Structure Elucidation of Major Metabolites from Medroxyprogesterone Acetate by P450

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We previously reported the separation and identification for the major metabolites from incubating medroxyprogesterone acetate (MPA) with P450. The structure assignments for these metabolites were tentatively assigned based on one-dimensional (1D) proton NMR. Unambiguous structure identification of these metabolites is critical to study biological pathways of P450. Here we report the complete structure elucidation by extensive two-dimensional (2D) NMR for the three major metabolites isolated in our earlier studies. The three major metabolites (namely M-2, M-3, M-4) were unambiguously identified to be 6β-, 1β-, and 2β-hydroxy MPA. The current work confirmed the speculated structures for these metabolites in our previous studies. More importantly, the unambiguous structural information and the establishment for their NMR chemical shifts of these metabolites can serve as reference standards for future studies.

Key words Medroxyprogesterone acetate metabolite; P450 enzyme; NMR structure elucidation

Medroxyprogesterone acetate (17α-acetoxy-6α-methylpregn-4-ene-3,20-dione; MPA, Fig. 1) is used extensively in conception and hormone replacement therapy.[1—3] However, due to its extensive metabolism, MPA usually has low bioavailability. Many side effects[4] are considered to be consequences of the generation of reactive metabolites from MPA.[5] Although extensive research has been done on the metabolic pathways of MPA, our current understandings of MPA metabolism are still very limited.[6,7]

We recently reported metabolism studies of MPA in human liver microsomes (HLMs), in minipig liver microsomes (PLMs) and in rat liver microsomes.[8] The aims of these studies were to identify the enzymes responsible for MPA metabolism and to compare potential species differences.[8] We obtained and purified three major mono-hydroxy MPA metabolites (namely M-2, M-3, M-4). However, due to the very small sample amount and instrument limitations, their structures were only speculated as 6β-, 1β-, and 2β-hydroxy MPA. Since structures of these metabolites are critical in understanding biological transformation of MPA by P450, it is vital to know their structures unambiguously. In addition, definitive structural information will enable these metabolites to serve as standard compounds or as basis for structure elucidation of further metabolites, without having to chemically synthesize authentic compounds which may be very difficult. Here we report the rigorous structural elucidation for these three metabolites using extensive NMR techniques and molecular modeling. These studies confirmed the previous structural assignments, and provided complete NMR chemical shift information for all the metabolites that may serve as reference standards for further studies of MPA metabolism by P450 enzymes.

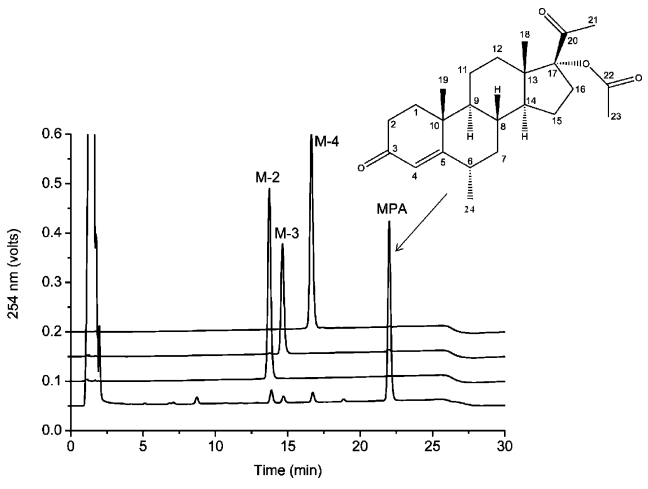

Fig. 1. Structure of MPA and HPLC of Purified Metabolites

Experimental

Generation and Purification of the Three Major Mono-hydroxy Metabolites All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Preparation and characterization of liver microsomes, generation and HPLC purification of the three major mono-hydroxy metabolites from MPA (M-2, M-3, M-4) were described in details in our previous report.[8] Briefly, MPA (200 μM) was incubated with PLMs (1.0 mg/ml) and NADPH-generating system (1 mM $NADP^+$, 10 mM glucose-6-phosphate, 1 unit/ml of glucose-6-phosphate dehydrogenase, and 4 mM $MgCl_2$) for 60 min at 37 °C. PLMs was selected because it resembled HLMs in MPA metabolism and because it's cheaper than HLMs.[8] The incubation system was scaled up to 500 ml to generate adequate metabolites. Approximately 5%, 3%, and 5% of MPA was converted to M-2, M-3, and M-4, respectively under these conditions. After protein precipitation by methanol, the reaction mixture was centrifuged at 9000×***g*** for 10 min. The supernatant was extracted with ethyl acetate and the organic layer was dried *in vacuo*. The residue was re-dissolved in ethyl acetate and separated by preparative TLC (Silica gel, 20×20 cm, 2 mm, Merck), which was developed by chloroform–acetone (9 : 1, v/v).[9] MPA and its metabolites were monitored under UV light at 254 nm. After the metabolites were isolated, they were further purified by HPLC before NMR analysis.

