Resonance Raman spectroscopy reveals that substrate structure selectively impacts the heme-bound diatomic ligands of CYP17

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Supporting Information

Part 1. The rR spectra

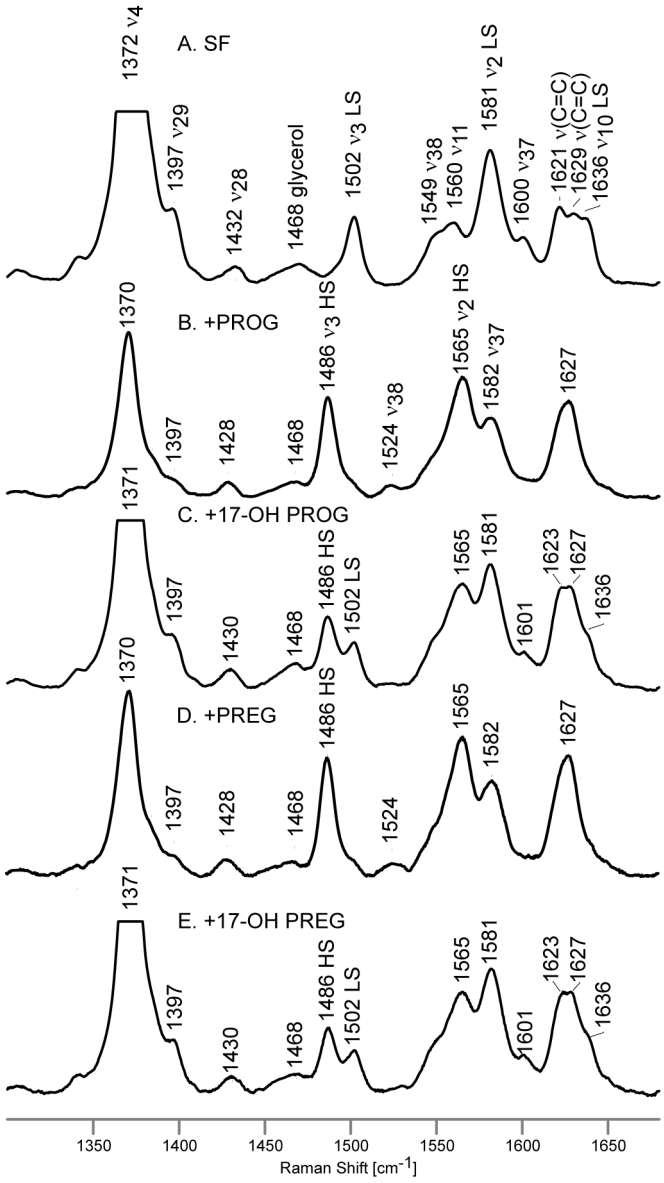

Figure S1. The high frequency RR data of ferric ND:CYP17 in substrate-free state (A) and with the following substrates: PROG (B), 17-OH PROG (C), PREG (D) and 17-OH PREG (E). The spectra were normalized to the glycerol mode observed at 1468 cm^{-1}.

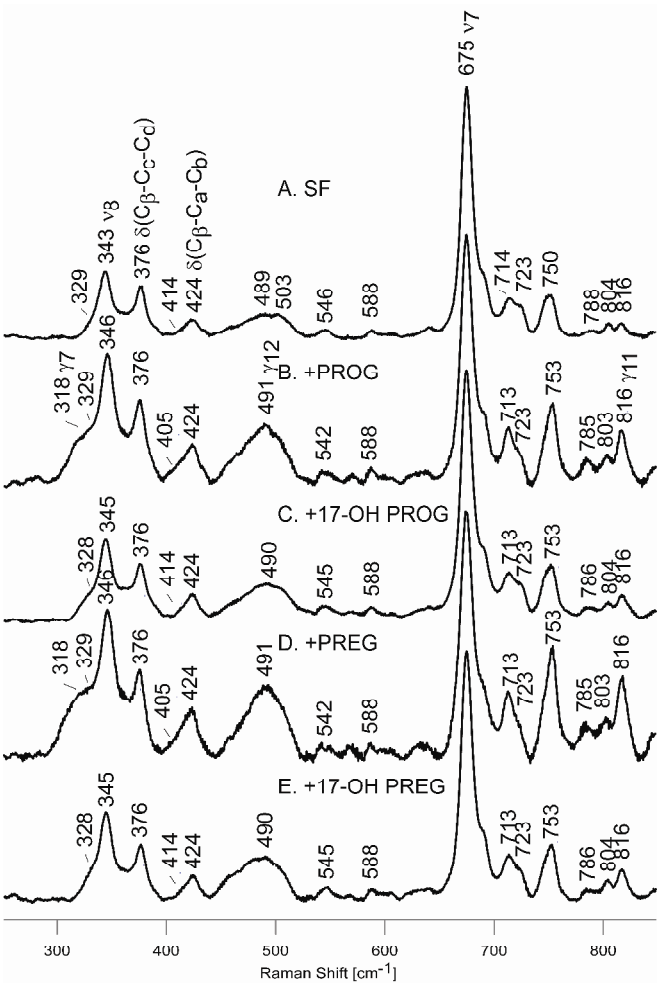

Figure S2. The low frequency RR data of ferric ND:CYP17 in substrate-free state (A) and with the following substrates: PROG (B), 17-OH PROG (C), PREG (D) and 17-OH PREG (E). The spectra were normalized to the ν_7 mode observed at 675 cm^{-1}.

