The Stereochemistry and Dynamics of Natural Products and Biopolymers from Proton Relaxation Spectroscopy: Spin-Label Delineation of Inner and Outer Protons of Gramicidin S Including Hydrogen Bonds

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Abstract: Since each conformational moiety of a natural product, polymer, or biopolymer has specific exposed and shielded protons, the parameter S_{1P} is proposed as a criterion for distinguishing conformational moieties; S_{1P} is defined as the enhancement of spin-lattice relaxation rates, R_1, per mole of added paramagnetic free radical. The specific effect of Tempo on all protons, NH, Hα, and side chain, of gramicidin S is reported here; the data semiquantitatively reflect proton-nitroxide distances and are consistent with random approach of these two solutes in Me_2SO-d_6 under the concentration used. Extension to other peptides, floppy peptides, and organic natural products, as well as accurate correlations with theory, is now underway.

I. Introduction

Delineation of hydrogen-bonded amide protons in peptides has involved many different approaches, including proton-deuterium exchange rates,[2] temperature dependence of chemical shifts,[3-6] solvent dependence of chemical shifts,[7-12] pH-rate profiles,[13] and solvent saturation.[14] Line broadening induced by the spin labeling with a stable nitroxide radical has been used[15,16] to detect the solvent-exposed vs. the solvent-shielded amide protons. In many of the above studies aimed at evolving NMR approaches to solution conformation, gramicidin S was the model compound used; its crystal[17] and solution structures are now well defined.[18,19]

Here we report extension and refinement of the use of paramagnetic reagents, specifically nitroxide spin-labels, in the following ways: (a) nitroxide radicals increase all proton spin-lattice relaxation rates (R_1) in the molecule—this includes Hα and side-chain protons as well as the previously reported line-broadening effects on amide protons;[15,16] (b) the nitroxide induced increase in R_1 values (designated R_{1P}) are smallest for inner (solvent-shielded) Hα and NH and largest for outer (solvent-exposed) protons of the backbone and side chains. The value of R_{1P} per mole of nitroxide is proposed as an index of the relative nitroxide effect, and hence solvent exposure. ESR spectra and the effect of temperature on R_{1P} are consistent with nitroxide molecules randomly approaching the peptide molecule with R_{1P} values reflecting the conformation in that solvent. Correlation of R_{1P} values with temperature coefficients of amide chemical shifts supports this view. Paramagnetic relaxation theories[20] predict that electron-proton distances of 5-20 Å can explain the data, but detailed quantitation is currently underway as well as extension to peptides of unknown or "floppy" structures and to the area of organic natural products.

II. Experimental Section

Gramicidin S hydrochloride was purchased from Sigma Chemical Co. and used without further purification. The solutions were prepared in 100% Me_2SO-d_6 and 99.8% CD_3OD (Aldrich). The amount of water was minimized by lyophilization of the samples. 2,2,6,6-Tetramethylpiperidine-1-oxyl (Tempo) (Molecular Probes Inc.) and 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrrolidine-1-yloxy (Aldrich) were used as soluble spin-labels.

The NMR spectra were recorded on a Bruker WH-270 equipped with a Nicolet 1180 computer; the temperature was controlled to ±1 °C by a Bruker unit. The spin-lattice relaxation rates were obtained with a $(180°-\tau-90°-t)_n$ pulse sequence and were calculated by linear fitting of the recovery curves of the longitudinal magnetization. The experimental precision in T_1 measurements was ±2% and the accuracy about 7%. Initial rate plots were used to give actual relaxation rates. The paramagnetic contribution (R_{1P}) was calculated by subtracting the relaxation rate in the blank solution (R_{1b}) from that obtained after addition of micro amounts of stock solution of the nitroxide (R_{1obsd}); care was taken to minimize volume changes.