HPLC and LC/MS Methods HPLC and LC/MS methods have been reported.[8] Briefly, MPA and its metabolites were separated by a Shim-pack (Shimadzu Corporation, Columbia, MD, U.S.A.) C_{18} column (4.6×150 mm, 5 μm). The mobile phases were solvent B, H_2O and solvent A, CH_3OH, with linear gradient from initially 52% A to 80% A over 20 min. The wavelength was set at 254 nm and the flow rate was 1 ml/min. For LC/MS detection, the HPLC eluent from UV detector was introduced into the mass spectrometer *via* a 1 : 4 split. The mass spectrometer was a TSQ triple quadrupole (Thermo Fisher Scientific, Waltham, MA, U.S.A.) equipped with an ESI in-

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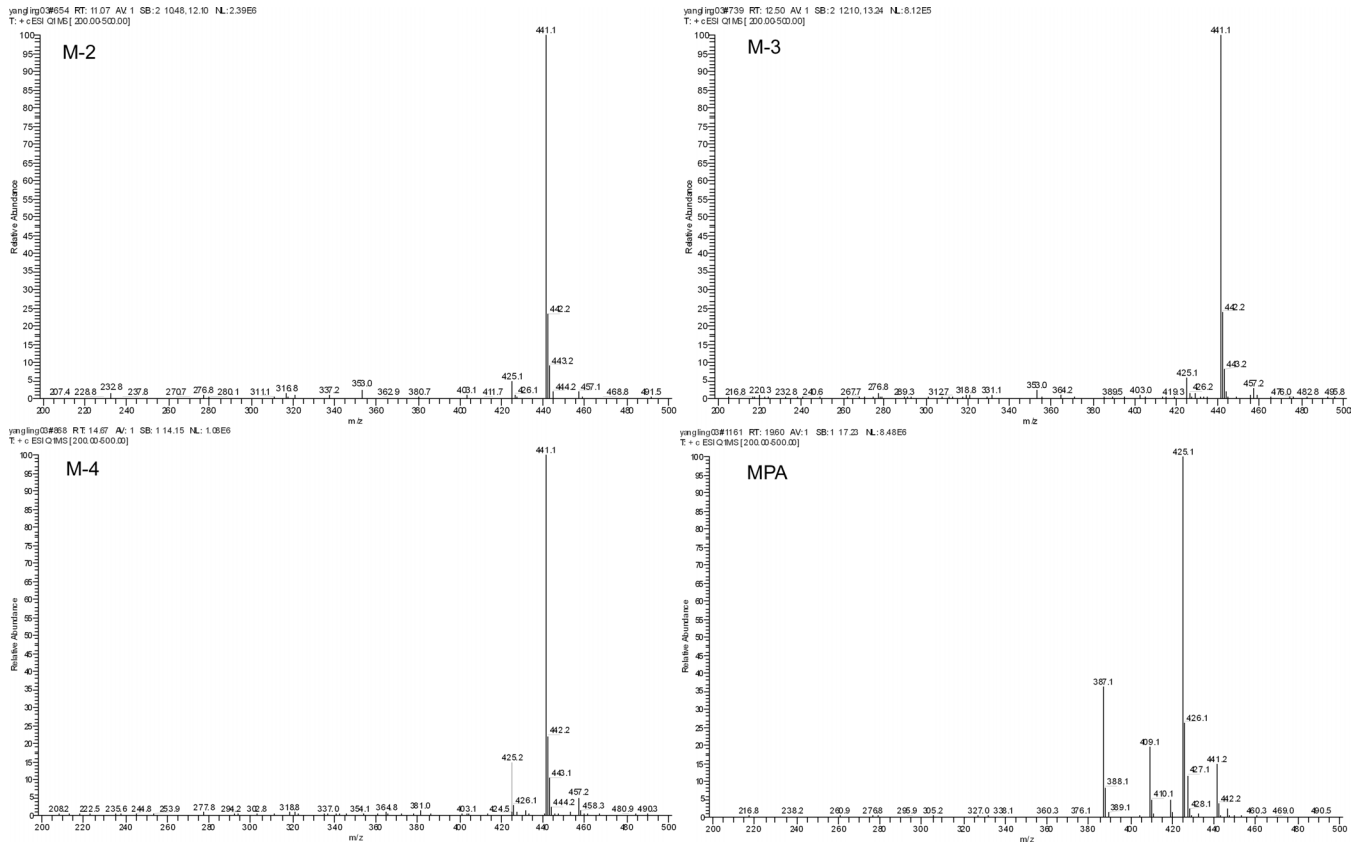

