An ENDOR and HYSCORE Investigation of a Reaction Intermediate in IspG (GcpE) Catalysis

Supporting Information

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Experimental Section

Materials. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used as provided. BL-21(DE3) competent cells were purchased from Stratagene (La Jolla, CA).

***E. coli* IspG Protein Production and Purification.** BL-21(DE3) cells overexpressing *E. coli* IspG (encoded in plasmid pASK-IBA5$^+$) and *isc* proteins (encoded in plasmid pDB1282) were grown in LB media supplemented with 100 mg/L ampicillin and 50 mg/L kanamycin at 37 °C, until the OD_{600} reached 0.3. Cells were then induced with 0.5 g L-arabinose to initiate overexpression of the *isc* proteins. Cysteine (0.5 mM) and $FeCl_3$ (0.1 mM) were supplemented, and cells were grown until the OD_{600} reached 0.6. At this point, 400 μg/L anhydrotetracycline was added to induce overexpression of *E. coli* IspG. Cells were grown at 21 °C for 24 hours, then were harvested by centrifugation (9000 rpm, 8 min, 4 °C) and were kept at -80 °C until use.

All purification steps were carried out in a Coy Vinyl Anaerobic Chamber (Coy Laboratories, Grass Lake, MI) with an oxygen level < 2 ppm, and all buffers were degassed by using a Schlenk line. Cell pellets were resuspended in 100 mM Tris-HCl, 150 mM NaCl buffer (pH 8.0). Lysozyme, Benzonase nuclease (EMD Chemicals, San Diego, CA) and phenylmethanesulfonyl fluoride were added, and stirred for 1.5 hour at 10 °C followed by sonication (Fisher Scientific Sonic Dismembrator, Model 500) with 4 pulses, each 7 sec duration at 35% power. The cell lysate was then centrifuged at 11,000 rpm at 10 °C for 30 min. The supernatant was purified by using Strep-tactin chromatography.[1] Fractions having a brown color were collected and desalted in pH 8.0 buffer containing 100 mM Tris-HCl and 150 mM NaCl.

Labeled MecPPs and HMBPP-epoxides. [u-^{2}H]-MEcPP, [u-^{13}C]-MEcPP (**14**) and [2,3-$^{13}C_2$]-MEcPP (**16**) were all prepared biosynthetically as described previously,[2] from ^{13}C-glucoses. [1,3,4-$^{13}C_3$] MEcPP (**15**) was from the batch whose synthesis was described previously.[3] HMBPP epoxides were prepared from labeled HMBPPs[4, 5] as described previously, by adding bromine water to form the bromohydrin, followed by ammonia, to form the corresponding epoxides.[2]

ENDOR/HYSCORE Sample preparation. All samples were prepared inside a Coy Vinyl Anaerobic Chamber with an oxygen level < 2 ppm. Samples were typically 1.0-2.0 mM in IspG, and glycerol was added as a glassing agent to 20% (v/v). 40 equivalents of sodium dithionite were added as a reducing agent, and ligands (MEcPP or HMBPP-epoxide) were added to 20 equivalents. To trap the reaction intermediate "X", samples in EPR tubes (706-PQ-9.50, Wilmad Labglass, Vineland, NJ) were frozen in liquid nitrogen ~30 sec after substrate injection at room temperature.

