an Gemini 200 spectrometer in $CDCl_3$ using TMS as an internal standard. The FT-IR spectra were measured on a Nicholet 500 spectrometer as KBr disks. Optical rotations were measured on a Jasco DIP-370 digital polarimeter.

(+)-Camphor-10-thiol (**4**). A solution of (+)-10-camphorsulfonyl chloride (**3**, 18.0 g, 71.8 mmol) in a mixture of 240 mL of dioxane and 60 mL of water was treated with triphenylphospine (75.3 g, 287 mmol). The clear mixture was stirred for 2 days at room temperature, and then refluxed for 1 h. The reaction mixture was concentrated in vacuo. The resulting syrupy residue was extracted with hot hexanes (200 mL ×3). As the hexanes solution cooled to room temperature, a white precipitate (phosphine oxide) was formed, which was discarded. The hexanes solution was extracted with 5 M NaOH solution (100 mL×5) and the combined NaOH extract cooled in an ice bath was acidified by careful addition of concentrated HCl (250 mL). The thiol was separated as a white solid, which was extracted with ethyl acetate (200 mL×3). The organic solution was dried (Na_2SO_4) and concentrated in vacuo to give a white solid. Finally, purification by vacuum sublimation (125-135 °C, 0.05 mmHg) yielded 9.47 g (72%) of the thiol **4** as a colorless crystal, mp 65-66 °C (lit.[7] 65-67 °C); $[\alpha]_D^{20}=+5.2$ (c=1.02, $CHCl_3$); ^1H NMR ($CDCl_3$) **d** 4.00-3.98 (m, 1H), 2.79 (dd, 1H, J=9.5 and 13 Hz), 2.58 (dd, J=5.3 and 13 Hz), 2.15 (bs, 1H), 1.28 (dd, 1H, J=9.5 and 5.3 Hz), 1.05 (s, 3H), 0.83 (s, 3H), and others; ^{13}C NMR ($CDCl_3$) δ 217.2, 60.3, 47.5, 43.4, 42.9, 26.7, 26.3, 21.0, 20.0, 19.5; IR (KBr) cm^{-1} 1731.

(1*S*)-(+)-10-Mercaptoisoborneol (**1**). To a solution of $NaBH_4$ (2.06 g, 54.2 mmol) in EtOH (100 mL), cooled in an ice bath was added a solution of ketone **4** (5.00 g, 27.1 mmol) in EtOH (20 mL) over 10 min under a nitrogen atmosphere. The whole mixture was stirred for 2 days. Then, the excess NaBH4 was destroyed with dilute HCl solution. The product was extracted with EtOAc (100 mL×2). The combined extract was washed with brine, dried (Na_2SO_4) and concentrated in vacuo. Finally, column chromatography of the residue (eluent; hexanes : EtOAc=20 : 1) on silica gel gave 4.73 g (93% yield) of the product as a solid, mp 73-74 °C (lit.[1] 76-78 °C; lit.[3] 7 0); $[\alpha]_D^{20}=-56.0$ (c=1.15, $CHCl_3$) (lit.[1] $[\alpha]_D^{24}=-55.4$; lit.[3] $[\alpha]_D^{24}=-57.44$ (c=10, $CHCl_3$); ^1H NMR ($CDCl_3$) δ. 3.97 (apparent t, 1H, J=4.7 Hz), 2.79 (dd, 1H, J=9.5, 13 Hz), 2.56 (dd, 1H, J=5.4, 13 Hz), 1.28 (dd, 1H, J=9.5, 5.4 Hz), 1.05 (s, 3H), 0.83 (s, 3H), and others; ^{13}C NMR ($CDCl_3$) δ 76.4, 52.9, 47.4, 45.7, 39.4, 30.3, 26.8, 23.7, 20.5, 19.9; IR (KBr) cm^{-1} 3467, 2950, 1393, 1373, 1071, 1033.

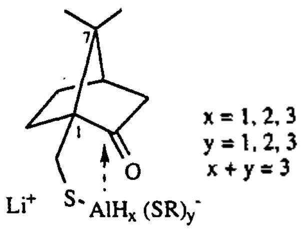

Figure 1.

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8. The high selectivity may be ascribed to the model above (Figure 1) where the carbon-sulfur bond is *anti* to the C1-C7 bond and the hydrogen atom is transferred intramolecularly to the less hindered si face of the carbonyl bond.

