17) Burger, R. M.; Peisach, J.; Blumberg, W. E.; Horwitz, S. B. *J. Biol. Chem.* **1979**, *254*, 10906.
(18) Pillai, R. P.; Lenkinski, R. E.; Sakai, T. T.; Geckle, J. M.; Krishna, N. R.; Glickson, J. D. *Biochem. Biophys. Res. Commun.* **1980**, *96*, 341.
(19) The ESR spectra were obtained by oxygen bubbling of 0.5 mM PYML-Fe(II) complex, 0.5 mM PYML-Fe(II) complex plus 5.0 mM $Na_2S_2O_4$, and 0.5 mM PYML-Fe(II)-CO complex in the presence of 0.08 M BPN. Conditions of ESR spectroscopy: microwave power, 10mW; modulation amplitude, 0.5 G; time constant, 0.03 s; scan time, 4 min. The radical spin concentration of the PYML-Fe(II) complex system was estimated to be approximately 20% of that of the BLM-Fe (II) complex system. Therefore, it is inferred that the sugar portion of BLM contributes to the more effectve dioxygen activation by the Fe(II) complex.

Use of Spy Nuclei for Relaxation Studies in Nuclear Magnetic Resonance

Y. Huang,[1] G. Bodenhausen, and R. R. Ernst*

Laboratorium für Physikalische Chemie
Eidgenössische Technische Hochschule
8092 Zürich, Switzerland

Received June 15, 1981

It is well-known that the study of spin-lattice relaxation in multilevel spin systems, such as coupled $I = {}^1/_2$ nuclei or quadrupolar nuclei, provides valuable information on molecular motional processes.[2] We should like to demonstrate how the measurement of the relaxation rates can be greatly simplified by the introduction of an additional nonparticipating "spy" nucleus.

The time evolution of the energy level populations P_i is governed by the master equation[3]

$$\frac{d}{dt}\mathbf{P}(t) = \mathbf{W}[\mathbf{P}(t) - \mathbf{P}_{eq}] \qquad (1)$$

where the relaxation matrix $\mathbf{W}$ contains all transition probabilities W_{ij} between pairs of energy levels. In conventional relaxation measurements, one is confined to measuring population *differences* as functions of an interval τ between initial perturbation and observation. Even within the initial rate approximation,[4] a difference $P_i - P_j$ is invariably affected by the transition probabilities W_{ik} and W_{jk} for all values of k. Their separation requires a laborious analysis based on extensive sets of experiments obtained with carefully chosen selective perturbations.[4]

Consider an additional nucleus (such as carbon-13) which is introduced into the spin system under investigation (e.g., a set of coupled protons). Provided the "spy" nucleus interacts through scalar or dipolar coupling with all nuclei in the original set, the spectrum of the spy will consist of a multiplet where each transition T_i corresponds to one particular state i of the spins under investigation. The migration of population from state i to state j resulting from an initial perturbation can now be observed through the migration of intensity between the transitions T_i and T_j of the "spy" nucleus. Experiments of this kind can be carried out most efficiently by two-dimensional exchange spectroscopy[5,6] which has hitherto been applied to chemical exchange[7,8] and spin diffusion or transient Overhauser effects.[9-11]

The spy nucleus is subjected to a pair of 90° pulses, separated by an evolution period t_1, and followed by a mixing interval τ_m which terminates with a 90° observation pulse with subsequent data acquisition. If signal intensity migrates from spy transition T_i to T_j in the τ_m period, a peak appears in the 2-D spectrum at $\omega_1 = \omega_i$ and $\omega_2 = \omega_j$. Within the limits of the initial rate approximation (neglecting relaxation actively involving the spy nucleus), the amplitude of such a peak is simply proportional to the transition probability, $A_{ij} \propto W_{ij}\tau_m$. With an appropriate form of difference spectroscopy,[12] it is also possible to obtain diagonal

(1) Physics Department, Huadong Normal University Shanghai, People's Republic of China.
(2) Vold, R. L.; Vold, R. R. *Prog. NMR Spectrosc.* **1978**, *12*, 79.
(3) Abragam, A. "The Principles of Nuclear Magnetism"; Clarendon Press: Oxford, England, 1961; Chapter VIII.
(4) Freeman, R.; Wittekoek, S.; Ernst, R. R. *J. Chem. Phys.* **1970**, *52*, 1529.
(5) Jeener, J.; Meier, B. H.; Bachmann, P.; Ernst, R. R. *J. Chem. Phys.* **1979**, *71*, 4546.
(6) Macura, S.; Huang, Y.; Suter, D.; Ernst, R. R. *J. Magn. Reson.* **1981**, *43*, 259.
(7) Meier, B. H.; Ernst, R. R. *J. Am. Chem. Soc.* **1979**, *101*, 6441.
(8) Huang, Y.; Macura, S.; Ernst, R. R. *J. Am. Chem. Soc.* **1981**, *103*, 5327.
(9) Macura, S.; Ernst, R. R. *Mol. Phys.* **1980**, *41*, 95.
(10) Kumar, Anil; Ernst, R. R.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **1980**, *95*, 1.
(11) Bösch, C.; Kumar, Anil; Baumann, R.; Ernst, R. R.; Wüthrich, K., *J. Magn. Reson.* **1981**, *42*, 159.
(12) Bodenhausen, G.; Huang, Y.; Ernst, R. R., to be published.

0002-7863/81/1503-6988$01.25/0