The EPR spectra were obtained with a Varian V4502 spectrometer operating at the microwave frequency of 9.46 GHz. Both ESR and

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Table I. Paramagnetic Effects Observed on Spin-Lattice Relaxation Rates (R) of Gramicidin S Protons in Me_2SO-d_6: Correlations with Temperature Coefficients of Amide Proton Chemical Shifts ($\Delta\delta/\Delta T$)

proton	chemical shift, ppm from TMS	relaxation rates, R, at different Tempo concentrations (M) 0	10^{-3}	5×10^{-3}	10^{-2}	2×10^{-2}	S_{1P}, s^{-1} M^{-1}	$\Delta\delta/\Delta T$,[a] ppb/°C
Leu NH	8.31	2.8_6	2.9_7	3.3_5	3.8_1	4.8_0	100 ± 6	−3.2
Leu Hα	4.64	1.9_3	2.1_8	3.3_4	4.1_5	6.3_4	105 ± 7	
Orn NH	8.62	2.7_7	2.9_2	4.4_3	6.4_7	10.0_6	380 ± 13	−5.3
Orn Hα	4.94	2.5_6	2.6_5	3.0_8	3.6_5	4.4_4	40 ± 10	
Phe NH	9.11	2.7_3	3.1_9	4.3_1	6.4_0	9.5_2	350 ± 15	−7.4
Phe Hα	4.47	1.8_2	2.2_4	3.5_2	4.9_5	8.5_0	235 ± 10	
Pro Hα	4.42	1.3_5	1.6_1	2.7_0	4.1_3	6.3_5	130 ± 6	
methyls	1.04	3.6_3	4.0_0	5.4_8	7.3_3	11.1_0	370 ± 14	
aromatics	7.20	1.2_0	1.5_8	3.2_4	5.3_2	9.3_0	410 ± 16	

[a] Data reported in ref 5.

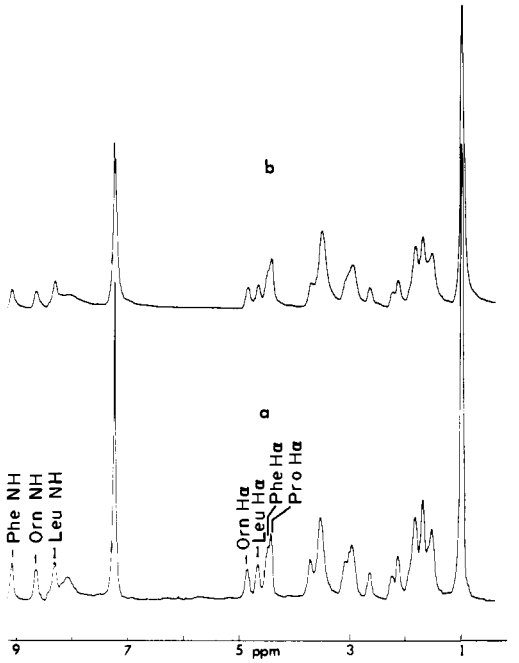

Figure 1. (a) 270-MHz ^1H NMR spectrum of gramicidin S (40 mM) in Me_2SO-d_6 at 299 K; (b) as in (a) in the presence of Tempo (10 mM).

NMR studies were performed at 26 °C.

III. Results and Discussion

1.1. Effect of Nitroxides on the NMR Spectrum of Gramicidin S. Figure 1 is similar to that obtained by Kopple and Schamper[15,16] (assignments are given in Table I): (a) all amide and amino proton signals are broadened by the presence of 20 mM Tempo; (b) the increasing selective broadenings of the Orn^2 NH_3^+ (δ 8.13 ppm), Phe^4 NH, and Leu^3 NH are readily seen (Val^1 NH is hidden by the Phe^4 ring proton signal); and (c) no significant chemical shift changes accompany nitroxide addition. However the line width ($1/T_2^*$) of all protons on the solvent, aromatic rings, and aliphatic Cα, Cβ, Cγ, and Cδ atoms are generally increased. Because of the above phenomena and the fact that T_2^* measurements from line broadening are not very accurate, it was decided to investigate pulse FT measurements of both T_1 and T_2; only T_1 is reported here (note: relaxation rate $R_1 = 1/T_1$).

