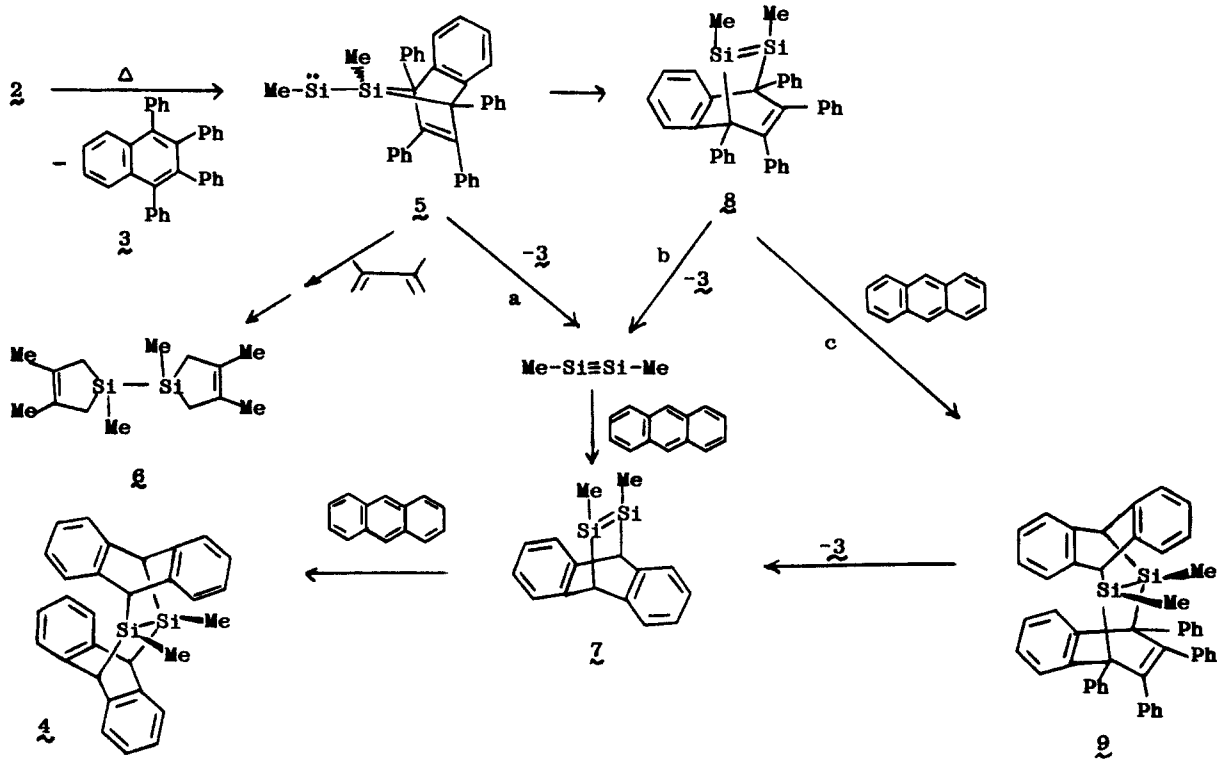
Scheme I



7-silanorbornadienes are known thermal precursors to silylenes,[13] a likely first step is fragmentation to give silylene **5** (Scheme I). Evidence for the intermediacy of **5** was obtained by thermolyzing **2** in the presence of 2,3-dimethylbutadiene, which afforded the five-membered ring compound bi(1,3,4-trimethyl-1-sila-3-cyclopenten-1-yl) (**6**) as final product in 13% yield.[14]

Several pathways are conceivable leading from **5** to the final product **4**. Silylene **5** might lose tetraphenylnaphthalene **3** according to pathway a to give dimethyldisilyne, which could add to anthracene successively to give **7** followed by **4**. Alternatively **5** could undergo ring expansion to disilene **8**.[15] The latter might fragment to give MeSi≡SiMe (pathway b) or might add anthracene to give the mixed compound **9**, which upon loss of **3** could produce **7** and ultimately **4** (pathway c).[16] Because of the possible involvement of the disilyne, the mechanism of this transformation is now of considerable interest.