Fig. 2. MS Spectra of MPA and Its Three Major Metabolites

terface. The spray voltage was set at 4.5 kV and the capillary temperature was 300 °C. Both nebulizing and auxiliary gas were nitrogen, and backpressure was set at 40 psi for nebulizing gas, and 20 (arbitrary units) for auxiliary gas. Initially, the mass spectrometer was programmed to perform full scans between *m*/*z* 200 and 500 in order to observe the $[M+H]^+$ and $[M-H]^-$ signals.

NMR Spectroscopy All NMR measurements were performed using either an inverse triple-resonance probe or a gHX high resolution magic angle spinning (HRMAS) Nanoprobe on a Varian Unity Inova 500 MHz spectrometer (Variannmr Inc., Palo Alto, CA, U.S.A.). Samples were dissolved in $CDCl_3$. Temperature was regulated at 22 °C and was controlled with an accuracy of ±0.1 °C. Chemical shifts were referenced to residual solvent peaks for $CDCl_3$ (7.27 ppm for proton and 77.0 ppm for carbon). Standard two-dimensional NMR experiments (^1H–^1H nuclear Overhauser effect spectroscopy (NOESY), ^1H–^{13}C heteronuclear single quantum correlation spectroscopy (HSQC), or ^1H–^{13}C heteronuclear multiple bond quantum correlation spectroscopy (HMBC)) were acquired in order to fully elucidate the structures of the metabolites. All data were processed using Varian's VNMR 6.1C software, with zero-filling in the direct dimension and linear prediction in the indirect dimension.

Molecular Modeling Molecular modeling was performed with Schrodinger suites 2008 (Schrodinger Inc., New York, NY, U.S.A.) running on a Dell Linux workstation. All possible structures based on NMR analysis were built and minimized in order to define the absolute configuration at the hydroxylation site based on NOE data.

Results and Discussion

Separation and Purification of Major Mono-hydroxy Metabolites Three major metabolites (M-2, M-3, and M-4) were prepared from incubation with PLM system, because PLM and HLM share similar metabolic profile. The purity of M-2, M-3, M-4 was about 99%, 97%, and 99% (Fig. 1), respectively. Their MS spectra suggest that the metabolites were products of mono-oxygenation, because *m*/*z* of the metabolites was 16 units higher than the parent compound, MPA (Fig. 2).

Identification of M-2 as 6β-Hydroxy MPA M-2 was the most abundant metabolite and its structure can be clearly assigned as 6β-hydroxy-MPA (Fig. 3). The most distinctive spectra changes were involved at position 6. The doublet of 24-methyl proton signal at 1.10 ppm (carbon at 18.3 ppm, Fig. 3A) in MPA was replaced by a singlet and shifted downfield to 1.44 ppm (carbon at 29.4 ppm, Fig. 3B) in M-2. The disappearance of vicinal coupling between 24-methyl and 6β protons in M-2 clearly indicated hydroxylation at C-6. Consistent with this, the one-bond ^1H–^{13}C correlation of 6-CH in MPA (Fig. 3A, square box) disappeared in M-2 (Fig. 3B, square box), indicating that C6 in M-2 became a quaternary carbon. The absolute configuration at position 6 in M-2 was unambiguously established to be 6β-configuration by analysis of its NOESY spectrum (Fig. 3C). Apart from strong NOE between 24-methyl protons and H-4 (δ=6.06 ppm), no NOE was detected between 24-methyl and 19-methyl (δ=1.42 ppm) protons (Fig. 3C, expanded region). This is only possible when 24-Me in M-2 remains at its original 6α-configuration in which the distance between the two methyl groups is 4.7 Å, whereas the distance between 24-Me and H-4 is 2.5 Å (Fig. 3D). If hydroxylation resulted in 6β-configuration for 24-Me which has a distance to 19-methyl of 2.5 Å, very strong NOE would be observed between 24- and 19-methyl protons, and very weak or no NOE would be observed between 24-methyl protons and H-4 (Fig. 3E). Collectively, M-2 was unambiguously identified as 6β-hydroxy-MPA as shown in Fig. 3D.