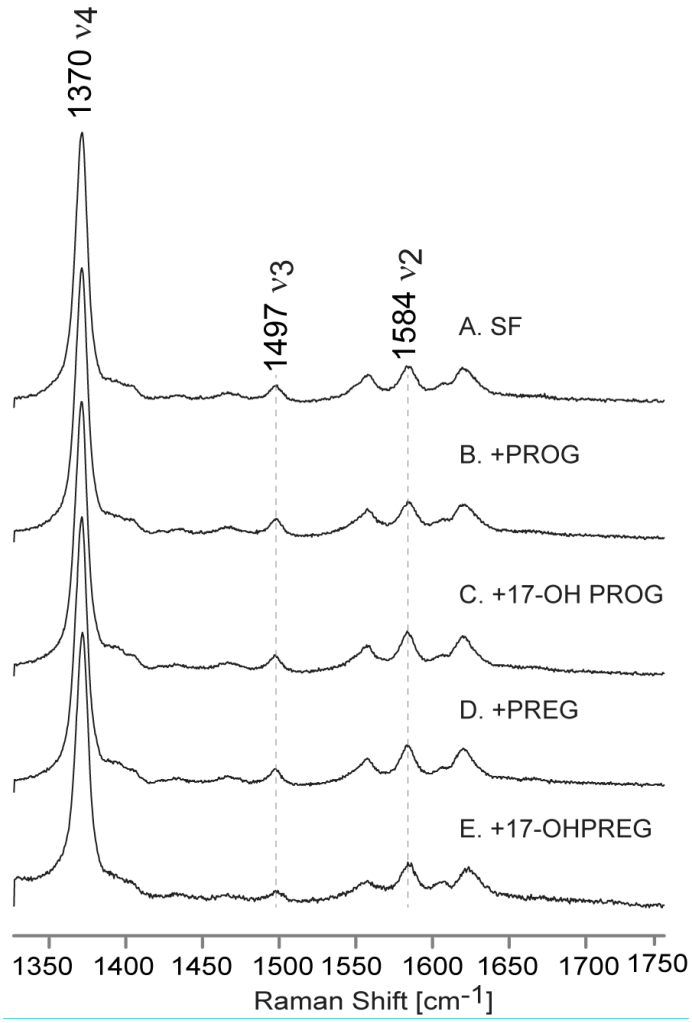

Figure S3. The high frequency RR data of ferrous CO ND:CYP17 in substrate-free state (A) and with the following substrates: PROG (B), 17-OH PROG (C), PREG (D) and 17-OH PREG (E). The spectra were normalized to the ν_4 mode observed at 1370 cm^{-1}.

Part 2. Validation of rR experimental procedures and data analysis.

Preparation of ferrous CO adducts of CYP17.

In order to prepare the ferrous CO adducts the ferric protein was transferred to a NMR tube sealed with rubber septum. 100 μL of 100 μM protein was saturated with CO gas for 15 minutes, while gently shaking in an ice bath. A 5-10 fold excess of sodium dithionite (2-4 μL) was added to CO-saturated solution of ferric protein and the formation of ferrous CO adducts monitored using electronic absorption spectroscopy. The ferrous to ferrous-CO transition is observed as a disappearance of 538 nm, 574 nm and 645 nm bands characteristic for ferric substrate-bound P450s and appearance of single Q band at around 552 nm representative for ferrous-CO adducts of P450s.

Three samples obtained from three different protein batches were prepared as described above and all three gave the same rR spectra. The samples were stable as observed by rR and UV-Vis spectroscopy for at least 8 hrs, sufficient for rR measurements. Furthermore, we modified the preparation of ferrous CO adduct to test various scenarios that might affect the formation of reduced sample. The following modifications were made:

A. The ferric sample was first reduced using 10-fold excess of sodium dithionite in anaerobic condition and the CO gas was added. The UV-Vis spectroscopy, in the Q-band region, showed the disappearance of 538 nm, 574 nm and 645 nm bands (ferric substrate-bound P450s) after the addition of sodium dithionite and the appearance of single band centered at around 542 nm was observed, which shifted by 10 nm to 552 nm upon addition of CO gas; this procedure was performed to test the possibility that the order of reduction and ligation might affect the spectral properties of the sample.

B. In another variation, a ferrous CO sample was prepared in same sequence as above (reduction → CO ligation), but allowing the protein to equilibrate with the dithionite solution for a longer period; e.g., the reduced protein was incubated under anaerobic conditions for 30 min, after which the CO gas was added. This method was used, because some cytochrome P450 samples require longer times to become fully reduced.