Highly Overlapping ^1H NMR Signal Assignments of 12,13-Diepimeric Coenzyme F430 by the Compensated ROESY Experiment

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Coenzyme F430 is a nickel(II)-containing cofactor of the methyl coenzyme M reductase (Component C which was found in the cells of methanogenic bacteria) that is involved in the bio-catalytic reduction of methyl coenzyme M (2-methylthioethanesulfonic acid, CH_3-S-CoM).[1,2] Coenzyme F430 is known to be mediated in the reductive demethylation of methyl coenzyme M, using reducing equivalents from 7-mercaptoheptanoylthreonine phosphate (HS-HTP). The products of this reaction are methane and the heterodisulfide of methyl coenzyme M and HS-HTP (CoM-S-S-HTP).[3-5]

The pentamethyl ester derivative of F430 (F430M) was chosen for earlier structural studies because this derivative is soluble in non-coordinating solvents where the low-spin form of Ni(II) dominates. In coordinating solvents such as D_2O or methanol, Ni(II) exists in a high-spin (paramagnetic) state and the molecule is not amenable to NMR investigations.[6-8] In earlier studies, only a partial ^1H NMR and ^{13}C NMR assignment was made for native F430 based on comparisons with the ^{13}C NMR spectrum of F430M. The general structural feature of F430 was deduced from extensive studies of the F430M involving biosynthetic methods and spectroscopic measurements, including selective ^{13}C-enrichment and traditional 1D-NOE difference NMR experiments.[7-10]

Structural work of native cofactors has been mostly accomplished depending on the NMR methods partly because of difficulties in obtaining the suitable crystal for X-ray crystallographic structure determination. ^1H NMR signals of native coenzyme F430 obtained using in deuterated trifluoroethanol (TFE-d_3) solvent at high magnetic field strength (11.75 T; 500 MHz) were sufficiently narrow to allow detailed investigations using recently developed two-dimensional (2D) NMR methods.[10-13] By confirming unambiguously structural aspects of native F430 from ^1H and ^{13}C NMR signal assignments, the solution state structure of native F430 and 12,13-diepimeric F430 were made by modern NMR techniques.[11-13] However, some of overlapping ^1H NMR signals were observed to be critical in determining the stereospecific macrocycle and the degree of characteristic puckering of the corphin ring (see saturated carbons associated with the D-five membered ring in Figure 1).[11-13] The characteristic saddle shape of the corphin ring is believed from the electrophilicity of Ni(II) and the size of ring, and the saturation of carbon macrocycle. However, the specific function of ring-puckering is not clearly known in F430-dependent biocatalysis.[11,14-17]

Assignment of the ^1H NMR signals was a crucial prerequisite for determining the atomic-level solution structure by using nuclear Overhauser effect (NOE) and NMR-based distance geometry techniques.[13,18,19] Current studies are designed to clarify the severely overlapping ^1H NMR singals (typically multiproton system associated with methylene geminal protons) that may enhance the determination of the degree of macrocyclic corphin ring-puckering by using NOE spectroscopy in the rotating frame (ROESY).[13,18,19]

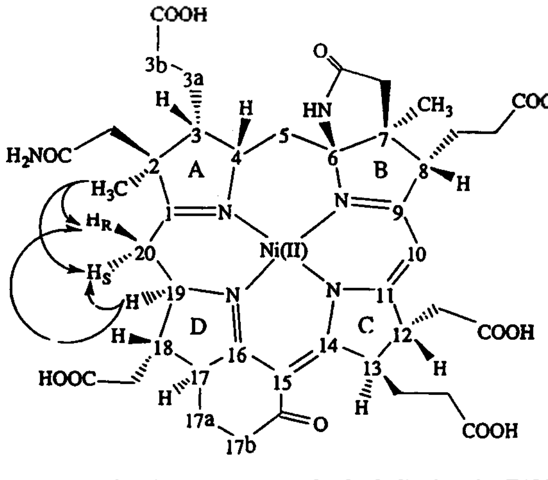

Figure 1. Molecular strucuture of 12,13-diepimeric F430 with numbering scheme. Arrows indicate NOE observations from Me2 (methyl group attached to C2) and H19 toward H20r,s

Experimental Section

Materials. Phenyl Sepharose CL-4B, QAE A-25 and DEAE-Sephadex A-25 were purchased from Pharmacia LKB Biotechnology Inc. C_{18} RP-HPLC (reverse phase high pressure liquid chromatography) columns were obtained from Waters. PM30 ultrafilteration membranes were purchased from Amicon. Deuterated trifluoroethanol (TFE-d_3) was purchased from Cambridge Isotopes. Bacterial cells were lab stock cultures.