ENDOR/HYSCORE Spectroscopy. Pulsed ENDOR/HYSCORE spectra were obtained on a Bruker ElexSys E-580-10 FT-EPR X-band and Q-band EPR spectrometer equipped with an Oxford Instruments CF935 cryostat. A Bruker RF amplifier (150 watts, 100 kHz - 250 MHz) was used for ENDOR experiments. Mims ENDOR used a three-pulse sequence $\pi/2_{mw}$ - τ - $\pi/2_{mw}$ – T – $\pi/2_{mw}$ - τ - echo; $\pi/2_{mw}$ = 16 ns, with π_{RF} (20 μs, 3 dB attenuation) applied during T. The X-band Mims ENDOR spectrum of "X" prepared using unlabeled HMBPP-epoxide was subtracted from those of "X" prepared using labelled HMBPP-epoxides. Davies ENDOR used a three-pulse sequence π_{mw} – T - $\pi/2_{mw}$ –τ – π_{mw} - τ - echo; $\pi/2_{mw}$ = 48 ns, with π_{RF} (10 μs, 3 dB attenuation) applied during T. HYSCORE used a four-pulse sequence $\pi/2_{mw}$ – τ - $\pi/2_{mw}$ – t_1 – π_{mw} – t_2 – $\pi/2_{mw}$ – echo; $\pi/2_{mw}$ = 16 ns and π_{mw} = 32 ns, 256 points for both t_1 and t_2, each using 16 ns steps. Time-domain data were baseline corrected using a 3rd order polynomial, then Hamming windowed, followed by zero-filling, 2D-Fourier transformation and symmetrization. The HYSCORE spectrum was simulated using the EasySpin program package.[6]

References

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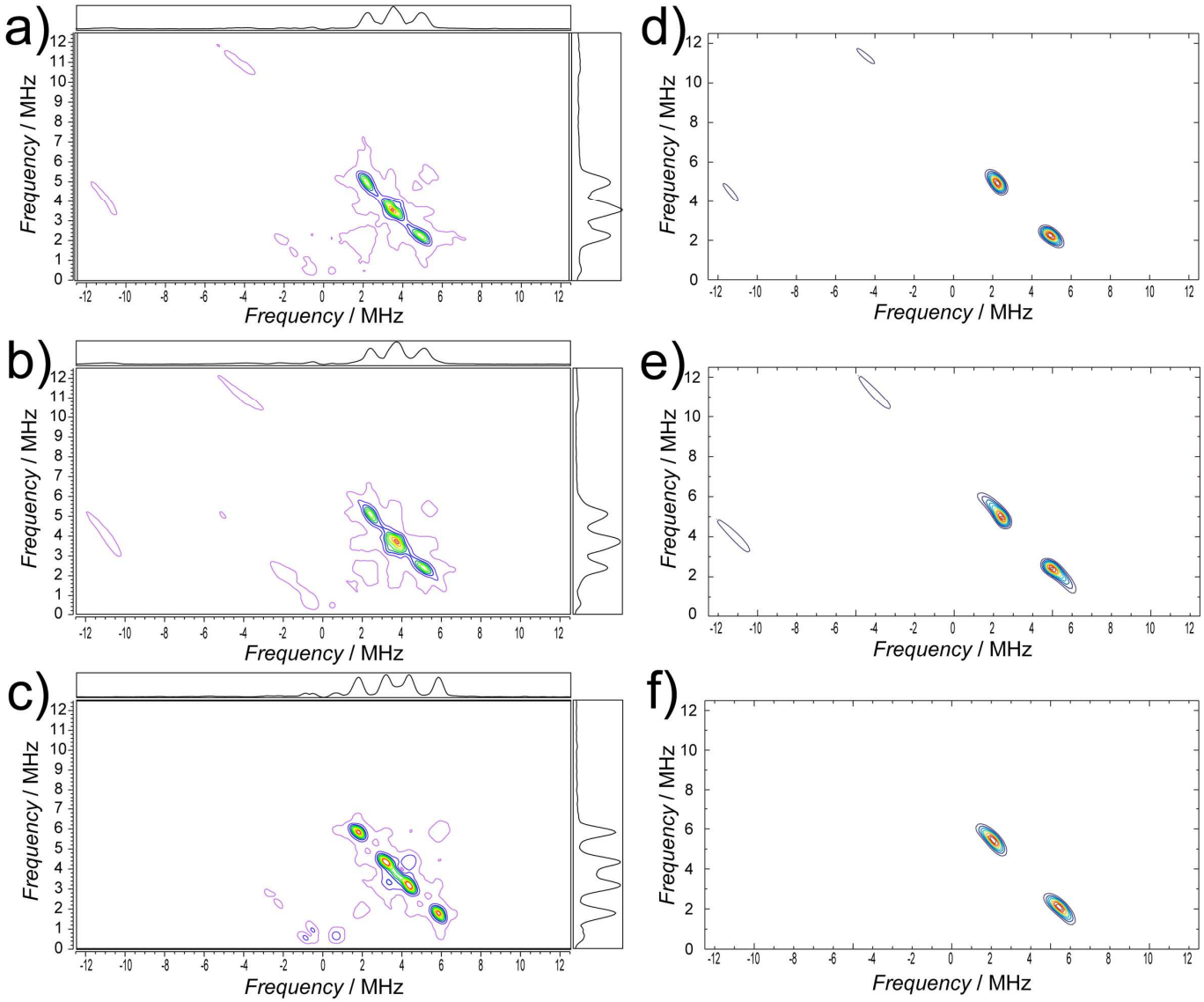

