Enantioselective α-Hydroxylation of 2-Arylacetic Acid Derivatives and Buspirone Catalyzed by Engineered Cytochrome P450 BM-3

Marco Landwehr,[a] Lisa Hochrein, [a] Christopher R. Otey,[a] Alex Kasrayan,[b] Jan-E. Bäckvall,[b] and Frances H. Arnold[a]

[a] *Division of Chemistry and Chemical Engineering 210-41, California Institute of Technology, Pasadena, CA 91125-4100, USA, and* [b]*Department of Organic Chemistry, Arrhenius Laboratory, Stockholm University, SE-106 91 Stockholm, Sweden.*

RECEIVED DATE (automatically inserted by publisher); frances@cheme.caltech.edu

Supporting Information:

Materials: All chemicals were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Ester substrates and product standards were synthesized in a standard esterfication reaction and purified by distillation. NADPH was obtained from BioCatalytics, Inc. (Pasadena, CA). Buspirone and racemic 6-hydroxybuspirone were provided by Bristol-Myers Squibb (BMS).

Protein expression and purification: Protein was expressed and purified as described (Peters et al. *J. Am. Chem. Soc.* **2003,** *125*, 13442-50).

Bioconversions: 900 µl bioconversions for determining regioselectivity and ee contained 50 or 500 nM protein, 5 mM substrate and 500 µM NADPH co-factor in 1% DMSO, 1% acetone in 100 mM Epps buffer, pH 8.2. Reaction time was 3 hours at RT. The reaction was acidified with 90 µl 7% perchloric acid to quench the reaction by precipitating protein. 1-Heptanol was added as internal standard. After centrifugation for 10 min at 13,000 x g, the product was extracted from the supernatant with dichloromethane and analyzed by GC. All reactions were set up at least in triplicate.
NADPH consumption rates were monitored under the same conditions as above but at lower substrate concentration (250 µM) at 340 nm using a Cary 100 BioSpectrophotometer. Rates were corrected for background NADPH consumption measured without substrate. All rates were measured in triplicate.

Bioconversions for measuring product formation followed the same conditions as described above and were quenched after one or two minutes by adding perchloric acid. To enhance TTN, bioconversions used an isocitrate regeneration system instead of supplying NADPH directly. Final concentrations for these reactions were: 1 U/ml isocitrate dehydrogenase, 0.05 mM $NADP^+$ and 25 mM isocitrate. 15 ml reactions were carried out with the concentrations described in the manuscript, stirred and supplied with air by bubbling directly into the reaction with a flow rate of about 2.5 ml/min. In addition to the standard buffer, 0.5% DMF was present in these reactions which seemed beneficial for enzyme stability.

GC Analysis: Identification and quantification of analytes were performed using product standards and 3-point calibration curves with internal standard. Sample injection volume was 1 µl. Analysis was performed in triplicate on a Hewlett-Packard 5890 Series II Plus gas chromatograph with a flame-ionization detector (FID). Direct analysis of the extract was performed on a chiral CycloSil-B column (Agilent Technologies). The temperature program for separating the components is as follows: 250°C inlet, 300°C detector, 100°C oven for 1 min, then 10°C/min gradient to 170°C, holding for 10 min, then 10°C/min to 250, holding for 3 min.

Retention times (in minutes) of substrates and products described in publication.

Substrate	Retention Time	Product	Retention Time (*S*)	Retention Time (*R*)