1.2. Effect of Tempo on Spin–Lattice Relaxation Rates. Typical partially relaxed proton spectra of gramicidin S in the presence and absence of nitroxide are seen in Figure 2; corresponding τ values are given in the figure caption. The R_1 values for the NH_3^+ protons is faster than those of the amide protons; the latter have approximately the same R_1 values as seen by their null point in the Figure 2a spectra. Addition of free radical markedly enhanced the R_1 values of these same protons (all spectra in Figures 2a and 2b have corresponding τ values in the 180°–τ–90° sequence to facilitate comparison); this can be qualitatively ascertained by comparing null points for each proton in Figures 2a and 2b. All protons relax much faster, as expected, but the solvent-exposed

Table II. Paramagnetic Effects Observed on Gramicidin S Protons in CD_3OD

proton	chemical shift,[a] ppm	S_{1P}, s^{-1} M^{-1}
Orn-Hα	4.92	40 ± 9
Leu-Hα	4.60	130 ± 7
Phe-Hα	4.45	235 ± 11
Pro-Hα	4.30	105 ± 6
Val-Hα	4.11	420 ± 17

[a] In ppm from internal HMS.[17,18]

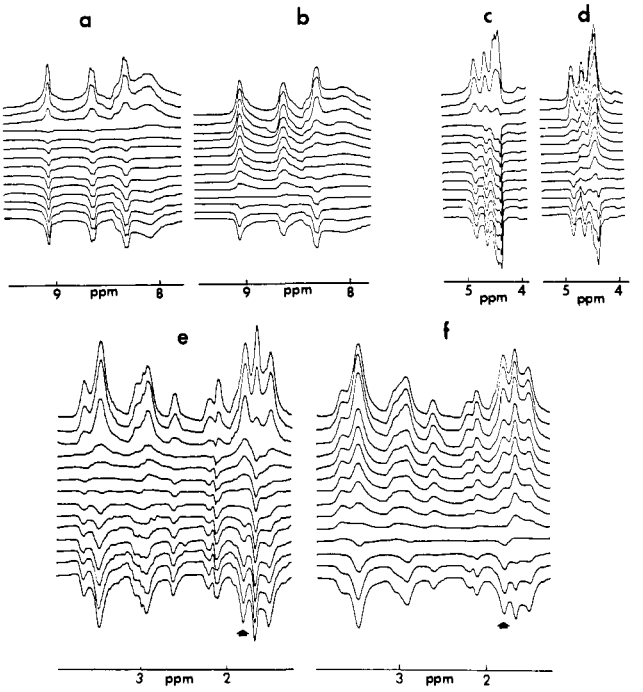

Figure 2. Partially relaxed proton spectra of selected regions of the gramicidin S spectrum under the same experimental conditions as Figure 1. The spectra on the right show the effects of 10 mM Tempo. All sequences refer to identical values; values from top to bottom in each section are 5000, 800, 400, 200, 180, 160, 140, 120, 100, 80, 60, 40, 20, 2 ms. Arrows of Figure 2e,f point at Orn Hγ's resonance.

NH and Hα, aromatic ring protons, and methyl protons have significantly more enhanced R_1 values than the inner directed Leu^3 NH and Orn^2 Hα. The actual spectra for α protons and the side-chain protons can be compared from Figures 2c and 2d and from Figures 2e and 2f, respectively. The partially relaxed spectra in the presence and absence of nitroxide reflect those changes expected from (a) the known model of gramicidn S,[17,18,22] (b) the

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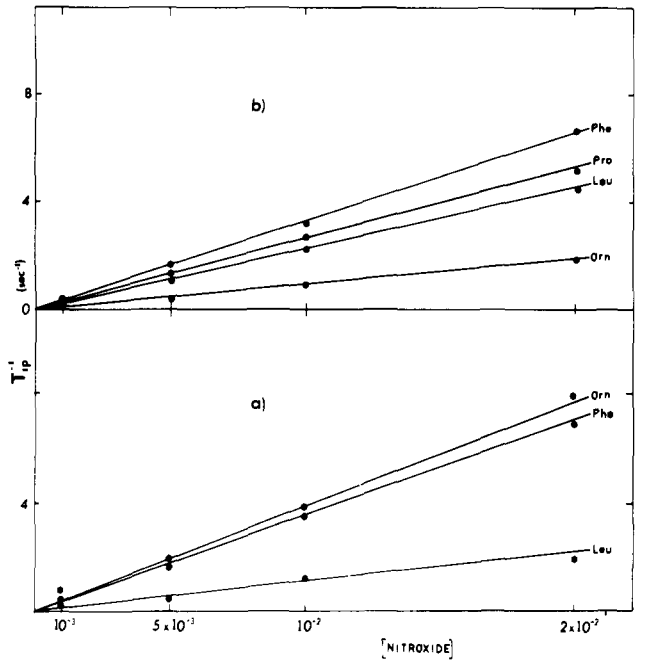