The anthracene adduct **4** is also a possible dimethyldisilyne precursor or synthon. In this connection the mass spectrum of **4** (EI, 30 eV) is suggestive; peaks are observed at *m/e* 442 ($M^+$, relative intensity 35), 264 ($M^+$ - anthracene 100), 249 ($M^+$ - anthracene - Me, 36), 178 (anthracene$^+$, 50). In addition to these, a peak with relative intensity 15 is found at *m/e* 86. The exact mass and isotope ratios of this peak show that it has composition $C_2H_6Si_2^+$.[17]

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(13) Gilman, H.; Cottis, S. G.; Atwell, W. H. *J. Am. Chem. Soc.* **1964**, *86*, 1596.

(14) $^1H$ NMR ($CDCl_3$) $\delta$ 0.15 (s, 6 H, SiMe), 1.34 (d, $J$ = 17.5 Hz, 4 H, ring proton), 1.53 (d, $J$ = 17.5 Hz, 4 H, ring proton), 1.68 (br s, 12 H, C=CMe); HRMS (EI, 30 eV), *m/z* 250.1588 (calcd for $C_{14}H_{26}Si_2$, 250.1573). This compound was independently prepared from the reaction of 1,2-dimethyl-1,1,2,2-tetrachlorodisilane with 2,3-dimethylbutadiene and Mg in HMPA.

(15) Ring expansion of silylenes is precedented, but in the published examples involving cyclopropylsilylene rearrangement to 1-silacyclobutene, higher temperatures are required (540–680 °C). (a) Ando, W.; Hamada, Y.; Sekiguchi, A. *J. Chem. Soc., Chem. Commun.* **1982**, 787. (b) Barton, T. J.; Burns, G. T.; Goure, W. F.; Wulff, W. D. *J. Am. Chem. Soc.* **1982**, *104*, 1149. (c) Burns, S. A.; Burns, G. T.; Barton, T. J. *J. Am. Chem. Soc.* **1982**, *104*, 6140. A rearrangement of a silylene to a disilene involving a methyl shift has recently been reported, again at higher temperature. Boo, B. H.; Gaspar, P. P. *Organometallics*, in press.

(16) An additional pathway might be considered involving addition of silylene **5** to anthracene to form a dibenzosilanorborandiene, which could undergo loss of tetraphenylnaphthalene followed by ring expansion to give **7**. However, this mechanism seems less likely because there is no precedent for 9,10-addition of silylenes to anthracene.

(17) HRMS for 86 peak 85.9995 (calcd for $^{12}C_2{}^1H_6{}^{28}Si_2$, 86.0008), 86.9980 (calcd for $^{12}C_2{}^1H_6{}^{28}Si^{29}Si$, 87.0004), 87.9969 (calcd for $^{12}C_2{}^1H_6{}^{29}Si_2$, 87.9976). The relative intensity of these peaks was 100:14:7, in agreement with the calculated ratio, 100:13:7.

## Second-Order Effects in Two-Dimensional Cross-Relaxation Spectra of Proteins: Investigation of Glycine Spin Systems

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The use of two-dimensional (2-D) nuclear Overhauser effect spectroscopy (NOESY) for biomolecule structure determination is becoming increasingly frequent.[1-3] In principle, the well-known $1/r^6$ distance dependence of the intensity of cross-relaxation peaks

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(1) Wider, G.; Marcura, S.; Ernst, R. R.; Wuthrich, K. *J. Magn. Reson.* **1984**, *56*, 207-234.

(2) Williamson, M. P.; Havel, T. F.; Wuthrich, K. *J. Mol. Biol.* **1985**, *182*, 295-315.

(3) Neuhaus, D.; Wagner, G.; Vasak, M.; Kagi, J. H. R.; Wuthrich, K. *Eur. J. Biochem.* **1985**, *151*, 257-273. (b) Wagner, G.; Neuhaus, D.; Worgotter, E.; Vasak, M.; Kagi, J. H. R.; Wuthrich, K. *J. Mol. Biol.* **1986**, *187*, 131-135.