Figure S1. HYSCORE spectra (left) of the reaction intermediate "X" prepared by using *E. coli* IspG and **14** at three different magnetic field strengths, together with simulations (right) of the 17.7 MHz and 3 MHz ^{13}C hyperfine signals. (a) Magnetic field strength = 330.8 mT (g = 2.08); (b) magnetic field strength = 342.3 mT (g = 2.02); (c) magnetic field strength = 345.5 mT (g = 2.00); (d) simulation of (a); (e) simulation of (b); (f) simulation of (c). The signals at ~ 3.6 MHz are the superposition of ^{13}C signals arising from very small hyperfine couplings (≤1 MHz) from the labeled substrates and the double quantum transition of protein ^{14}N, and are not simulated. Experimental parameters are: microwave frequency = 9.684 GHz, τ = 136 ns and T = 20.0 K. Spectra were simulated using A_{ii} = [14.5, 12.0, 26.5] MHz with Euler angles $\alpha = 0^{\circ}$, $\beta = 18^{\circ}$, $\gamma = 0^{\circ}$ for C2, and A_{ii} = [1.8, 2.0, 5.1] MHz with Euler angles $\alpha = 40^{\circ}$, $\beta = 30^{\circ}$, $\gamma = 0^{\circ}$ for C3.

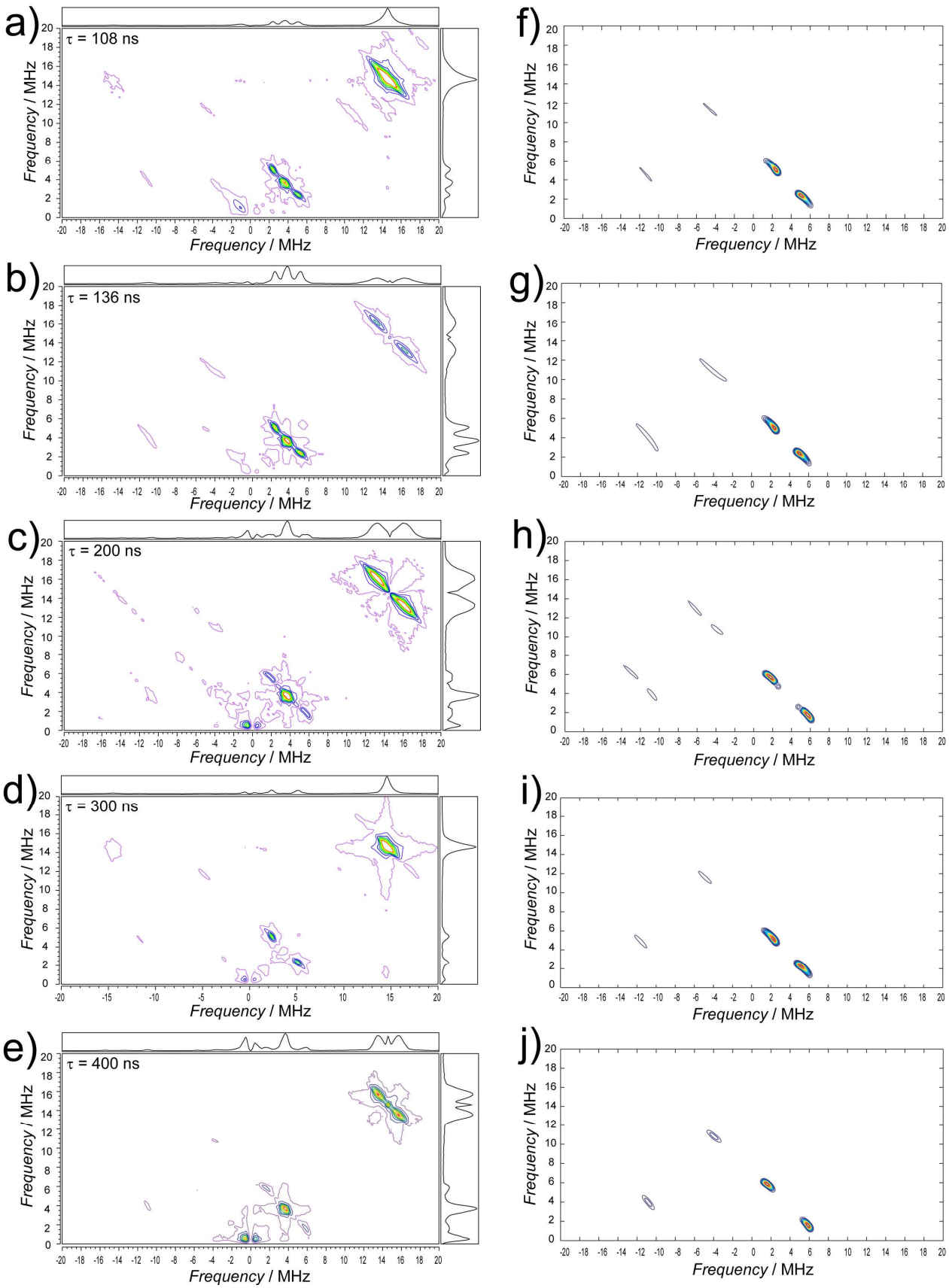

Figure S2. HYSCORE spectra (left) at g_2 of the reaction intermediate "X", prepared by using **14**, at different τ values, together with simulations (right) of the 17.7 MHz and 3 MHz signals. (a) - (e) are experimental results at τ = 108, 136, 200, 300 and 400 ns, respectively. (f) - (j) are simulations of (a) - (e). In (a) - (e), the signals at ~ 3.6 MHz are the superposition of ^{13}C signals with very small hyperfine couplings (≤1 MHz) from the labeled substrates and the protein ^{14}N double quantum transition, and are not simulated; the signals centered at 14.5 MHz are proton signals and are also not simulated. Experimental parameters are: microwave frequency = 9.674 GHz, magnetic field strength = 342.5 mT and T = 20.0 K. Simulation parameters are listed in the Figure Caption of Figure S3.