C. In an additional modification, a sub-stoichiometric amount of sodium dithionite was used and the samples was allowed to incubate for 30 min and then the CO gas was added; this approach was used to test the unlikely possibility that the 5-10 fold excess of reduction agent can affect the substrate.

All these three preparations of ferrous-CO sample gave the same rR spectral pattern, eliminating any concern that the methods used to prepare the ferrous CO adducts are problematical.

The ferrous-CO photodissociation studies

The rR measurements of photo labile ferrous CO adducts are typically performed with low laser power (~ 1.0 mW), the sample is placed in NMR tube that is constantly spun during rR measurements, and the laser beam is focused onto the sample using cylindrical lens to spread the laser power over larger area. These experimental conditions allow collection of spectra of ferrous CO adducts and minimize photodissociation of the CO molecules. Although it is unexpected that the presumably small amounts of photolyzed P450 would contribute to the 442-excited rR spectrum (because the ferrous form Soret band is near 408 nm), the unusual spectral pattern of 17-OH PREG bound samples prompted us to explore the possible contributions of unwanted photodissociation. Figure S6 shows the typical spectrum measured with 1.0 mW laser power (A) compared with spectrum measured with lower power of 0.4 mW (B). Both spectra exhibit the same spectral pattern and the intensities of ν(Fe-C) stretches relative to the ν_7 mode are the same, proving that the 1.0 mW on the sample doesn't cause photodissociation of CO. Moreover, a significantly higher power was used to acquire the rR spectrum of the photodissociated sample (Figure S6, C) and showed a spectral pattern that is completely different from that of ferrous CO adducts (traces A and B). Though the 484 cm^{-1} feature in trace C could conceivably contribute to traces A and B, the absence of the equally strong bands below 300 cm^{-1} in traces A and B, eliminates this possibility. When the power was turned down following the 35 mW excitation (trace D), the same spectral pattern observed in traces A and B returned.

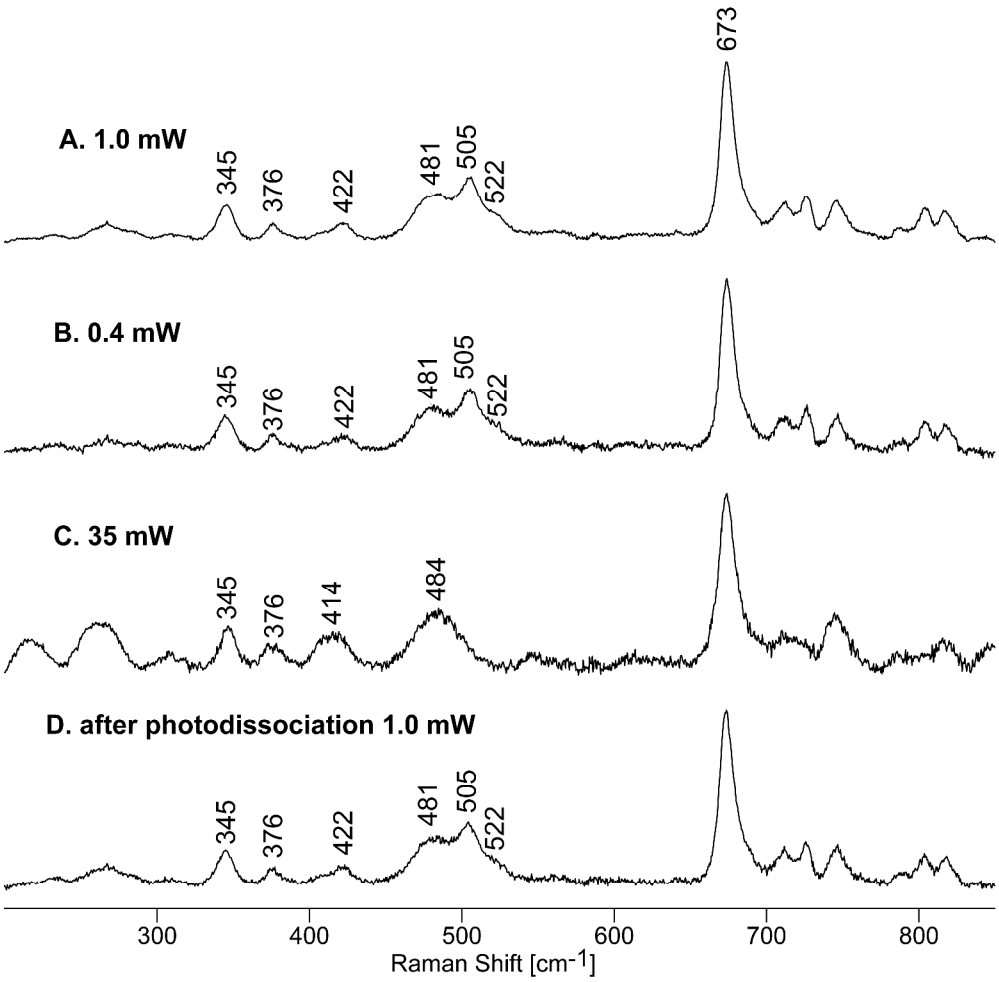

Figure S4. The RR spectra of the ferrous CO adducts of 17-OH PREG bound ND:CYP17 measured with 1.0 mW (A), with 0.4 mW (B), with 35 mW (C) and 1.0 mW after photolysis (D).

