Enzymology:

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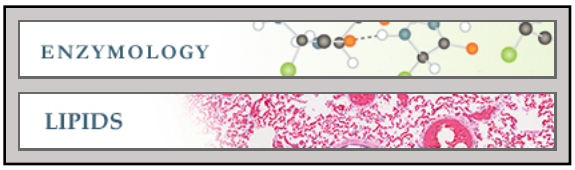

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Oxidation of Endogenous *N*-Arachidonoylserotonin by Human Cytochrome P450 2U1

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Running title: *P450 2U1 oxidation of N-arachidonoylserotonin*

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Background: Cytochrome P450 (P450, CYP) 2U1 is an 'orphan' enzyme, only reported to catalyze some fatty acid oxidations.
Results: Metabolomic screening led to the identification of *N*-arachidonoylserotonin as a substrate.
Conclusion: P450 2U1 catalyzes oxygenation at C-2 of the indole ring.
Significance: The localization of P450 2U1 and its metabolism of an inhibitor of fatty acid amide hydrolase may indicate a function in brain.

ABSTRACT

Cytochrome P450 (P450) 2U1 has been shown to be expressed, at the mRNA level, in human thymus, brain, and several other tissues. Recombinant P450 2U1 was purified and used as a reagent in a metabolomic search for substrates in bovine brain. In addition to fatty acid oxidation reactions, an oxidation of endogenous *N*-arachidonoylserotonin was characterized. Subsequent NMR and mass spectrometry and chemical synthesis showed that the main product was the result of C2 oxidation of the indole ring, in contrast to other human P450s that generated different products. *N*-Arachidonoylserotonin, first synthesized chemically and described as an inhibitor of fatty acid amide hydrolase, had previously been found in porcine and mouse intestine; we demonstrated its presence in bovine and human brain samples. The product (2-oxo) was 4-fold less active than *N*-arachidonoylserotonin in inhibiting fatty acid amide hydrolase. The rate of oxidation of *N*-arachidonoylserotonin was similar to that of arachidonic acid, one of the previously identified fatty acid substrates of P450 2U1. The demonstration of the oxidation of *N*-arachidonoylserotonin by P450 2U1 suggests a possible role in human brain and possibly other sites.

Cytochrome P450 (P450) enzymes play important roles in the metabolism of steroids, drugs, carcinogens, and numerous other compounds in mammals (1). Included among the substrates are compounds of both endogenous and exogenous (xenobiotic) origin (2). Some of the human P450 enzymes can rather clearly be defined as using only certain endogenous substrates (e.g., steroids, prostanoids) while others only use xenobiotic substrates. Some P450s use both both, e.g. P450 1B1, P450 46A1 (3-5). Of the 57 human P450 genes, ~ one-fourth can still be classified as "orphans" in that these were, in general, discovered through the Human Genome Project and our understanding of their biological functions is still rather limited (6).

The functional annotation of gene products is a central problem in biology today (7,

8). Several approaches can be applied to the issue. One involves prediction from a crystal structure, but this is problematic with P450s because their conformations usually change upon ligand binding (9). Another is a biased (i.e., trial-and-error) approach, sometimes guided by similarities to enzymes of known function. A third approach is the use of limited libraries of compounds to find leads, which can be explored in further chemical space (10). Sequence similarity may be useful in identifying function, e.g. as in the case of bacterial operons. However, that approach has had limited value in the assignment of functions (and redox partners) with the *Streptomyces coelicolor* P450s (11). There is considerable substrate diversity in the human P450 2C Subfamily, even among these four proteins with >80% sequence identity (3). Still another approach is a "metabolomics" one, involving an "unbiased" search in an extract of a tissue in which the enzyme is normally found (12, 13). Some results with the human P450s have been compiled (14, 15). It is also possible that a particular P450 may not have true endogenous substrates, e.g. have a role in a general protective mechanism against the toxic effects of xenobiotics (16).