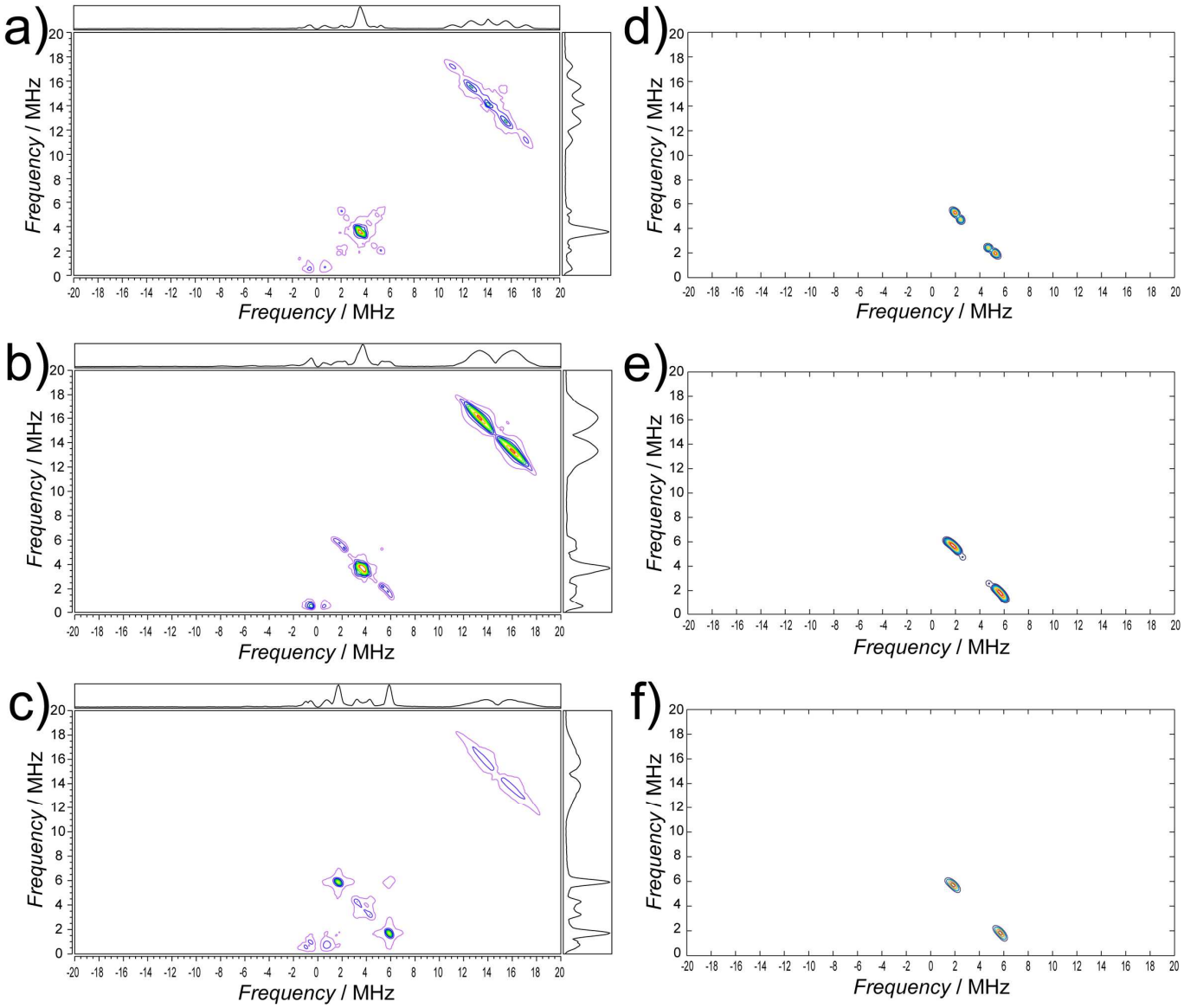

Figure S3. HYSCORE spectra of the reaction intermediate "X" prepared by using *E. coli* IspG **15** at three different magnetic field strengths (left) and simulations of the 3 MHz ^{13}C hyperfine signals (right). (a) Magnetic field strength = 331.0 mT (g = 2.08); (b) magnetic field strength = 342.86 mT (g = 2.02); (c) magnetic field strength = 346.0 mT (g = 2.00); (d) – (f) are simulations of (a) - (c), respectively. In (a) - (c), the signals at ~ 3.6 MHz are superpositions of ^{13}C signals arising from very small hyperfine couplings (≤1 MHz) from the labeled substrates and protein ^{14}N double quantum transitions and are not simulated; the signals centered at 14.5 MHz are proton signals and are also not simulated. Experimental parameters are: microwave frequency = 9.684 GHz, τ = 200 ns and T = 20.0 K. Spectra were simulated using A_{ii} = [1.8, 2.0, 5.1] MHz with Euler angles $\alpha = 40^{\circ}$, $\beta = 30^{\circ}$, $\gamma = 0^{\circ}$ for C3.