Identification of M-3 as 1β-Hydroxy MPA Analysis of proton and HSQC spectrum of M-3 indicated that all the

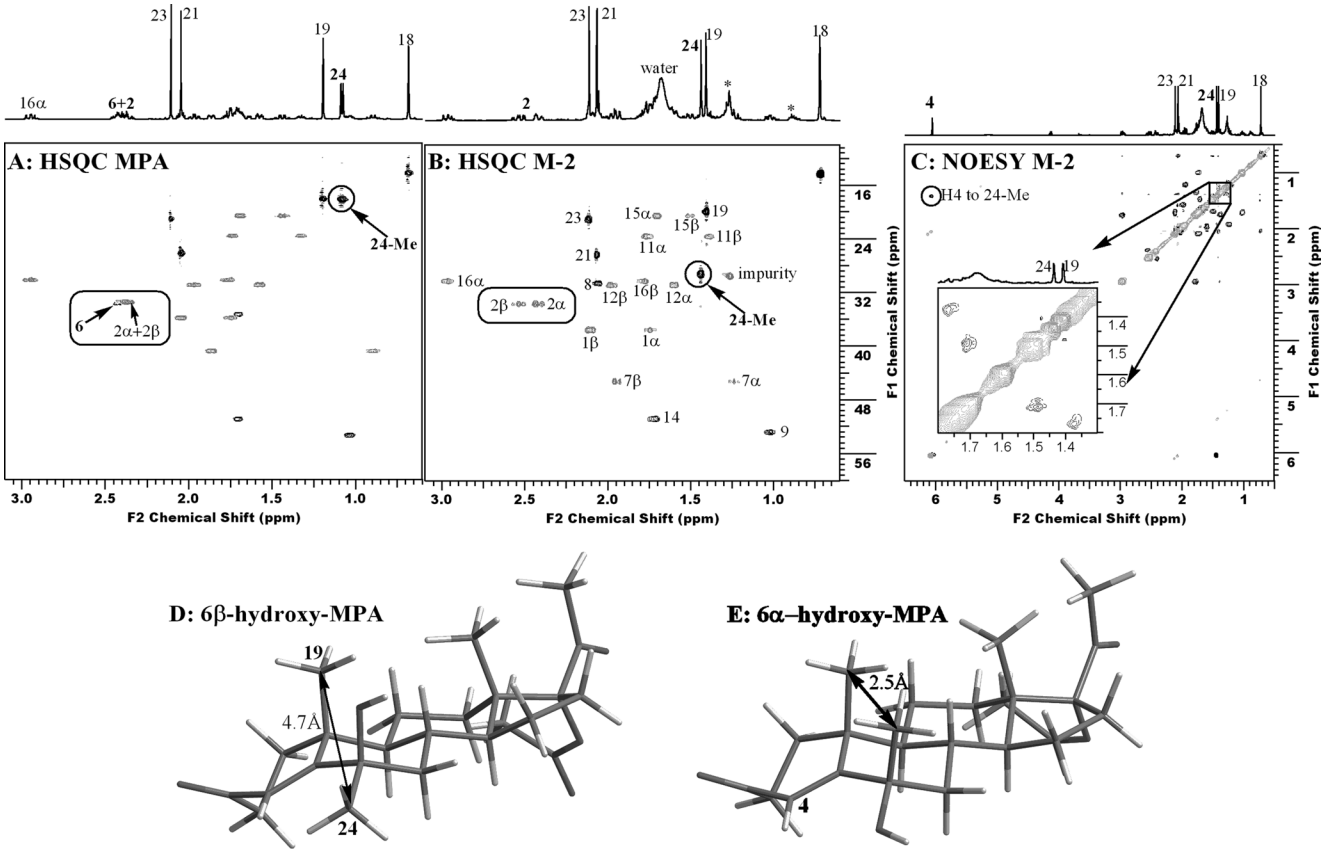

Fig. 3. Identification of M-2 as 6β-Hydroxy-MPA

Expansion of ^{1}H–^{13}C HSQC spectra for MPA (A), M-2 (B), ^{1}H–^{1}H NOESY in M-2 (C), optimized structure of 6β-hydroxy-MPA (D) and 6α-hydroxy-MPA (E). 6-CH correlation in MPA disappeared in M-2 due to the hydroxylation. The 24-Me doublet in MPA became a singlet in M-2 with concurrent downfield shifts for both its proton and carbon signals. The absence of NOE between 24-Me and 19-Me clearly indicate the 6β-hydroxylation in M-2 (insert inside C). The numbers indicate the corresponding position in MPA structure. Impurities presented in M-2 are labeled with *, these are most likely fatty acid chains from microsomes or bleeding from HPLC column materials.

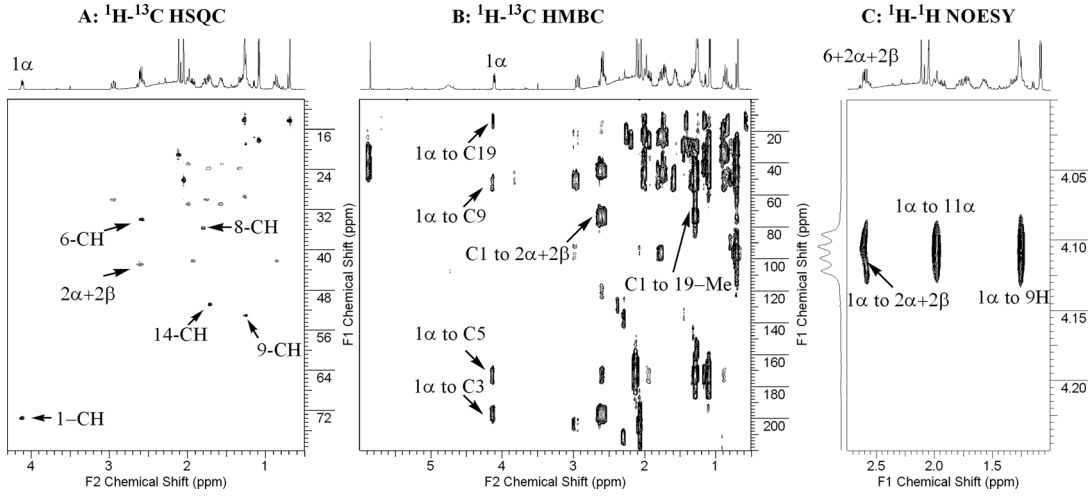

Fig. 4. Identification of M-3 as 1β-Hydroxy-MPA

Expansion of ^{1}H–^{13}C HSQC (A), ^{1}H–^{13}C HMBC (B), and ^{1}H–^{1}H NOESY (C) spectra.

methyl and methine groups were intact, and the hydroxylation occurred on a methylene group in MPA. A new carbinol proton at 4.12 ppm (dd, J=8.67, 4.34 Hz) appeared which is attached to a carbon at 73.6 ppm (Fig. 4A). Analysis of HMBC indicated that this proton has strong long range coupling to C19 (14.6 ppm), C9 (53.3 ppm), C5 (173.2 ppm) and C3 (197.5 ppm), and the corresponding carbon (δ= 73.6 ppm) has a strong correlation to 19-methyl protons (δ=1.29 ppm) and the two protons (2.62 ppm) at C2 (Fig. 4B). This is only possible when this carbinol proton is at C1. Analysis of NOESY spectrum for M-3 showed strong NOE from this carbinol proton to protons at position 2 (δ= 2.62 ppm), 9 (δ=1.27 ppm), and 11α (δ=2.00 ppm), but no NOE to 19-Me (δ=1.29 ppm) (Fig. 4C). Therefore, the