Studies of the ferrous-CO P420 forms

The ferrous CO samples of P450 are usually stable for hours; however, over time, some part of the protein might undergo conversion into inactive form of cytochrome, the so-called P420 form. The Soret band of ferrous CO P420 is observed at 420-423 nm and the ν(Fe-C) stretching mode is seen at around 490 cm^{-1}. [1,2] Measurements of ferrous CO P450 with the 442 nm excitation line usually allows selective excitation of modes associated with the native enzyme, even when small fraction of P420 are present. However, if the sample contains mainly P420 form, some of its bands might be seen in the 442 nm spectra. To test whether the P420 form could contribute to the spectra of ferrous CO P450, the samples of ferrous CO adducts of P450 were intentionally converted to P420 by incubation at 4 ˚C for approximately 72 hrs, as determined by electronic absorption spectroscopy. The samples were then measured with a 413 nm excitation line and their spectra are shown in Figure S7. The spectrum of the substrate-free sample exhibits the ν(Fe-C) stretching mode at 493 cm^{-1} and samples containing the substrates exhibited the same 493 cm^{-1} frequency. A similar lack of substrate effect on the ν(Fe-C) frequency has been reported previously for CYP101 in its P420 form.[1,2] Most importantly, the frequency of the ν(Fe-C) seen for P420 CYP17 fails to correspond with either of the ν(Fe-C) frequencies of the P450 form of the 17-OH PREG sample, eliminating the possibility of P420 form contributing to the spectrum of that sample.

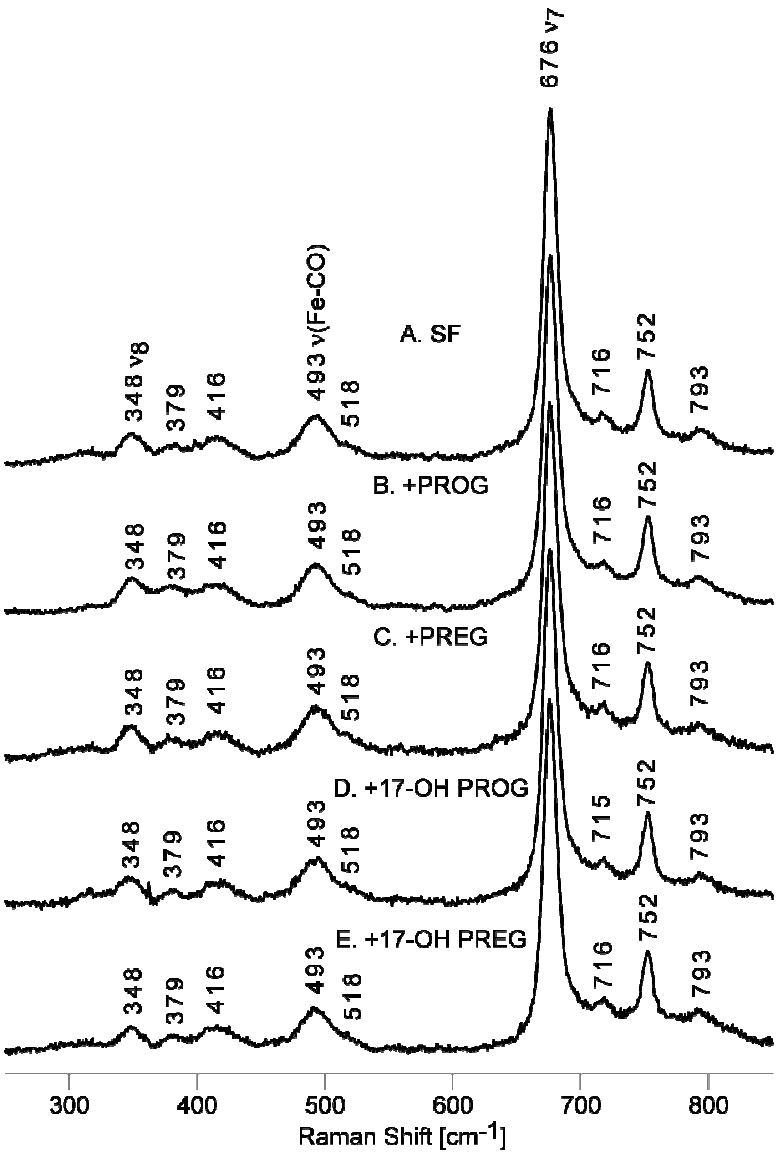

Figure S5. The RR spectra of the P420 ferrous CO adducts of substrate-free ND:CYP17 (A) and with PROG (B), PREG (C), 17-OH PROG (D) and 17-OH PREG (E). The spectra were normalized to the ν_7 mode observed at 676 cm^{-1}.

Part 3. Validation of purity of 17α-hydroxy pregnenolone substrate.