One of the human P450s generally grouped under the heading of "orphans" is P450 2U1 (3, 15). In several reports the mRNA has been found to be preferentially expressed in thymus, brain, and several other tissues (17-19). Recently the expression of P450 2U1 (mRNA and protein) has also been reported in human platelets (20). Although the expression profiles are known in mice, rats, and humans, relatively little is known about any biological function of P450 2U1. A recombinant (baculovirus-infected insect cells) protein was demonstrated to catalyze ω- and ω-1 hydroxylation of several fatty acids (18).

In this report we expressed human P450 2U1 in *Escherichia coli* and utilized the purified enzyme in unbiased metabolomic searches with bovine brain extract. In addition to some fatty acids, we identified *N*-arachidonoylserotonin as a substrate (Fig. 1). *N*-Arachidonoylserotonin was originally studied as a synthetic molecule (21) designed to inhibit fatty acid amide hydrolase, an enzyme that hydrolyzes anandamide ligands of cannabinoid receptors (22). In 2011 *N*-arachidonoylserotonin was identified in hog and mouse intestine (23). We characterized the site of oxidation by P450 2U1, on the indole moiety of *N*-arachidonoylserotonin (Fig. 1), and found that this oxidation attenuated the fatty acid amide hydrolase inhibitory activity 4-fold. *N*-Arachidonoylserotonin was also identified in human brain. The localization of P450 2U1 in brain and the loss of the inhibitory function of *N*-arachidonoylserotonin following oxidation suggest possible roles in mammalian brain function.

EXPERIMENTAL PROCEDURES

Chemicals—Arachidonic acid, *N*-arachidonoylserotonin, serotonin-HCl, and a fluorescence-based (coumarin derivative) fatty acid amide hydrolase inhibitor screening assay kit were purchased from Cayman Chemicals (Ann Arbor, MI). *tert*-Butyldimethylsilyl chloride (TBDMS) was purchased from Oakwood Chemicals (West Columbia, SC). Tetrahydrofuran (THF) with butylated hydroxytoluene (BHT), added as stabilizer, was purchased from SigmaAldrich (St. Louis, MO). L-α-1,2-Dilauroyl-*sn*-glycero-3-phosphocholine and serotonin-C were purchased from SigmaAldrich. Organic solvents for chromatographic and mass spectrometric methods were from Fisher Scientific (Waltham, MA).

Enzymes and Tissue Samples—Catalase (bovine liver), superoxide dismutase (bovine erythrocyte), and other reagents were purchased from SigmaAldrich. Catalase was dialyzed before use to remove the inhibitor thymol. Rat NADPH-P450 reductase was expressed in *E. coli* and purified as previously described (24). Cytochrome b_5 (human) was expressed in *E. coli* and purified (25, 26).

Human liver samples (from organ donors) were obtained from Tennessee Donor Services and stored at -70 °C. Human brain samples were obtained through the Vanderbilt Ingram Cancer Center/Cooperative Human Tissue Network. Bovine brains (trimmed of meninges) were purchased from Pel-Freez (Rogers, AR).

Instrumentation—LC-MS separations (in untargeted metabolomics and stable isotope experiments) were performed with a Waters Acquity UPLC system in tandem with a Thermo

LTQ XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA). A Waters SYNAPT Q-TOF high-resolution mass spectrometer was used for determination of accurate masses. A Bruker NMR spectrometer (600 MHz for ^{1}H) with a 5 mm Z-gradient TCI cryo-probe was used to acquire spectra, and Topspin software was used to analyze the data.

Optimization of the P450 2U1 Nucleotide Sequence for Heterologous Expression and PCR-Based Gene Synthesis—Codon optimization was performed for the whole coding sequence to improve the expression level in *E. coli*, and oligonucleotides were designed for PCR-based gene synthesis with DNAWorks 2.4 (27) as described previously (28, 29). A $(His)_6$ tag was added to the C-terminal of the synthesized gene to facilitate purification. The optimized coding sequence was cloned into a "monocistronic" pCW vector (not coding for NADPH-cytochrome P450 reductase (30)) for expression.