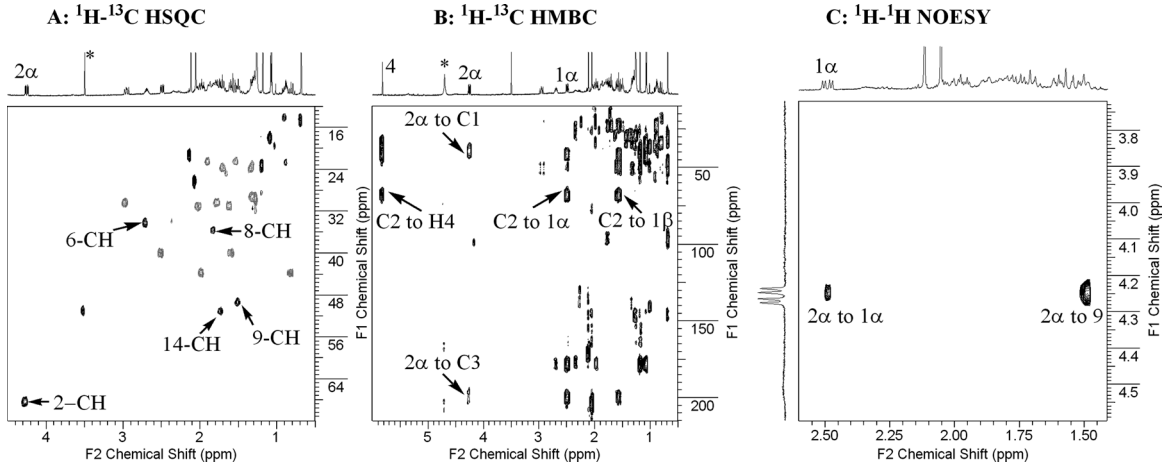

Fig. 5. Identification of M-4 as 2β-Hydroxy-MPA

Expansion of ^{1}H–^{13}C HSQC (A), ^{1}H–^{13}C HMBC (B), and ^{1}H–^{1}H NOESY (C) spectra.

Table 1. NMR Chemical Shift Assignments for MPA and Its Metabolite M2 by Analysis of Their 2D NMR Spectra

Atom	MPA			M2 (6β-OH)		
	^{1}H	Multiplicity and coupling, J (Hz)	^{13}C	^{1}H	Multiplicity and coupling, J (Hz)	^{13}C
1α	2.06	m 1H	35.92	2.11	m 1H	37.68
1β	1.77	m 1H	35.92	1.76	m 1H	37.68
2α	2.40	m 1H	33.63	2.42	m 1H	33.85
2β	2.40	m 1H	33.63	2.55	m 1H	33.85
3	NA	NA	199.71	NA	NA	200.85
4	5.80	d 1H, J=1.1 Hz	121.36	6.06	s 1H	123.15
5	NA	NA	173.86	NA	NA	170.04
6	2.44	m 1H	33.77	NA	NA	71.62
7α	0.91	m 1H	40.86	1.24	m 1H	45.31
7β	1.88	ddd 1H, J=12.7, 4.2, 2.8 Hz	40.86	1.95	dd 1H, J=13.7, 3.4 Hz	45.31
8	1.70	m 1H	35.42	2.07	m 1H	30.76
9	1.05	m 1H	53.32	1.03	m 1H	52.92
10	NA	NA	38.84	NA	NA	38.94
11α	1.71	m 1H	20.78	1.71	m 1H	20.74
11β	1.46	tq 1H, J=13.2, 13.1, 4.2 Hz	20.78	1.52	m 1H	20.74
12α	1.60	dt 1H, J=12.7, 3.4 Hz	31.01	1.61	m 1H	31.08
12β	1.99	dt 1H, J=13.0, 4.3 Hz	31.01	1.99	m 1H	31.08
13	NA	NA	46.75	NA	NA	46.95
14	1.70	m 1H	50.92	1.74	m 1H	50.95
15α	1.74	m 1H	23.76	1.78	m 1H	23.77
15β	1.33	m 1H	23.76	1.40	m 1H	23.77
16α	2.94	m 1H	30.22	2.98	m 1H	30.41
16β	1.78	m 1H	30.22	1.79	m 1H	30.41
17	NA	NA	96.60	NA	NA	97.17
18	0.70	s 3H	14.39	0.74	s 3H	14.64
19	1.21	s 3H	18.26	1.41	s 3H	20.07
20	NA	NA	204.02	NA	NA	204.12
21	2.06	s 3H	26.32	2.07	s 3H	26.48
22	NA	NA	170.60	NA	NA	171.00
23	2.12	s 3H	21.20	2.12	s 3H	21.27
24	1.10	d 3H, J=6.4 Hz	18.33	1.44	s 3H	29.36

NA=quaternary carbon, no proton attached.

carbinol proton must be 1α-configuration, and M-3 was unambiguously assigned as 1β-hydroxy-MPA.