The purity of 17-OH PREG was verified by electrospray ionization mass spectrometry (ESI-MS), electron-impact mass spectrometry following gas chromatographic separation (GC/MS), ^{1}H and ^{13}C NMR.

Electrospray ionization mass spectrometry (ESI-MS): Mass spectra were obtained at the University of Illinois at Urbana Champaign School of Chemical Sciences Microanalysis Laboratory. ESI-MS analysis of 17-OH PREG was carried out on a Waters ZMD 2000 mass spectrometer in positive ion mode. Consistent with previously reported data,[3] the $[M+H]^+$ parent ion was observed at 333.4 m/z and dehydration products $[MH - 18]^+$ and $[MH - 36]^+$ were present at 315.3 and 297.4 m/z.[3] Additionally, the common ESI sodium and ammonium adducts were observed at 350.4 and 355.3 m/z (Figure S8).

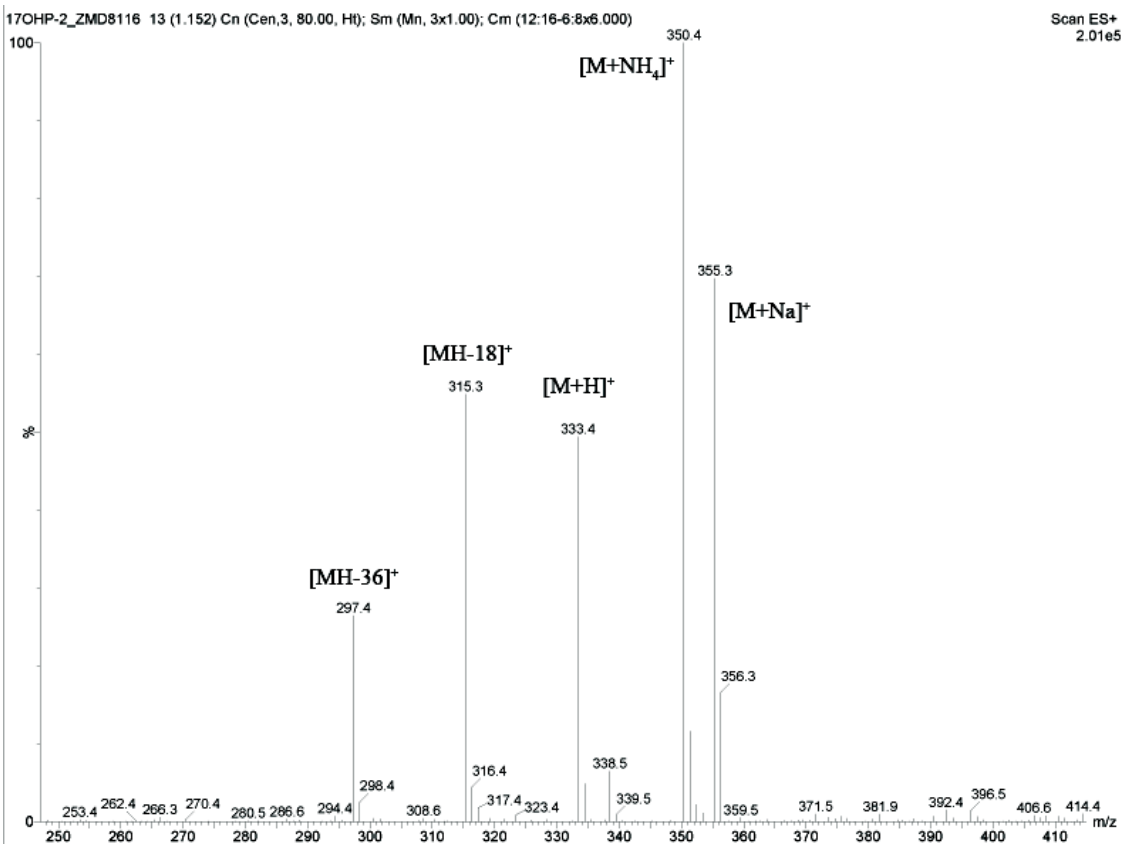

Figure S6. Positive mode ESI-MS spectra of 17-OH PREG.

Gas chromatography/electron-impact mass spectrometry (GC-MS): GC-MS was performed on a sample of underivatized GC-MS samples were analyzed on a Waters Premier CGT^{TM} system using a TG-5MS column (Thermo Scientific). Underivatized 17-OH PREG in methanol was injected using a 250°C injector temperature and the initial oven temperature was held at 100°C for 1.5 minutes, ramped to 275°C at 30°C/min, and held for 15 minutes. Retention time of OH-PREG was 12.26 min and time-of-flight mass spectra revealed the presence of a parent ion m/z: 332.2 [M^+] with a variety of fragmentation products (Figure S9).