Bacterial Expression of Human P450 2U1—The constructed plasmid and a plasmid containing the gene for the *E. coli* molecular chaperone GroEL/ES (31) were transformed into *E. coli* Top10 competent cells. Competent cells were incubated in Super Optimal Broth medium with Catabolite repression (S.O.C. medium) in a gyrorotary shaker (INFORS Multitron) for 1 h at 37 °C and 225 rpm and then transferred onto LB medium plates enriched with ampicillin (100 μg/ml) and kanamycin (50 μg/ml). Cells were grown overnight at 37 °C, and subsequently single colonies were used for preparing starting cultures in LB medium containing ampicillin and kanamycin.

Starting cultures were incubated overnight at 37 °C and 220 rpm in a gyrorotary shaker. For long term storage, 600 μl of bacterial starting culture was mixed with 300 μl of 50% glycerol (v/v) and stored at -80 °C. For preparation of a fresh overnight culture, 60 μl of a glycerol stock of *E. coli* cells was added into 300 ml of Luria-Bertani (LB) media containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml), which was incubated overnight in a gyrorotary shaker (*vide supra*) at 37 °C and 220 rpm. Large scale expression was done with 9 liters of Terrific Broth (TB) media enriched with the previously mentioned antibiotics. Cultures were incubated at least for 4 h with gyrorotary shaking at 37 °C and 220 rpm, checked by measuring OD_{600}. Expression of P450 2U1 was initiated by the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside, 1 mM 5-aminolevulinic acid, and 6 mM arabinose (all added at OD_{600} 0.8). Cultures were incubated for 24 h at 28 °C and 190 rpm, followed by determination of P450 concentration (32).

Purification of P450 2U1—Cells were harvested by centrifugation at 3,500 rpm (Sorvall RC3B centrifuge, H-6000A/HBB-6 rotor) for 10 min and recovered in 1 liter of TES buffer (0.10 M Tris-acetate buffer, pH 7.4, containing 0.5 M sucrose and 0.5 mM EDTA). Lysozyme was added (1.0 ml of a 1.0 mg/ml solution per 500-ml bottle), and the suspension of cells was incubated on ice for 30 min. The resulting spheroplasts were recovered by centrifugation at 3,500 rpm for 10 min (*vide supra*) and the pellet from each (1-liter) bottle was resuspended in 50 ml of sonication buffer (0.10 M potassium phosphate buffer, pH 7.4, 16% glycerol (v/v), 9 mM magnesium acetate, and 100 μM dithiothreitol (from a freshly dissolved stock)). Membranes containing P450 2U1 were solubilized with 25 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), with subsequent purification of enzyme using a Ni^{2+}-nitrilotriacetic acid column (elution of enzyme with 200 mM imidazole). A typical yield of P450 2U1 enzyme was 350 nmol (from 9 liters culture).

P450 concentrations were estimated spectrally using the method of Omura and Sato (32) (Fig. 2).

Proteomic Analysis of Purified P450 2U1—Purified P450 2U1 samples (1 μg of protein loaded onto gel) were separated by SDS-polyacrylamide gel electrophoresis (7.5%, w/v) (33). The gel was stained using a sensitive Blue Silver staining protocol (34) and the two major gel bands (Fig. 3) were subjected to proteomic analysis in the Vanderbilt facility. Fragments of peptides obtained with tandem mass spectrometry proteomic techniques were compared with those peptides for P450 2U1 included in the SEQUEST database (Fig. 4).

Edman N-terminal analysis was done by Jodie Franklin in the Synthesis and Sequence Facility at Johns Hopkins Univ. (Baltimore, MD) (Table 1, Fig. 2*B*).

Tissue Extracts Preparation—Human liver extracts were prepared as previously described (35). Human brain tissue samples (0.6 g) from four different donors were homogenized in 12 ml of a $CHCl_3/CH_3OH$ mixture (2:1, v/v), stirred for 30 min at 23 °C, and filtered through filter paper. The resulting extract was mixed with 0.2 volume of deionized H_2O and centrifuged for 20 min at 7,000 × *g*. The organic layer was collected, evaporated under a stream of nitrogen, and immediately purged with argon and stored at -70 °C. On the day of use, samples were reconstituted in 2 ml of C_2H_5OH and sonicated 3 × 30 s. The same procedure was used for preparation of bovine brain tissue extracts, on a larger scale.