Isolation of native F430 and its epimers. *Methanobacterium thermoautotrophicum* strain ΔH, (DSM 1053) were grown in a 250 *l* fermenter (B. Braun) at 60 °C, pH=7.3 with H_2 and CO_2 as carbon and energy sources, respectively. The medium was reduced with H_2S (to *ca* 440 mV *vs.* NHE) before inoculation. During the fermenter running, H_2S, H_2 and CO_2 flow rates were adjusted manually and via computer to maintain a constant pH (0.15). As the cells reached the end of exponential growth (but before stationary phase) they were aerobically harvested (Sharples centrifuge). The cell paste was then immediately transfered into Wheaton bottles and made anaerobic by several nitrogen gas flushing cycles in an air lock of an anaerobic hood (Coy). The cells were stored under N_2 at -20 °C either as a cell paste or as a cell suspension of whole cells in 50 mM potassium phosphate (pH 7.0) buffer (1 : 1).

Native F430 and epimers were purified by utilizing hydrophobic interaction chromatography (Phenyl Sepharose) and anion exchange chromatography (QAE A-52 and DEAE A-52) as described previously.[9,12] Two additional HPLC systems were used for further purification of native F430. The eluent was monitored at both 560 nm and 430 nm using HPLC systems (I and II): (HPLC SYSTEM I: Waters C18 μBondapak 3.9 mm × 30 cm; 25 min linear gradient; 10% MeOH (50 mM NH_4CO_2H, pH 7.0) to 50% MeOH (50 mM NH_4CO_2H, pH 7.0); 0.5 mL/min); (HPLC SYSTEM II: Waters C18 μBondapak 7.8 mm × 30 cm; 20 min linear gradient; 10% MeOH (50 mM NH_4CO_2H, pH 7.0) to 60% MeOH (50 mM NH_4CO_2H, pH 7.0); 1 mL/min.). Final purity of F430 was checked with UV/Vis spectrometric analyses and mass spectrometry. The characteristic absorbance ratio of A430/A275 was 1.05, and fast atom bombardment (FAB) cation mass spectrum of native F430 gave a m/z value (=905). The elemental composition was determined to be $C_{42}H_{51}O_{13}N_6Ni$ by high resolution mass spectrometry (70-SE-4F, four sector 8-kV mass spectrometer). Detail methods and procedures of sample purification were described in privious paper.[11]

NMR data. NMR spectral data were obtained by using GE GN-500 MHz and Bruker AM-600 MHz spectrometer. Sample conditions were as follows: 1.1 mM 12,13-diepimeric F430 in F_3CCD_2OD (TFE-d_3) solvent; T=25 °C. Raw NMR data were transferred *via* ethernet to Silicon Graphics Personal Iris computers, converted to FTNMR file format by using an in-house program and processed by using FELIX. ^1H chemical shifts were referenced to internal TFE-d_3 (3.88 ppm, ^1H). Homonuclear correlated spectra were zero-filled to final matix sizes of 1024×1024 real points. Data acquisition and processing paramaters for individual 2D experiments (defined below) were as follows.

ROESY. 2×256×1024 raw data matrix size; 64 scans per t1 increment; 2.4 s repetition delay; 150 ms continuous wave spin lock period; 6.25 kHz spin lock field strength, corresponding to 40 μs 90° pulse width; 6 Hz Gaussian and 90° shifted squared sine bell filtering in the t2 and t1 domains, respectively.[20-22]

NOESY. 2×256×1024 raw data matrix size; 64 scans per t1 increment; 2.8 s repetition delay period; 10, 50, 100, 300, 500 ms mixing period for NOE buildup profile; 6 Hz Gaussian and 90° shifted squared sine bell filtering in the t2 and t1 domains, respectively.[23,24]

Results and Discussion

NMR methods. The methods and procedure for ^{1}H and ^{13}C NMR signal assignments were obtained by utilizing ^{1}H-^{1}H homonuclear and ^{1}H-^{13}C heteronuclear NMR methods.[11-13] Through space ^{1}H-^{1}H dipolar connectivity were obtained from NOESY and ROESY NMR methods.[21,22] The advantage of ROESY experiment is to distinguish peaks arising from multiple quantum artifacts by indentifying spin diffusion peak with opposite phase to the NOE cross peak. However, ROESY experiment for strongly coupled protons (interproton pair located 2 or 3 bond away) does give poor distance information due to severe Hartmann-Hahn transfer.[25-27] The identification of relay NOESY cross peak among overlapping peaks are important in NMR-based structure determination.[18]