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**Table I.**[a] Volume Integral Ratios[e] for AX and BX Cross-Peaks

| Gly | $J/\delta$[f] | $\tau_{mix}$, ms | first-order prediction | exptl observation | simulated | simulated with $J_{AB} = 0$ |
|---|---|---|---|---|---|---|
| MT 47[b] | 0.05 | 150 | 0.345 | 0.483 | 0.478 | 0.485 |
| ACP 74 | 0.09 | 80 | 0.277 | 0.363 | 0.366 | 0.357 |
| ACP 12 | 0.17 | 150 | 0.330 | 0.700 | 0.666 | 0.624 |
| ACP 12 | 0.17 | 80 | 0.316 | 0.502 | 0.530 | 0.500 |
| ACP 33 | 0.52 | 150 | 0.287 | *d* | 0.497 | 0.451 |
| ACP 33 | 0.52 | 50 | 0.270 | *c* | 0.490 | 0.352 |

[a] Experimental 2-D pure absorption NOESY spectra were recorded on a 490 MHz. NMR spectrometer operating in the Fourier transform mode. Typically 500 experiments with 4K points per experiment were acquired. A sweep width of 5000 Hz was employed and the data were processed by applying a 20° phase-shifted sine-bell weighting function in both dimensions. Data processing and simulation were performed on a Vax 11/750 computer equipped with a CSPI minimap array processor using software written by D. Hare. [b] Reference 3b. [c] Data sets not available. [d] Quantitation impossible due to strong second-order nature of the cross-peaks. [e] Volume ratio = (volume integral of smaller $\alpha$-CH–amide cross-peaks)/(volume integral of larger $\alpha$-CH–amide cross-peaks). [f] Spectral parameters input for $\delta(\alpha\text{-CH}_1)$, $\delta(\alpha\text{-CH}_2)$, $\delta$(NH), $J_{AB}$ (Hz), $J_{AX}$ (Hz), $J_{BX}$ (Hz), $r_{AB}$ (Å), $r_{AX}$ (Å), $r_{BX}$ (Å), $\tau_c$ (ns): 4.39, 3.62, 7.39, 17.8, 2.0, 10.1, 1.75, 2.49, 3.00, 1.7 for MT; 4.08, 3.71, 7.78, 15.7, 7.0, 7.1, 1.75, 2.92, 2.34, 2.1 for ACP 74; 3.86, 3.70, 8.41, 14.2, 2.6, 8.0, 1.75, 2.82, 2.31, 2.1 for ACP 12; 3.97, 3.93, 7.37, 13.6, 5.9, 7.2, 1.75, 2.92, 2.34, 2.1 for ACP 33.

between isolated pairs of nuclei allows a highly accurate determination of internuclear distances. These distances can be combined to produce three-dimensional structures. In recognition of the marginal quality of much 2-D data and the numerous assumptions necessary for quantitative determination of distances, early studies did not attempt a truly quantitative interpretation of cross-peak intensities but, instead, relied on theories which required only specifications of upper and lower bounds on internuclear distances.[4] As experimental methods have improved, however, there is an increasing temptation to make use of more precisely determined distances.[5,6] A careful consideration of some of the underlying assumptions in the interpretation of cross-peak volumes on the basis of of a simple $1/r^6$ dependence is thus a timely subject. Such considerations have been given to one-dimensional cross-relaxation data in cases where large numbers of spins interact,[7,8] but they have been largely neglected for 2-D sets on molecules with spin systems showing strong $J$ coupling as well as multiple dipolar interactions. In this paper we demonstrate that second-order processes significantly complicate the interpretation of NOESY cross-peak intensities in terms of molecular structure when data are accumulated under experimental conditions typically used for proteins. We can, however, provide some useful guidelines in choice of mixing times that allow first-order interpretations for systems showing moderate degrees of scalar coupling.

We have examined these second-order effects as they relate to cross-peaks for glycine residues in two representative proteins: acyl carrier protein (ACP, 8850 daltons)[9] and metallothionein (MT, 6900 daltons).[3] Most glycines in these proteins present ABX spin systems in which the X spin is an amide proton and the A and B spins are $\alpha$ protons. A range of scalar couplings from strong to weak are represented. The A–B dipolar coupling is quite strong in glycine and potentially complicates interpretation of A–X and B–X interactions. Glycines are usually rigidly fixed in the protein backbone structure and avoid complications which exist with residues having many internal degrees of freedom. In the cases chosen for examination the glycines show sufficient resolution to allow geometry determination through application of the Karplus relations.[10]