Identification of M-4 as 2β-Hydroxy-MPA M4 was identified as 2β-hydroxy-MPA based on similar analysis of its 2D spectra. Again, the five methyl groups and the four methine groups were intact, only one of the methylene groups in MPA was replaced by a methine group (proton δ=4.27 ppm, dd, J=14.23, 5.56 Hz; carbon, δ=68.5 ppm) in M-4 due to hydroxylation (Fig. 5A). Analysis of HMBC spectrum (Fig. 5B) indicated strong correlation from this carbinol proton to C1 (δ=40.0 ppm) and C3 (δ=200.1 ppm), while this carbon has strong correlation to protons at 1β (δ=1.61 ppm), 1α (δ=2.50 ppm), and 4 (δ=5.83 ppm). This is only possible that the carbinol proton is at position 2. The stereochemistry can be easily identified as 2β-hydroxy configuration, since the large vicinal coupling constant (^3J= 14.23 Hz) can only be possible between axial 2α-proton and axial 1β-proton. This is further confirmed by the strong NOE

Table 2. NMR Chemical Shift Assignments for MPA Metabolites M3 and M4 by Analysis of Their 2D NMR Spectra

Atom	M3 (1β-OH)			M4 (2β-OH)		
	^{1}H	Multiplicity and coupling, J (Hz)	^{13}C	^{1}H	Multiplicity and coupling, J (Hz)	^{13}C
1α	4.12	dd 1H, J=8.7, 4.3 Hz	73.64	2.50	dd 1H, J=14.2, 5.6 Hz	39.99
1β	—	—	—	1.61	m 1H	39.99
2α	2.62	m 1H	43.19	4.27	dd 1H, J=14.2, 5.6 Hz	68.51
2β	2.62	m 1H	43.19	—	—	—
3	NA	NA	197.47	NA	NA	200.05
4	5.87	d 1H, J=1.5 Hz	120.44	5.83	d 1H, J=1.5 Hz	115.84
5	NA	NA	173.16	NA	NA	178.14
6	2.59	m 1H	33.77	2.71	m 1H	34.25
7α	0.87	m 1H	42.48	0.82	m 1H	43.73
7β	1.94	m 1H	42.48	1.98	m 1H	43.73
8	1.70	m 1H	35.42	1.82	m 1H	35.56
9	1.27	m 1H	53.32	1.50	m 1H	49.41
10	NA	NA	45.07	NA	NA	41.91
11α	2.00	m 1H	23.30	1.90	m 1H	22.69
11β	1.58	m 1H	23.30	1.53	m 1H	22.69
12α	1.59	m 1H	31.26	1.62	m 1H	31.11
12β	2.00	m 1H	31.26	2.02	m 1H	31.11
13	NA	NA	47.06	NA	NA	47.34
14	1.70	m 1H	50.92	1.72	m 1H	51.02
15α	1.74	m 1H	24.06	1.71	m 1H	23.95
15β	1.35	m 1H	24.06	1.33	m 1H	23.95
16α	2.97	m 1H	30.37	2.97	m 1H	30.57
16β	1.78	m 1H	30.37	1.78	m 1H	30.57
17	NA	NA	96.76	NA	NA	96.92
18	0.72	s 3H	14.66	0.70	s 3H	14.65
19	1.29	s 3H	14.58	1.20	s 3H	23.41
20	NA	NA	204.51	NA	NA	204.16
21	2.02	s 3H	26.5	2.07	s 3H	26.32
22	NA	NA	170.87	NA	NA	171.07
23	2.13	s 3H	21.46	2.13	s 3H	21.37
24	1.11	d 3H, J=6.5 Hz	18.67	1.10	d 3H, J=6.2 Hz	18.07

NA=quaternary carbon, no proton attached.

correlation from 2α-proton to protons at 1α (δ=2.50 ppm) and 9 (δ=1.50 ppm) (Fig. 5C). Collectively, M-4 was unambiguously assigned as 2β-hydroxy-MPA.

Tables 1 and 2 summarized detailed chemical shifts for all atoms for MPA and all three metabolites. Chemical shifts for protons and protonated carbons were obtained from HSQC spectra, while chemical shifts for quaternary carbons were obtained from HMBC spectra.

Conclusion

Using detailed NMR analysis, we have rigorously elucidated structures for the three major metabolites from MPA incubation with P450. The current work confirmed the speculated structures for these metabolites in our previous studies. More importantly, the unambiguous structural information and the establishment for their NMR chemical shifts of these metabolites could serve as reference standards for future studies.

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