Five geminal protons, including H3b,b' (2.28 ppm), H7a, a' (2.48 ppm), H12a,a' (2.24 ppm), H18a,a' (2.47 ppm), and H20,20' (3.00 ppm), were assigned to be severely overlapping peaks in the 12,13-diepimeric F430 corphin ring. The complex coupling patterns from H19 to H20,20' geminal protons are critical in the determination of the degree of ring-puckering. In order to resolve the severely overlapping geminal protons, two calibrated 90° soft pulses were added during the spin-lock(SL) period (compensated ROESY) to minimize Hartmann-Hahn effect [90°-t$_1$-90°-SL (τ_m)-90°].[27] In addition, various mixing period τ_m (50-200 ms) were applied to identify multiple coupling patterns.

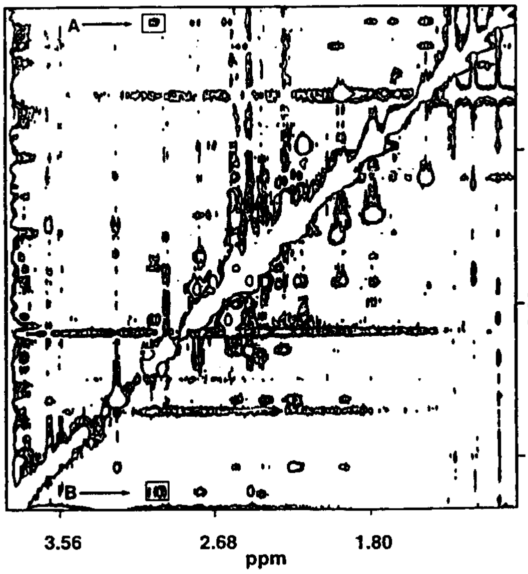

Figure 2. Portion of 2D ROESY spectrum obtained for 12,13-diepimeric F430 in TFE solution with a mixing time of 150 ms. Spectrum are shown with the positive and negative contour plot in the range of 0.8 to 3.8 ppm. A indicates a cross peak from Me2 to H20r,s whereas B points out a cross peak from H19 to H 20r,s.

Spectra from compensated ROESY experiment. Portion of 2D ROESY spectrum obtained for 12,13-diepimeric F430 with a mixing time of 150 ms is shown in Figure 2. Both positive and negative contour plot in the range of 0.8 to 3.8 ppm are exhibited. Symbol A indicates a long range cross peak from Me2 to prochiral H20r,s whereas symbol B points out a strongly coupled cross peaks from H 19 to H20r,s those exhibit direct and indirect coupling patterns (see four multiplets resolved in Figure 2). In order to clarify the correlation between multiplets of 1D spectrum and ROESY spectrum, two cross section of 2D ROESY spectrum with a range of 2.0-3.5 ppm and 1D spectrum are presented in Figure 3. Multiplets appeared at 2.9-3.1 ppm are from the strongly coupled H20r,s methylene protons to neighboring H19 in 1D spectrum. Symbol A is a cross section of 2D ROESY spectrum from Me2 to H20r,s indicating that Me2 is geometrically bisecting the two protons attached to C20 carbon (no spin diffusion). However, sym-