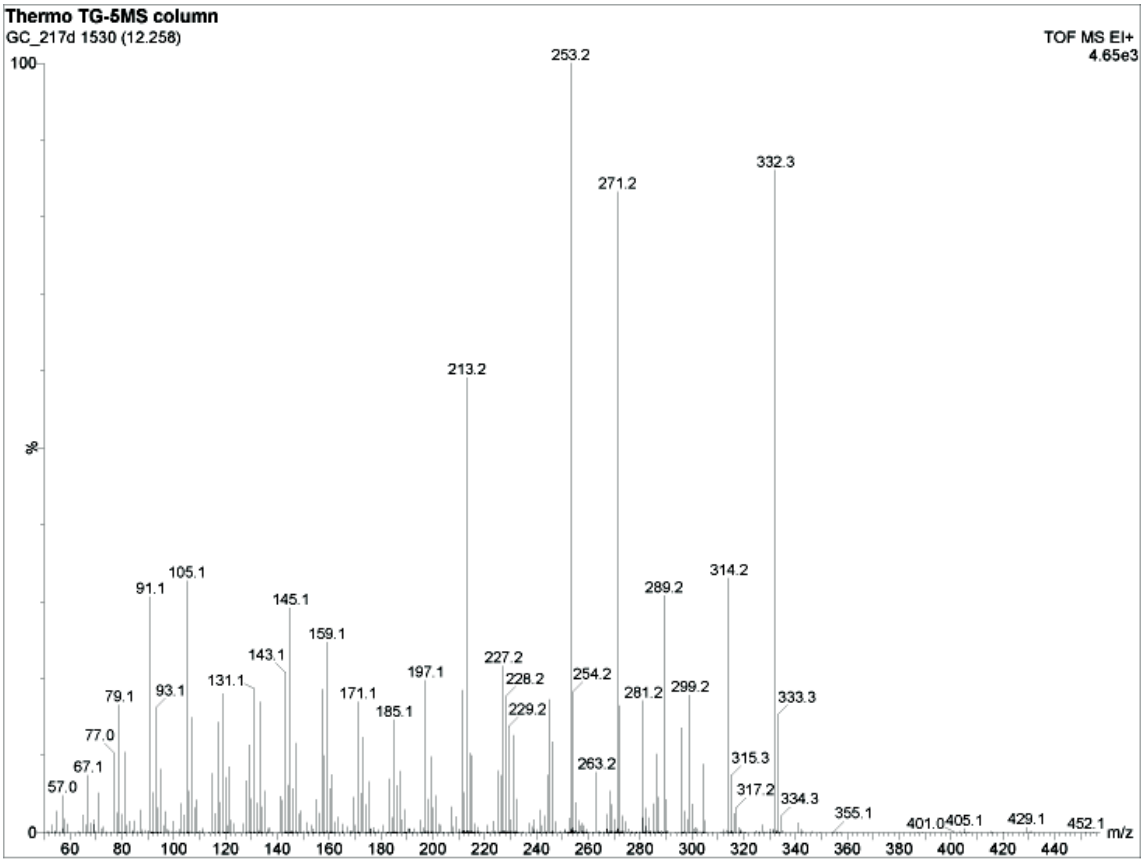

Figure S7. The GC-MS of 17-OH PREG. Extracted mass spectrum of 17-OH PREG peak retained at 12.26 minutes.

Nuclear magnetic resonance: The ^{1}H NMR (600 MHz, DMSO-d_6, 20 ºC, TMS) and ^{13}C NMR (100.5 MHz, DMSO-d_6, 20 ºC) spectra were recorded on a Varian 600 and 400 MHz NMR spectrometer and were in precise agreement with reported data (Figures S10 and S11).[4]