In less well-defined systems distances between amide and $\alpha$ protons would provide valuable pieces of information for the determination of secondary structure if they could be quantitated. We have investigated conditions under which quantitation might be possible by comparing experimental data to data predicted by a simple first-order theory and data simulated by using a theory which includes both strong scalar coupling and multiple dipolar relaxation interactions. Several treatments of cross-relaxation between weakly coupled spin systems in NOESY experiments have appeared in the literature.[11,12] We use a slightly different treatment based on Redfield theory which allows the inclusion of strongly scalar coupled systems.[13] Details concerning the theoretical approach and its implementation in a general multipulse simulation program are discussed in a previous publication.[14] Simulated data were obtained by using the resulting program and parameters such as chemical shifts and coupling constants obtained from pure absorbtion scalar coupling correlated spectra on ACP and MT samples. The correlation time for each protein was calculated from the Stokes–Einstein relation. These parameters are summarized in the legend to Table I.

Table I shows cross-peak intensity data for glycines with spin systems varying from AMX to ABX. To avoid the necessity of using an internal intensity standard, ratios of volume integrals are reported. Note that the ratios simulated by using the full theory agree well with experiment. However, in most cases, there are deviations of a factor of 2 or more between the simulated NOE intensity ratios and the ratios predicted on the basis of first-order theory (NOE $\alpha$ $1/r^6$).

These deviations are the result of either of two magnetization transfer processes: (i) successive through-space magnetization transfers (spin diffusion) so that a fraction of the magnetization transferred between NH and $\alpha$-$CH_i$ is subsequently transferred through space to $\alpha$-$CH_j$ due to the close proximity of spins $i$ and $j$; (ii) coherent transfer of magnetization from $\alpha$-$CH_i$ to $\alpha$-$CH_j$ due to the strong coupling (in the ABX cases) between protons $i$ and $j$. This strong coupling mixes the zero-order wavefunctions of spins $i$ and $j$ so that the eigenstate of proton $j$ is a linear combination of the zero-order eigenstates of protons $i$ and $j$. In this way a portion of the magnetization transferred from NH to $\alpha$-$CH_i$ contributes to the resonances associated with $\alpha$-$CH_j$.

Simulation of spectra with the geminal $\alpha$-$CH_2$ couplings set at zero eliminates the coherent pathway and provides an assessment of the relative importance of the two second-order transfer pathways. Spectral simulation in this limit using a 150-ms mixing time indicates that successive through-space magnetization transfers are responsible for the majority (>80%) of the deviation between first-order cross-peak intensity ratios and experimental ratios even in highly $J$ coupled spin systems ($J/\delta = 0.5$). At shorter mixing times (50 ms) and for strongly coupled spin systems the importance of second-order dipolar transfer processes are reduced and the effects of coherent transfer processes are more easily seen. For example, the ABX spin system of glycine 33 from ACP with $J/\delta = 0.52$ shows that approximately 60% of the de-

(4) Brown, L. R.; Braun, W.; Kumar, A.; Wuthrich, K. *Biophys. J.* **1982**, *37*, 319–328.
(5) States, D. J.; Haberkorn, R. A.; Ruben, D. J. *J. Magn. Reson.* **1982**, *48*, 286–292.
(6) Olejniczak, E. T.; Hoch, C. J.; Dobson, C. M.; Poulsen, F. M. *J. Magn. Reson.* **1985**, *64*, 199–206.
(7) Noggle, J. *J. Magn. Reson.* **1979**, *35*, 95–109.
(8) Dobson, C. M.; Olejniczak, E. T.; Poulsen, F. M.; Ratcliffe, R. G. J. *Magn. Reson.* **1982**, *48*, 97–110.
(9) Mayo, K. H.; Tyrell, P. M.; Prestegard, J. H. *Biochemistry* **1983**, *22*, 4485–4493.
(10) Bystrov, V. F. *Prog. Nucl. Magn. Reson. Spectrosc.* **1976**, *10*, 41–81.
(11) Macura, S.; Ernst, R. R. *Mol. Phys.* **1980**, *41*, 95–117.
(12) Keepers, J. N.; James, T. L. *J. Magn. Reson.* **1984**, *57*, 404–426.
(13) Redfield, A. G. *IBM J. Res. Div.* **1957**, *1*, 19.
(14) Kay, L. E., Scarsdale, J. N.; Hare, D. R.; Prestegard, J. H. *J. Magn. Reson.*, in press.

viation between first-order predicted and simulated cross-peak ratios is due to this effect at a mixing time of 50 ms.