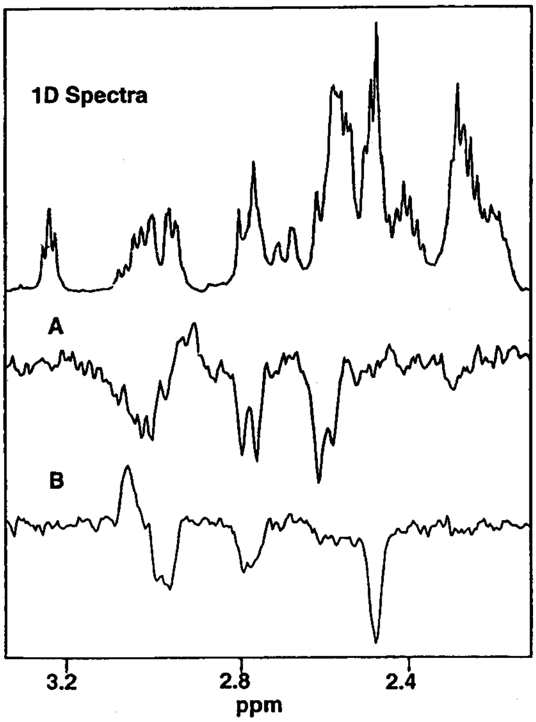

Figure 3. 1D spectrum of 12,13-diepimeric F430 in the range of 2.0-3.5 ppm. Multiplets appeared at 2.9-3.1 ppm are from the strongly coupled H20r,s geminal protons to neighboring H19. A is a cross section of 2D ROESY spectrum from Me2 to H20r,s implying that Me2 is geometrically bisecting the two protons attached to C20 carbon. B is a cross section of 2D ROESY spectrum from H19 to H20r,s indicating that a peak at 3.05 ppm is a spin diffusion peak while a peak at 2.95 ppm is from direct coupling.

bol B is a cross section of 2D ROESY spectrum from H19 to H20r,s indicating that a peak at 3.05 ppm is a spin difussion peak while a peak at 2.95 ppm is from direct coupling.

NOE buildup curves from H19 to neighboring protons exhibit a scalar (J) coupling effect on H19-H20r,s curve at lower mixing time (10-100 ms) due to the contribution of both through bond and through space coupling (see Figure 4). NOE buildup profiles were archieved to check behavior of spin system during the dipolar relaxation, and to compare with NOEs from NMR-based distance geometry structure. Experimental NOE volume integral at five different mixing time (10, 50, 100, 300, and 500 ms) were obtained by integrating off-diagonal cross peaks, and were then normalized with the integrated value of some well-resolved diagonal peaks.[13,18] The fast slope and negative values of NOE buildup curve for H19-H20,20' indicate that multiplets are influenced by COSY effect (see the NOE buildup in Figure 4). The comparison of experimental NOE buildups with those of solution state strucuture is a major criteria in the evaluation of generated NMR-based structure. The comparison of 2D-NOESY back-calculations between 12,13-diepimeric coenzyme F430 and pentamethlated ester form (F 430M) were made in Figure 5. Peak intensities associated with cross peaks H19, Me2, and D-ring which is most important in the determination of the degree of corphin macrocyclic ring-puckering are appeared to be same. In addition, the spectral comparison made for H4 (A-ring) and H 10 (B,C-ring) to cross peaks are appeared to be very similar. The analysis of ROESY peaks and NOE buildup profiles enabled to clearly distinguish each multiplets of H20r,s.[13,18] Results of peak analysis are summarized by differenciating coupling patterns ($^3J_{H19-H20r,s}$ and $^2J_{H20r-H20s}$) in Figure 6.

In addition, prochiral assignments for 14 out of 18 methylene groups were made on the basis of the identification of relay peak by comparing NOESY and compensated ROESY spectra. Although H20r,s peaks are severely overlapped, current mehod provided structural feature which is useful for the determination of the degree of puckering of the corphin-ring. However, some of severely overlapping methylene ^1H signals arising from side chain rotation (H3br,s, H12ar,s, and H18ar,s) are still needed to be assigned.

Degree of corphin ring-puckering. Solution state structure of 12,13-diepimeric F430 previously made by NOE-derived distance restraints those were obtained via qualitative assessment of NOE cross peak volume was evaluated in cur-

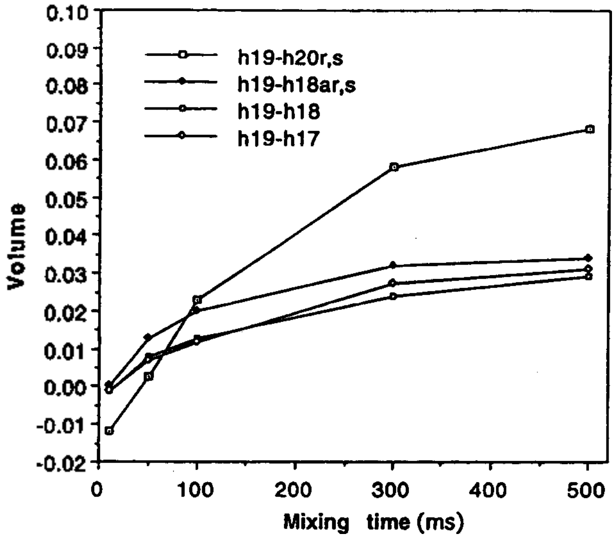

Figure 4. NOE buildup curves from H19 to neighboring protons. Severe correlated spectroscopy (COSY) effect were appeared on H19-H20r,s at lower mixing time (10-100 ms) due to the contribution of both through bond and through space coupling. Negative NOEs and fast slope of NOE buildup is in general from COSY effect.