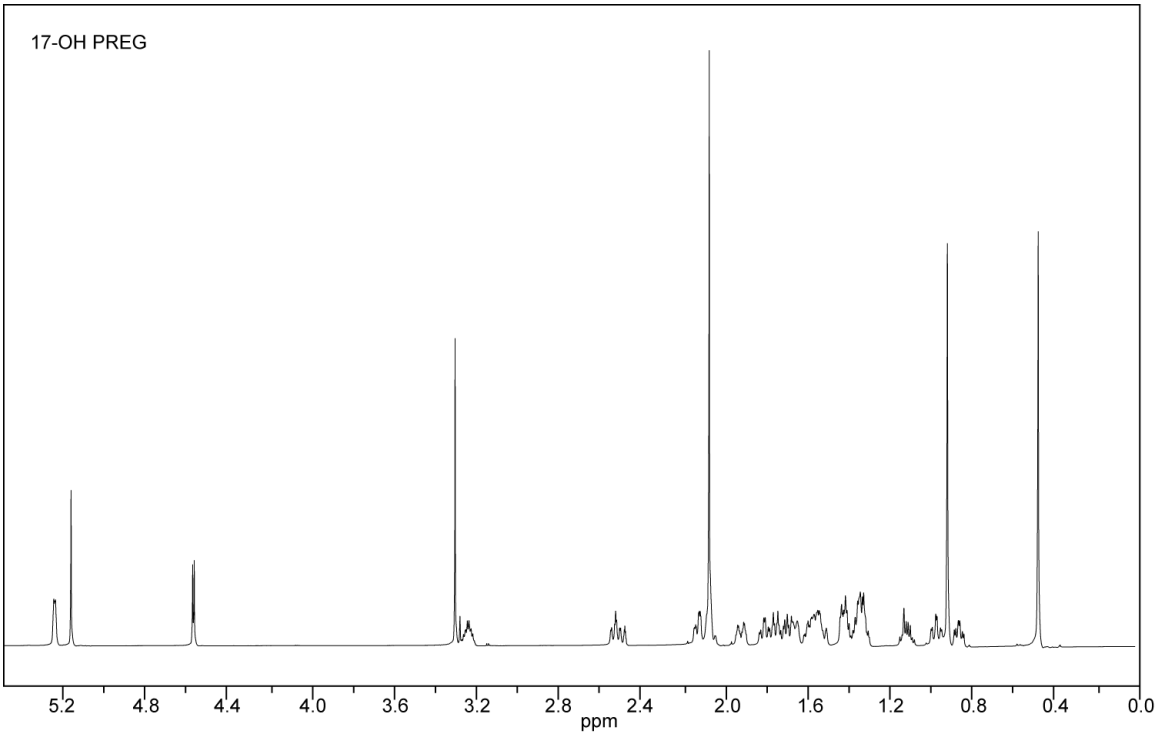

Figure S8. The ^{1}H NMR spectra of 17-OH PREG.

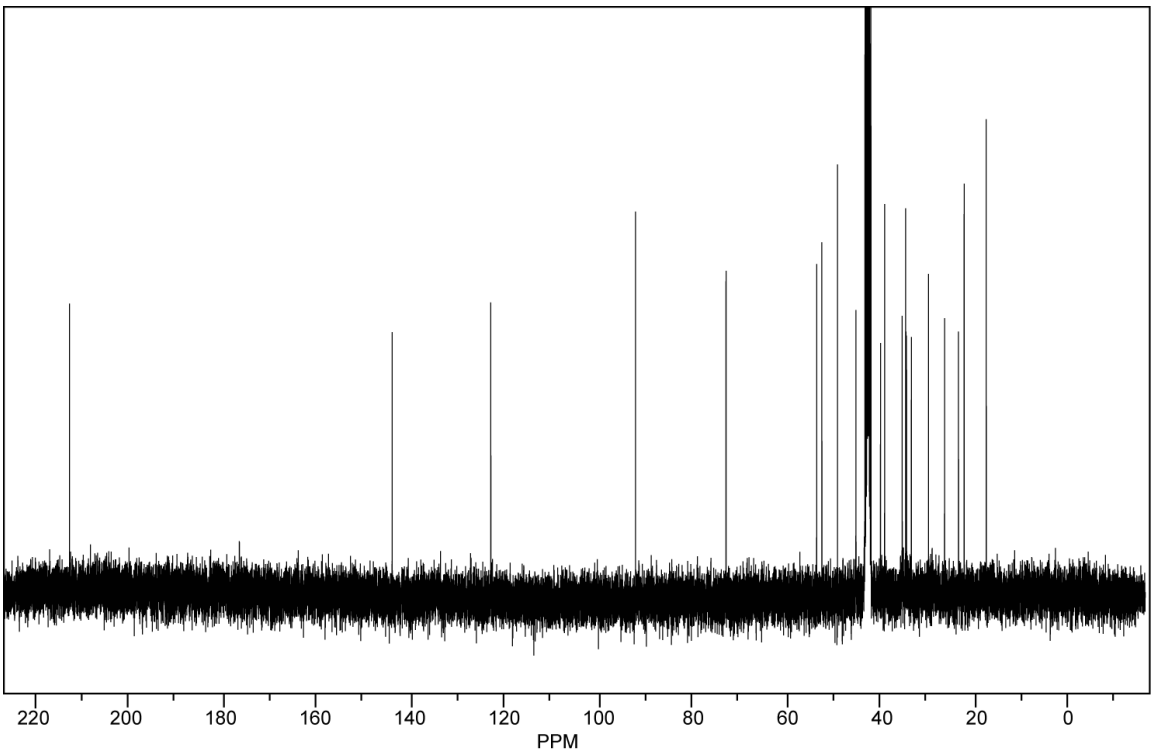

Figure S9. The ^{13}C NMR spectra of 17-OH PREG.

Table S1. The frequencies of ν(Fe-C) and ν(C-O) stretching modes for CYPs and NOS's. The numbers in the right column represents points in Figure 7.

		ν(Fe-C)	ν(C-O)	ref.
CYP				
CYP101				
1.	substrate-free	464	1963	1,5,6,7
2.	+ norcamphor	473	1947	7,8
3.	+ adamantanone	474	1942	7,9
4.	+ camphoroquione	476	1941	7,8
5.	+ fenchone	480	1945	7,8
6.	+ camphor	481	1940	1,5,6,7,10,11
7.	+ tetramethylcyclohexanone	485	1934	7,8
CYP17				
8.	substrate-free	472	1957	tw
9.	+ 17-OH pregnenolone	481	1953	tw
10.	substrate-free	485	1946	tw
11.	+ 17-OH progesterone	491	1938	tw
12.	+ progesterone	498	1932	tw
13.	+ pregnenolone	498	1940	tw
14.	+ 17-OH pregnenolone	505	1928	tw
NOS				
nNOS				
15.	substrate-free	487	1949	12
16.	substrate-free	501	1930	12
17.	+ Arg	503	1929	12
18.	+ HO-Arg	502	1928	12
$iNOS_{FL}$				
19.	substrate-free	487	1945	13
20.	+ Arg	512	1906	13
$iNOS_{oxy}$				
21.	substrate-free	491	1946	14
22.	+ Arg	512	1907	14
23.	+ H_4B	490	1944	14
24.	+ Arg / H_4B	512	1905	14

tw: this work

References:

(1) Wells, A. V., Li, P., Champion, P. M., Martinis, S. A., and Sligar, S. G. (1992) Resonance Raman investigations of Escherichia coli-expressed Pseudomonas putida cytochrome P450 and P420, *Biochemistry 31*, 4384-4393.

(2) Tian, W. D., Wells, A. V., Champion, P. M., Di Primo, C., Gerber, N., and Sligar, S. G. (1995) Measurements of CO geminate recombination in cytochromes P450 and P420, *J. Biol. Chem. 270*, 8673-8679.

(3) Shindo, N., Yamauchi, N., Murayama, K., Fairbrother, A. and Korlik, S. (1990) Identification of 17-hydroxyprogesterone and other steroid hormones in saliva from a normal child and patients with congenital adrenal hyperplasia by plasmaspray liquid chromatography/mass spectrometry, *Biomed.Chrom. 4*, 171-174.

(4) Wishart, D. S., Knox, C., Guo, A. C., Eisner, R., Young, N., Gautam, B., Hau, D. D., Psychogios, N., Dong, E., Bouatra, S., Mandal, R., Sinelnikov, I., Xia, J., Jia, L., Cruz, J. A., Lim, E., Sobsey, C. A., Shrivastava, S., Huang, P., Liu, P., Fang, L., Peng, J., Fradette, R., Cheng, D., Tzur, D., Clements, M., Lewis, A., De Souza, A., Zuniga, A., Dawe, M., Xiong, Y., Clive, D., Greiner, R., Nazyrova, A., Shaykhutdinov, R., Li. L., Vogel, H. J., and Forsythe, I. (2009) HMDB: a knowledgebase for the human metabolome, *Nucleic Acids Res. 37*, D603-D610.

(5) Uno, T., Nishimura, Y., Makino, R., Iizuka, T., Ishimura, Y., and Tsuboi, M. (1985) The resonance Raman frequencies of the iron-carbon monoxide stretching and bending modes in the carbon monoxide complex of cytochrome P-450cam, *J. Biol. Chem. 260*, 2023-2026.

(6) O'Keefe, D. H., Ebel, R. E., Peterson, J. A., Maxwell, J. C., and Caughey, W. S. (1978) An infrared spectroscopic study of carbon monoxide bonding to ferrous cytochrome P-450, *Biochemistry 17*, 5845–5852.

(7) Jung, C., Hoa, G. H. B., Schroeder, K. L., Simon, M., and Doucet, J. P. (1992) Substrate analog induced changes of the carbon-oxygen-stretching mode in the cytochrome P450cam-carbon monoxide complex, *Biochemistry 31*, 12855–12862.

(8) Bangcharoenpaurpong O. (1987) Resonance Raman Studies of Myoglobin, Cytochromes P450 and Chloroperoxidase, A Dissertation, Northeastern University.

(9) Hu, S., and Kincaid, J. R. (1991) Resonance Raman characterization of nitric oxide adducts of cytochrome P450cam: the effect of substrate structure on the iron-ligand vibrations, *J. Am. Chem. Soc. 113*, 2843–2850.

(10) Nagano, S., Shimada, H., Tarumi, A., Hishiki, T., Kimata-Ariga, Y., Egawa, T., Suematsu, M., Park, S.-Y., Adachi, S.-I., Shiro, Y., and Ishimura, Y. (2003) Infrared Spectroscopic and Mutational Studies on Putidaredoxin-Induced Conformational Changes in Ferrous CO-P450cam, *Biochemistry 42*, 14507–14514.

(11) Unno, M., Christian, J. F., Sjodin, T., Benson, D. E., Macdonald, I. D. G., Sligar, S. G., and Champion, P. M. (2002) Complex formation of cytochrome P450cam with putidaredoxin. Evidence for protein-specific interactions involving the proximal thiolate ligand, *J. Biol. Chem*. *277*, 2547-2553.

(12) Wang, J., Stuehr, D. J., and Rousseau, D. L. (1997) Interactions between Substrate Analogues and Heme Ligands in Nitric Oxide Synthase, *Biochemistry 36*, 4595–4606.

(13) Fan, B., Wang, J., Stuehr, D. J., and Rousseau, D. L. (1997) NO Synthase Isozymes Have Distinct Substrate Binding Sites, *Biochemistry 36*, 12660–12665.

(14) Li, D., Stuehr, D. J., Yeh, S.-R. and Rousseau, D. L. (2004) Heme Distortion Modulated by Ligand-Protein Interactions in Inducible Nitric-oxide Synthase, *J. Biol. Chem. 279*, 26489-26499.