Figure 3. Dependence of R_{1P} ($=1/T_{1P}$) on Tempo concentration for (a) NH protons and (b) Hα protons of gramicidin S (40 mM in Me_2SO at 299 K).

supposition that the nitroxide will enhance the relaxation of all protons, and (c) the fact that inner directed vs. outer directed protons will experience relaxation enhancements whose magnitudes reflect relative approach distances between the free radicals and the proton. This effect allows the delineation of solvent exposed protons, and vice versa, provided the solute–nitroxide interaction is as strong as the solute–solvent one. As a consequence, the local conformation of gramicidin S governs the extent of free-radical enhancement of the relaxation rates of each proton. The best examples of protons that are solvent shielded in the conformation of gramicidin S are (a) Orn[2] Hα, (b) Leu NH and Orn Hγ; exposed protons are (a) Leu[3] Hα, Pro[5] Hα, and Phe[4] Hα, (b) Val CH_3's and Leu CH_3's, and (c) Phe[4] ring protons.

1.3 Concentration Effects. Figure 3 shows the effect of nitroxide concentration on R_{1P} for the amide and α protons of gramicidin S in Me_2SO-d_6. The dependence on concentration is a linear and easily discernible event at low concentrations where T_2* effects on line width were barely visible. The selective effects induced by Tempo on the relaxation rates of gramicidin S protons, previously outlined by a vis-à-vis analysis of partially relaxed spectra, can be accurately quantitated on the basis of R_{1P} vs. Tempo concentration plots in Figure 3. The enhancement in relaxation rate per mole of nitroxide, S_{1P}, for each proton, or group of protons, was obtained from these graphs and is an excellent criterion of shielded vs. exposed protons as shown in Table I. S_{1P} values and $\Delta\delta/\Delta T$, the temperature coefficient of amide proton chemical shifts, exhibit a close correlation, confirming, at least for the amide protons, that S_{1P} values reflect solvent exposure.

1.4 Solvent Effects. The general picture described above in Me_2SO-d_6 pertains for the Hα protons of gramicidin S in CD_3OD. The viscosity and hence correlation times for motion in these two solvents are not the same and so perfect correspondence was not expected. Figure 4 shows that the R_{1P} values for Orn Hα are relatively insensitive to nitroxide concentration, while the other Hα protons are increasingly sensitive.

2. Nitroxide–Peptide–Solvent Interaction. Before soluble nitroxide could be used as criteria for conformational analysis, as described above, it was necessary to investigate the nature of the interactions taking place in the nitroxide–peptide–solvent mixtures. The data are consistent with the hypothesis that nitroxide interacts nonspecifically and randomly with all parts of the peptide. The unpaired electron therefore reflects the relative distances between the free radical and the individual protons. The evidence for this hypothesis comes from (a) ESR spectroscopy, (b) effect of temperature on the paramagnetic contribution to the individual proton (called S_{1P}) relaxation rates Figure 5, and (c) comparison of the temperature dependence of chemical shifts and R_{1P} values for amide protons. (Table I).

2.1. ESR Spectroscopy. The characteristic three-line ESR spectrum of Tempo in dimethyl sulfoxide was indistinguishable from that of Tempo–gramicidin S–dimethyl sulfoxide solutions, indicating that the free radical isotropically rotates with a τ_c (nitroxide) $\approx 5 \times 10^{-11}$ s^{-1} [21] in both solutions; the motion of Tempo is not significantly affected by the slower motion of gramicidin S which was evaluated, under these conditions,[22] to have a correlation time of τ_c (gramicidin S) $\approx 1 \times 10^{-9}$.