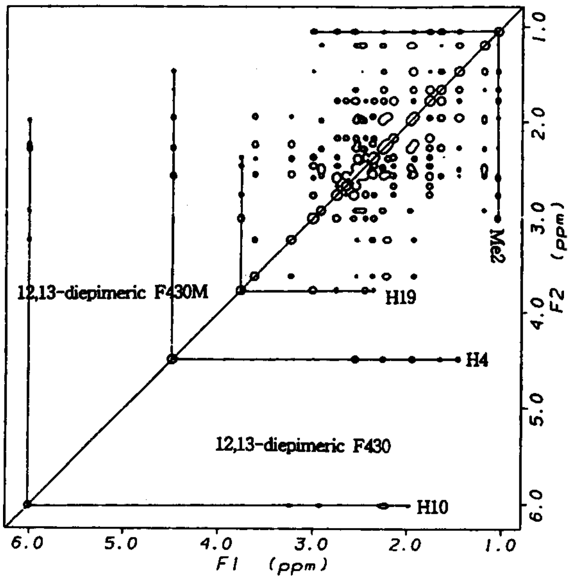

Figure 5. Comparison of the back-calculatd 2D-NOE spectra of 12,13-diepimeric F430 (NMR-based saolution structure) and 12, 13-diepimeric F430M (X-ray crystallographic structure) at 300 ms mixing period. Several consistencies for several protons H10, H4, H19, and Me2 are labeled.

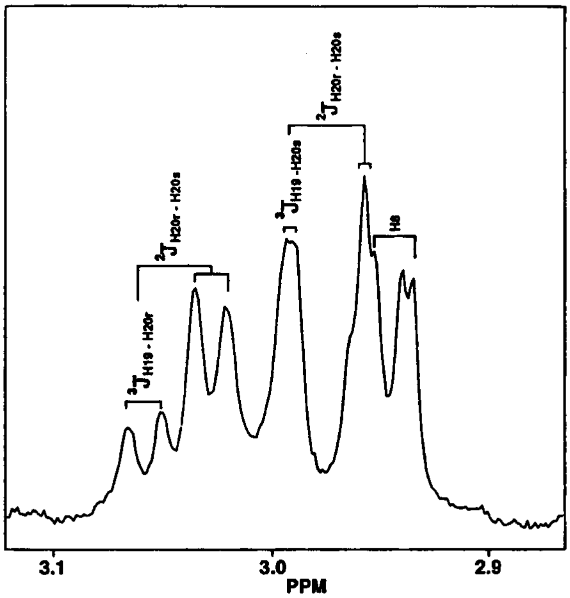

Figure 6. Direct and indirect coupling patterns associated with H19 and H20r,s geminal protons. ^{3}JH19-H20r,s and ^{2}JH20r-H20s stand for three-bond and two-bond away coupling patterns, respectively.

rent studies.[13] The spin-locked NOE (ROESY) experiment was specially useful to distinguish direct NOE effects from indirect (or relay) effects.[19-24] The compensated ROESY experiment with two additional 90° pulses added on spin-lock period was sufficient enough to resolve the strongly coupled multiplet patterns (through-bond and through-space coupling effects are mixed).[25-27] The range of spin-lock period of 50-200 ms with 6.25 kHz field strength resulted in the least contribution of Hartmann-Hahn transfer, and it leads to identify severely overlapping methylene peaks on C20 which is critical in the determnation of the degree of corphin macrocyclic ring-puckering. Attempt to fit spin diffusion cross-peak distinguished from direct dipolar cross relaxation were made. For example, prochiral assignment of H20r,s were made by using compensated ROESY, and subsequently chemical shifts of overlapping geminal H20,20' were corrected to be H20s (2.97 ppm) and H20r (3.06 ppm), respectively. Interproton distance constraints (H19-H20s, 2.25 Å; H19-H20r, 3.34 Å, and H18-H20r, 3.25 Å) and NMR-based distance geometry resulted in specific saddle-shaped corphin conformation in terms of dihedral angle [*ca* 28.75° with standard deviation of 4.07° for N1-N2-N3-N4 binding to Ni (II)] for 20 NMR-based structures. The stereospecific conformations of A-, B-, C-, and D-ring were obatined as follow: Me2(axial)-H3(axial)-H4(equatorial), H12(equatorial)-H13 (equatorial), and H17(axial)-H18(axial)-H19(axial).

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