It is difficult to generalize the above results to assess the magnitude of error that would be introduced in internuclear distance calculations based on first-order theory because of the many different spin systems, motional time scales, and internuclear distance combinations encountered in NOESY spectra of proteins. We can, however, provide some crude guidelines for analysis of cross-peaks involving geminal proton pairs in moderately sized proteins. From the above discussion it is clear that secondary through-space transfers are capable of producing large errors in distances calculated between either member of the geminal pair and a neighboring proton. For example, simulation of cross-peak intensities of glycine residue 33 in ACP in the limiting case where $J_{AB}/\delta = 0$ (see Table I) shows that first-order interpretation would lead to an error in the NH-$\alpha$-CH distance ratio of 8.5% for a mixing time of 150 ms. This error can be reduced to 4.5% by using a mixing time of 50 ms. When strong scalar coupling exists, first-order interpretation of the data can also produce distance errors. The effects due to strong $J$ coupling, however, tend to be obscured by secondary through-space transfers except at short mixing times and for very strongly coupled spins ($J/\delta = 0.5$). These effects are probably not of great consequence since in general it is not possible to integrate accurately cross-peaks that arise from very strongly coupled spins. Thus, first-order theory is appropriate for most $J/\delta$ ratios for which resolvable cross-peaks arise but interpretation of distances from cross-peaks involving geminal proton pairs should be relegated to data sets collected with short mixing times.

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## Determination of Vinyl Orientation in Resting State and Compound I of Horseradish Peroxidase by the $^1$H Nuclear Overhauser Effect

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There exists a large body of indirect evidence that the heme pocket of horseradish peroxidase, HRP, is stereochemically more rigid and buried than in myoglobin or hemoglobin.[2-6] One of the manifestations of this influence is the clamping of the heme vinyls so as to restrict their oscillatory mobility and force a more in-plane orientation than in other hemoproteins.[3-7] Such in-plane orientations have been indirectly supported by both $^1$H NMR[3-5] and resonance Raman,[6,7] RR, spectral interpretations and rationalized to enhance the stability of the doubly oxidized reactive intermediate, compound I, HRP-I. The inability to grow adequate single crystals, however, has prevented the usual confirmation of such structural details by X-ray diffraction. It would be useful to have not only more direct evidence for such in-plane orientations but also to distinguish among two types of in-plane orientations, *cis* or *trans* (as depicted for the 4-vinyl and 2-vinyl groups, respectively, in F of Figure 1). RR studies on reduced HRP have indicated *cis* orientations for both vinyls.[6]