2.2. Effect of Temperature. Figure 5 is a plot of R_{1P} vs. (T/η). The negative slopes exclude a contribution from exchange of the nitroxide between different sites. The fact that R_{1P} varies linearly with (T/η) is consistent with the Hubbard theory[20] of the diffusion-limited nuclear relaxation; the relaxation rate changes proportionally to D, which is the mean diffusion coefficient of the radical and the peptide:

$$D = \tfrac{1}{2}(D_{FR} + D_{GS})$$

$$D_{FR} = (k/8\pi)(T/\eta)(1/a_{FR})$$

$$D_{GS} = (k/8\pi)(T/\eta)(1/a_{GS})$$

Here, k is the Boltzman constant; a_{FR} and a_{GS} are the hydrodynamic radii of the free radical and gramicidin S; D_{FR} and D_{GS}

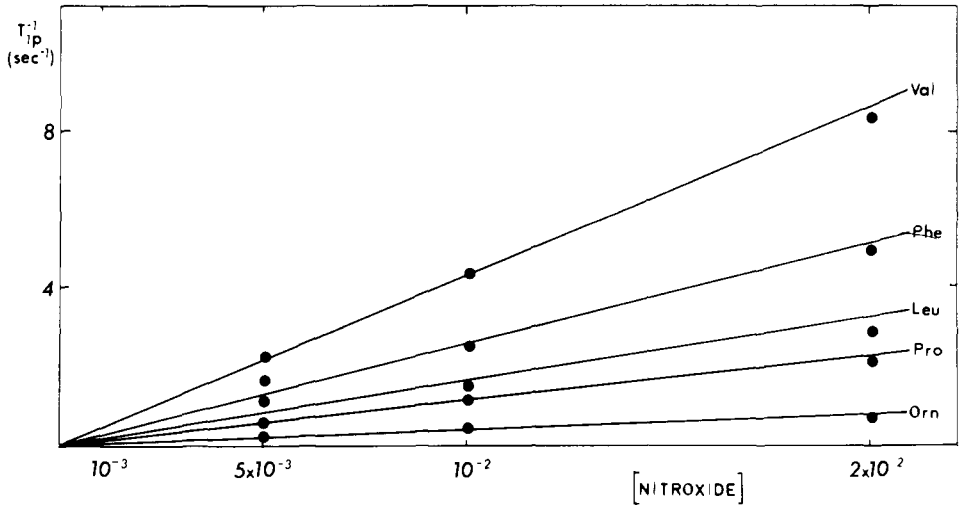

Figure 4. Dependence of R_{1P} ($=1/T_{1P}$) on nitroxide concentration for the Hα protons of gramicidin S (50 mM in CD_3OD at 304 K).

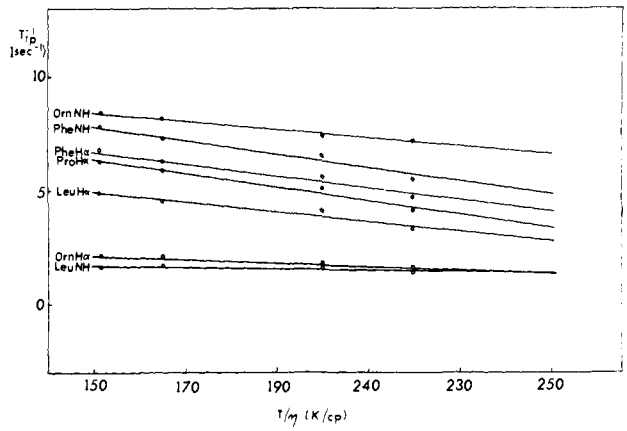

Figure 5. Dependence of R_{1P} ($=1/T_{1P}$) on T/η for the NH and Hα protons of gramicidin S (40 mM in Me_2SO). The temperature was varied from 299 to 329 K.

are the coefficients for self-diffusion of free radical and gramicidin S.

2.3. Correlation of R_{1P} with Temperature Dependence of Amide Chemical Shifts ($\Delta\delta/\Delta T$). $\Delta\delta/\Delta T$ is regarded as one of the best criteria for distinguishing solvent-exposed and solvent-shielded amide protons. R_{1P} appeared to vary linearly with $\Delta\delta/\Delta T$ for Orn[2] NH, Leu[3] NH and D-Phe[4] NH (see Table I).

IV. Conclusions

In general, free radicals increase the relaxation rates of all protons, not just amide protons, in gramicidin S. Protons having greater solvent exposure, and hence access to the unpaired electron of the nitroxide radical, have more enhanced relaxation rates compared to inner protons of the molecule; the latter include hydrogen-bonded amides, solvent-shielded C Hα's and Orn Hγ's.