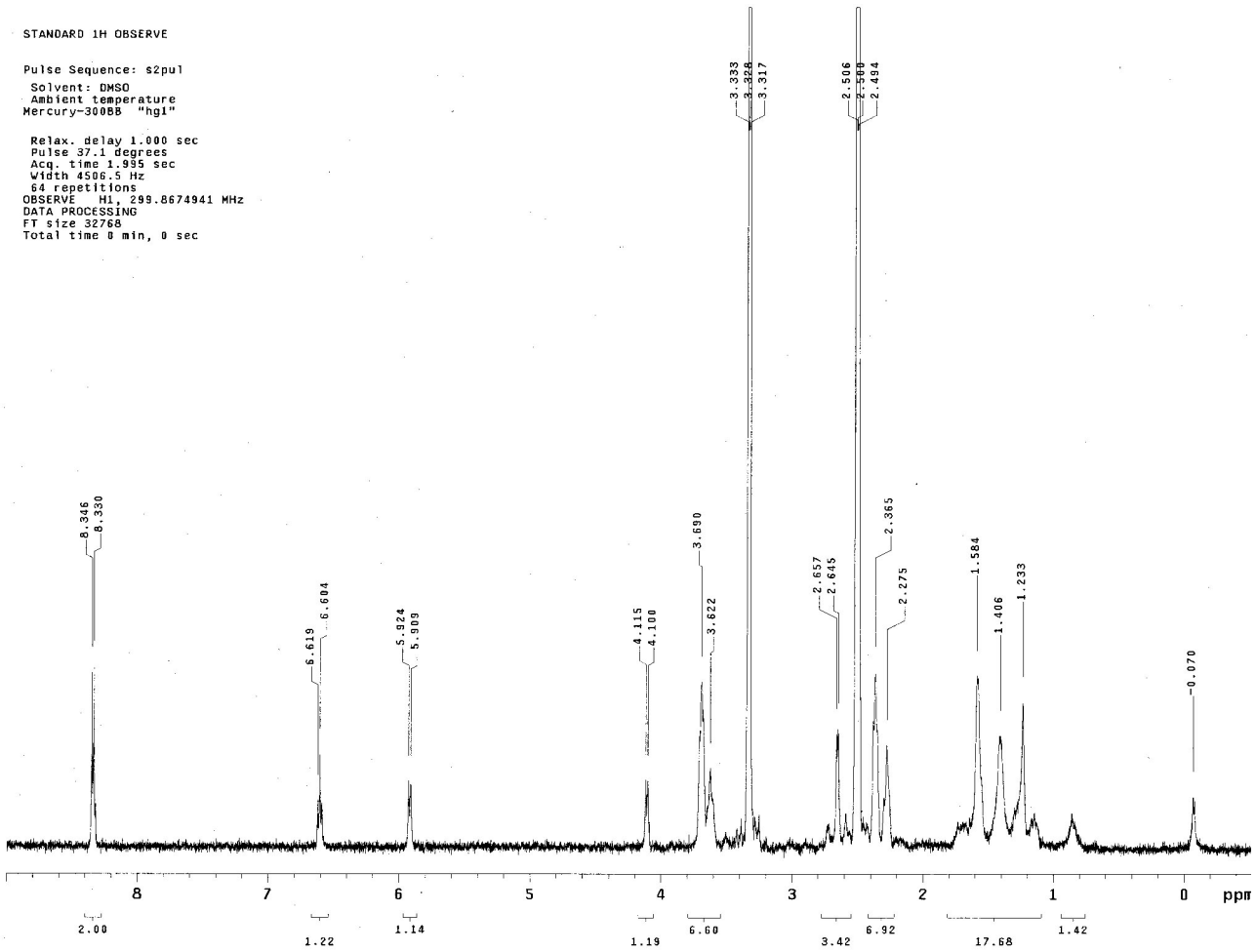
1a (Me)	6.82	**2a** (Me)	9.23	9.28
1b (Et)	7.48	**2b** (Et)	9.74	9.83
1c (Pr)	8.60	**2c** (Pr)	11.32	11.41
1d (Bu)	10.03	**2d** (Bu)	13.67	13.74
3b (Et)	10.20	***m*-chloro ethyl mandelate**	13.95	14.35
3c (Pr)	12.05	***m*-chloro propyl mandelate**	17.04	17.48
3d (Bu)	14.79	***m*-chloro butyl mandelate**	20.34	20.53

Buspirone Bioconversions: 0.5 ml bioconversion for determining regioselectivity and *ee* were set up with 50 or 500 nM protein, 2 mM buspirone, and 500 μM NADPH co-factor in 1% DMSO, 1% acetone and 100 mM Epps buffer, pH 8.2. Reaction time was 3 hours at RT. The sample was extracted by adding 0.5 THF, 50 μl 8.5% phosphoric acid, 166 μl saturated sodium chloride solution, and 125 μl MTBE. 400 μl of the organic layer was removed after centrifugation at 20,800 x g for 3 minutes and combined with 0.5 ml of a second extraction (0.5 ml THF and 125 μl MTBE). The organic phase was concentrated by evaporation to about 30 μl. 10 μl DMSO, 10 μl acetone, and 480 μl 0.1 M Epps, pH 8.2 were added to the concentrated sample. A 15 ml bioconversion using the regeneration system described above was used to generate the material for NMR analysis.