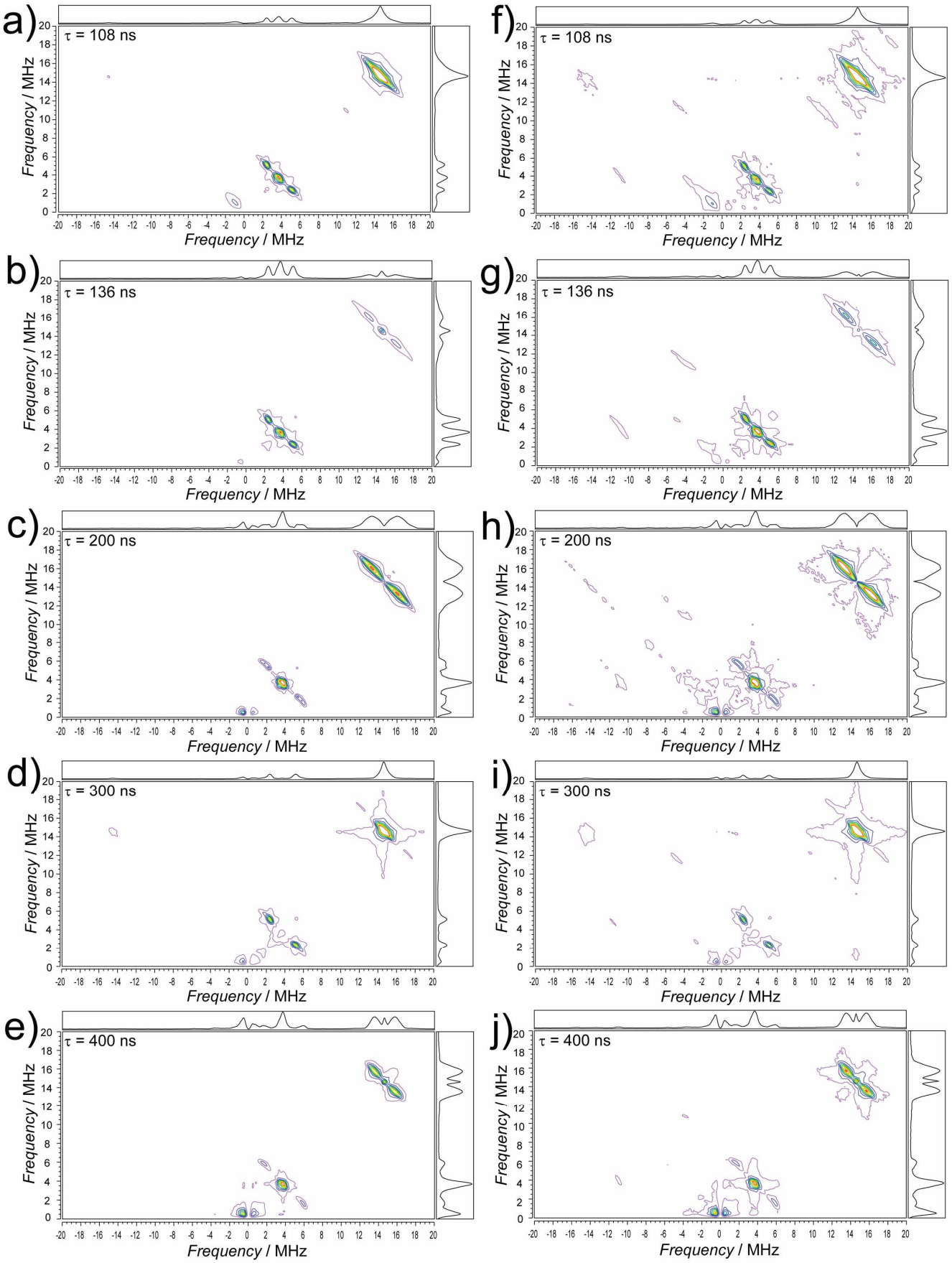

Figure S4. HYSCORE spectra at g_2 of the reaction intermediate "X" prepared by using ^{13}C-labeled MEcPP at different τ values. (a) - (e) are from the sample prepared using **15** and (f) - (j) are from the sample prepared using **14**. The signals at ~ 3.6 MHz are superpositions of ^{13}C signals having very small hyperfine couplings (≤1 MHz) from the labeled substrates, and the protein ^{14}N double quantum transitions. The signals centered at 14.5 MHz are proton signals. Microwave frequency 9.684 GHz for (a) - (e) and 9.674 GHz for (f) - (j). Magnetic field strength: 342.86 mT for (a) - (e) and 342.5 mT for (f) - (j). T = 20.0 K.