The enhancement of spin–lattice relaxation rates R_{1P} was measured as a function of radical concentration and solvent (Me_2SO-d_6 and CD_3OD); R_{1P} vs. concentration curves reflect exposed vs. shielded protons. The paramagnetic relaxation[20,21] theories predict that electron–proton distances in the range 5–20 Å should produce the differential enhancement reported here. We are currently determining ratios of distances and actual distances from the proton relaxation rate enhancements per mole, S_{1P}; these values provide an excellent criterion for evaluating the relative solvent exposure of a proton on a molecule provided no specific nitroxide–molecule complexes exist.

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Registry No. Gramicidin S, 113-73-5; Tempo, 2564-83-2.

Diazoalkane Complexes of Molybdenum and Tungsten

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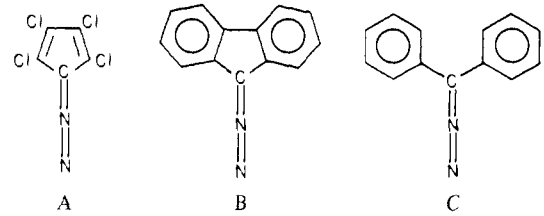
Abstract: Ordinary diazoalkanes, N_2CHR' and $N_2C(CH_3)R'$ (R' = C_6H_5, p-$CH_3C_6H_4$), were found to react with $M(CO)_3(S_2CNR_2)_2$ (M = Mo, W; R = CH_3, C_2H_5; R_2 = $(CH_2)_4$) at room temperature to form stable complexes of the type $M(CO)(N_2CHR')(S_2CNR_2)_2$ which were conveniently isolated in good yields. Oxidation of $M(CO)(N_2CHR')(S_2CNR_2)_2$ with 1 equiv of Cl_2 or Br_2 produced $MX_2(N_2CHR')(S_2CNR_2)_2$ (X = Cl, Br) which were isolated as chloroform solvates. The addition of excess HBr to $W(CO)(N_2CHR')(S_2CNMe_2)_2$ gave a red solid which analyzed for $WBr_2(N_2H_2CHR')(S_2CNMe_2)_2$. Physical and spectroscopic data suggest that the diazoalkane ligands in the above complexes behave as terminal, singly bent, four-electron donor ligands. The NMR chemical shifts of the methine proton in N_2CHR' ligands indicate that the $M(S_2CNR_2)_2$ unit is strongly electron withdrawing. Syntheses for new tungsten compounds, $W(CO)_3(S_2CNR_2)_2$, $W(CO)_2(S_2CNR_2)_2$, $W(CO)_2(L)(S_2CNR_2)_2$ (L = p-$CH_3C_6H_4NHNH_2$, 4-$CH_3C_5H_4N$), $WBr_2(S_2CNR_2)_2$, $W(S_2CNR_2)_4$, and $WBr_3(S_2CNR_2)_2$, are also reported.

Diazoalkanes ($R_2'C{=}N{=}N$) are reactive compounds which find a wide range of synthetic uses as carbene ($R_2'C$:) precursors or as electron-deficient nitrene-like ($R_2'C{=}\ddot{N}$-$\ddot{N}$:) molecules. It is not at all surprising that the interactions of transition-metal complexes with diazoalkanes often lead to the catalytic or stoichiometric evolution of N_2 and the production of free or coordinated carbene reaction products.[1] Indeed, it was not until 1972 that the first isolable mononuclear complexes of diazoalkanes were reported.[2] It is noteworthy that the only published reports of isolable diazoalkane complexes prepared from diazoalkanes and transition metals involve diazoalkanes which are stabilized by strongly electron-withdrawing groups (R' = –CN, $-CF_3$, $-CO_2R$, –C(O)R) or groups which can resonance-stabilize negative charges such as those found in diazotetrachlorocyclopentadiene (A), diazofluorene (B), and diazodiphenylmethane (C).



A B C

Interest in diazoalkane complexes not only stems from the plethora of possible new metal-assisted and metal-hindered reactions of the reactive compounds but also from the stereochemistry of the R'_2CNN ligand and its many modes of bonding. A survey of recent chemical literature indicates that at least six

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