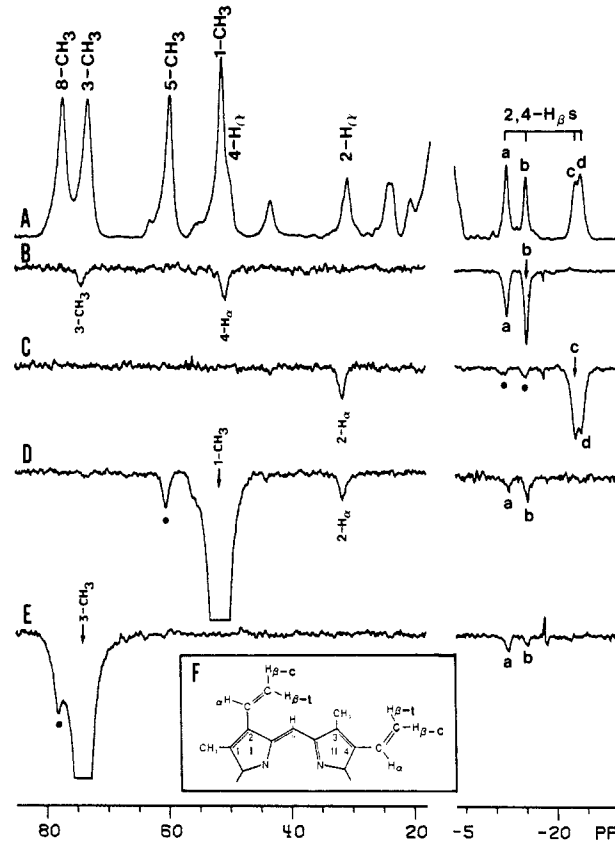


**Figure 1.** 360-MHz $^1$H NMR spectrum of (A) 3 mM HRP-I in $^2H_2O$ at 15 °C, pH 7.0. Previously assigned resonances[4] are labeled. (B)-(E) are the NOE difference spectra generated by subtracting the reference spectrum with the decoupler off-resonance from a similar spectrum of the same sample in which the desired resonance was saturated for 30 ms with a 50-mW decoupler pulse. Spectra were collected by the Redfield 21412 pulse sequence. The upfield portions were recorded with the carrier at −18 ppm while the downfield portions were collected with the carrier centered at 55 ppm. In each of the difference spectra (B)-(E), an arrow indicates the peak being saturated. A filled circle denotes off-resonance power spillage. (B) Saturate b; note NOEs to a and 4-H$_\alpha$. (C) Saturate c; note NOE to 2-H$_\alpha$. (D) Saturate 1-CH$_3$; note NOE to 2-H$_\alpha$; the NOEs to a and b are due to the partial saturation of the overlapping 4-H$_\alpha$ peak. (E) Saturate 3-CH$_3$; note NOEs to peaks a and b. (F) Portion of hemin possessing the two vinyl groups; the 2-vinyl is depicted in the *trans* and the 4-vinyl in the *cis* orientations.

In principle, the homonuclear Overhauser effect,[8] NOE, lends itself particularly well to detailed determination of vinyl orientations.[9-11] The fractional change in intensity of spin $i$ upon saturating spin $j$ is given by[8] $\eta_{j\to i} = \sigma_{ij}/\rho_i$, where the cross-relaxation rate $\sigma_{ij} \propto r_{ij}^{-6}\tau_c$ ($r$ is the distance between spins $i$ and $j$ and $\tau_c$ is the protein tumbling time) and $\rho_i$ is the relaxation rate for spin $i$. For a 30° dihedral angle with the heme plane, $r$(CH$_3$-H$_\alpha$) ~ 4.3 Å and $r$(CH$_3$-H$_{\beta\text{-t}}$) ~ 2.1 Å for the cis and ~2.8 Å and ~5.2 Å for the *trans* orientation, respectively. The shorter distance in each case is consistent with the detection of a NOE in a protein.[8-11] In a strictly out-of-plane (perpendicular)

(1) (a) Department of Chemistry. (b) UCD NMR Facility.
(2) Dunford, H. B.; Stillman, J. S. *Coord. Chem. Rev.* **1976**, *19*, 187-251.
(3) La Mar, G. N.; de Ropp, J. S.; Smith, K. M.; Langry, K. C. *J. Biol. Chem.* **1980**, *255*, 6646-6652.
(4) La Mar, G. N.; de Ropp, J. S.; Smith, K. M.; Langry, K. C. *J. Biol. Chem.* **1981**, *256*, 237-243.
(5) La Mar, G. N.; de Ropp, J. S.; Smith, K. M.; Langry, K. C. *J. Am. Chem. Soc.* **1983**, *105*, 4576-4580.
(6) Desbois, A.; Mazza, G.; Stetzkowski, F.; Lutz, M. *Biochim. Biophys. Acta* **1984**, *785*, 161-176.
(7) Terner, J.; Sitter, A. J.; Reczek, C. M. *Biochim. Biophys. Acta* **1985**, *828*, 73-80.
(8) Noggle, J. H.; Shirmer, R. E. *The Nuclear Overhauser Effect*; Academic Press: New York, 1971.
(9) Mabbutt, B. C.; Wright, P. E. *Biochim. Biophys. Acta* **1983**, *744*, 281-290.
(10) Mabbutt, B. C.; Wright, P. E. *Biochim. Biophys. Acta* **1985**, *832*, 175-185.
(11) Cooke, R. M.; Wright, P. E. *Biochim. Biophys. Acta* **1985**, *832*, 365-372.

0002-7863/86/1508-4244$01.50/0 