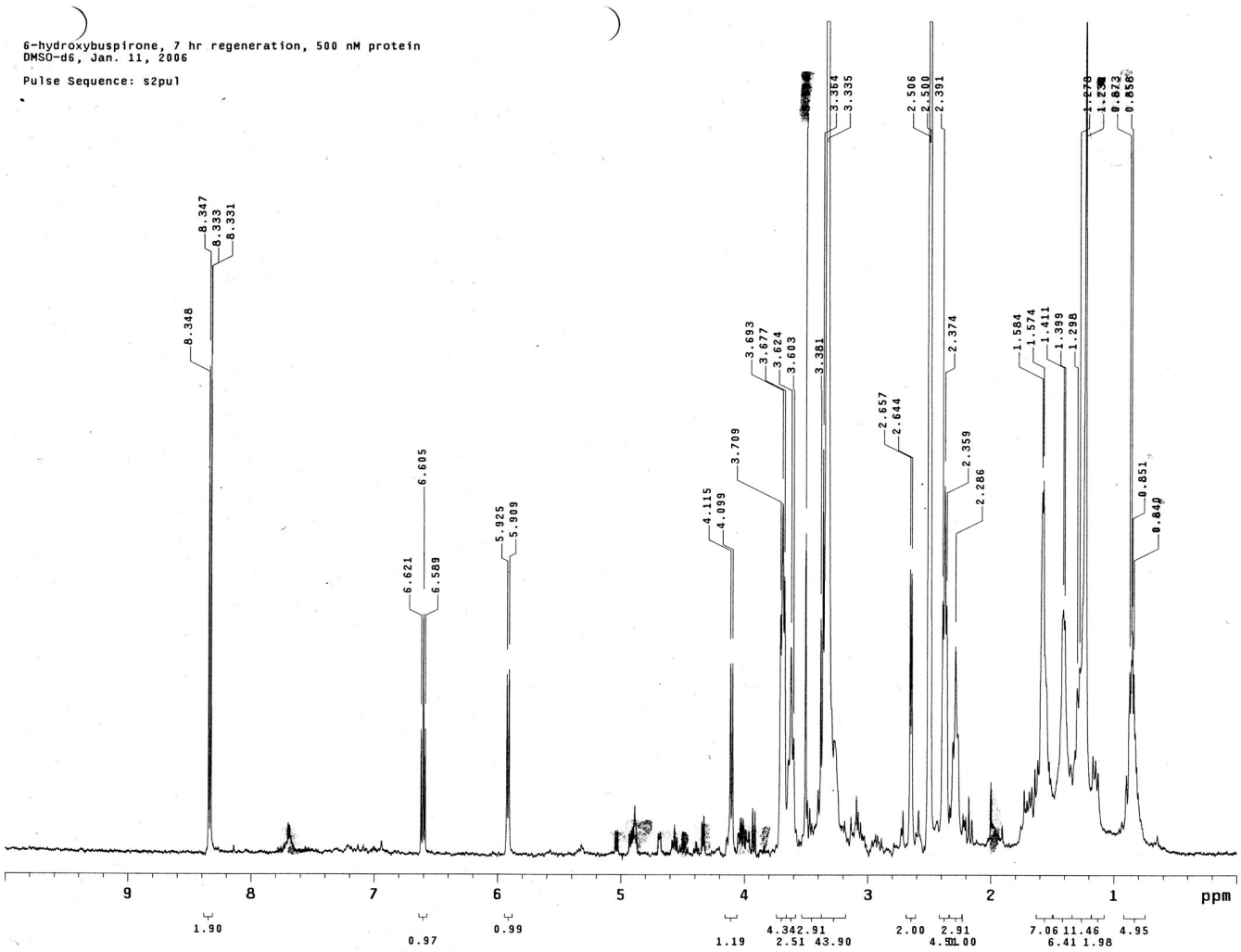
HPLC Analysis: Analysis of the products was carried out on a Waters 2690 Separation Module with a Waters 996 Photodiode Array Detector using a chiral Chiralpak AD column (250 x 4.6 mm, 20 μm particle size). The mobile phase was 85% ethanol/15% acetonitrile/0.06% triethylamine with a flow rate of 1 ml/min. A 20 °C column temperature and a 20 μl injection volume were used. The detection was at 243 nm. The retention times are as follows: buspirone: 12.8 min., (R)-6-hydroxybuspirone: 8.1 min., (S)-6-hydroxybuspirone: 13.6 min.

NMR: The 15 ml bioconversion was extracted with 15 ml chloroform twice. The organic layer was concentrated under reduced pressure using a rotary evaporator. Thin-layer chromatography (TLC) was carried out on in 4:1 chloroform:hexane with 0.1% THF. The product band was removed and eluted from the silica in chloroform. The samples were dried and suspended in DMSO-d6. 1H NMR spectra were recorded on a Mercury 300 Spectrometer (300 MHz) and are internally referenced to residual protic solvent signals (DMSO = 2.50 ppm). ^{1}H NMR (DMSO-d6, ppm) δ 1.406 – 1.584 (m, 12H), 2.275 – 2.275 (m, 6H), 2.645 - 2.657 (m, 2H), 3.603 (t, J=6.3 Hz, 2H), 3.693 (t, J=4.8 Hz, 4H), 4.108 (d, J =4.5 Hz, 1H), 5.917 (d, J=4.5 Hz, 1H), 6.612 (t, J=4.5 Hz, 1H), 8.338 (d, J=4.8 Hz, 2H)

Reference: Full author list for reference 17(d): DeSantis, G.; Zhu, Z.; Greenberg, W. A.; Wong, K.; Chaplin, J.; Hanson, S. R.; Farwell, B.; Nicholson, L. W.; Rand, C. L.; Weiner, D. P.; Robertson, D. E.; Burk, M. J., *J. Am. Chem. Soc.* **2002,** *124*, 9024-5.



^{1}H NMR spectrum of 6-hydroxybuspirone standard



^{1}H NMR spectrum of TLC separated product of bioconversion of buspirone