Oxidation of Arachidonic Acid by P450 2U1—Incubations with arachidonic acid were performed in 100 μl of 100 mM Tris-HCl buffer (pH 7.4) with 0.3 μM purified P450 2U1, 3 μM NADPH-P450 reductase, 75 μM L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine, 10 mM $MgCl_2$, 10 mM isocitrate, 0.2 unit ml^{-1} isocitrate dehydrogenase, and 50 μM [1-^{14}C]-arachidonic acid (58 mCi $mmol^{-1}$). In some cases *E. coli*-expressed recombinant human cytochrome b_5 (0.3 or 1.0 μM) was included. The enzyme reaction was initialized by addition of 1 mM NADPH. Samples were then incubated for 30 min at 37 °C in a shaking water bath. Reactions were stopped by addition of 100 μl of CH_3CN containing 0.2% CH_3CO_2H (v/v) and 0.005% BHT (w/v), followed by mixing with a vortex device and centrifugation at 1.5 × 10^4 × *g* for 10 min. The supernatant (175 μl) was injected onto a Varian octadecylsilane (C_{18}) column (4.6 mm × 160 mm) at 23 °C, with a flow rate of 0.8 ml min^{-1}. Products were separated with a gradient of mobile phase A (H_2O and 0.1% CH_3CO_2H, v/v) and mobile phase B (CH_3CN and 0.1% CH_3CO_2H (v/v). Gradient conditions were as follow: 50% mobile phase B (v/v), with a linear gradient increasing to 100% mobile phase B over the next 40 min, held for 5 min, then returned back to initial conditions over 5 min followed by column equilibration for 10 min.

LC-MS Metabolomic Experiments—*In vitro* incubation of purified P450 2U1 with tissue extracts (human liver or bovine brain) was performed in 1.0 ml of 100 mM potassium phosphate buffer (pH 7.4). Each reaction mixture contained 1 μM purified enzyme, 2 μM NADPH-P450 reductase, 150 μM L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine, and 1% (v/v) ethanolic solution of a tissue extract (equivalent to 10 mg tissue). For longer incubation times (> 20 min), catalase (100 units per reaction mixture) was added to each test tube (to prevent degradation by H_2O_2) (36). The enzymatic reaction was started by the addition of 100 μl of an NADPH-generating system including 100 μl of 100 mM glucose 6-phosphate, 50 μl of 10 mM $NADP^+$, and 2 μl of a 1 mg ml^{-1} solution of yeast glucose 6-phosphate dehydrogenase (37). Samples were then incubated for 2 h at 37 °C. Reactions were stopped by addition of 2 ml of CH_2Cl_2, followed by mixing for 1 min with a vortex device and centrifugation at 2,000 × *g* for 10 min. The lower organic layer was carefully removed and evaporated under a stream of nitrogen. Samples were reconstituted in 100 μl of C_2H_5OH, transferred into vials, and subjected to LC-MS analysis. LC separations were performed with a Waters Acquity UPLC system (Waters, Milford, MA) and an Acquity BEH octadecylsilane (C_{18}) column (1.7 μm, 2.1 mm × 100 mm) at 35 °C with a flow rate of 0.3 ml min^{-1}. Samples (20 μl) were injected into system in the full-loop mode and were separated with a gradient. For detection of analytes using a linear ion trap mass spectrometer (LTQ XL, Thermo Fisher Scientific, Waltham, MA) with electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI), both positive and negative modes were used with mobile phase A, consisting of 10 mM $NH_4CH_3CO_2$ in a 5:95 (v/v) mixture of CH_3CN and H_2O (v/v), and mobile phase B, consisting of 10 mM $NH_4CH_3CO_2$ in a 95:5 (v/v) mixture of CH_3CN and H_2O (v/v). Separation of analytes (in case of the APCI mode) was performed using a gradient of mobile phase A, consisting of 0.1% HCO_2H (v/v) in a 5:95 mixture (v/v) of CH_3CN and H_2O, and mobile phase B, consisting of 0.1% HCO_2H (v/v) in a 95:5 mixture of (v/v) of CH_3CN and H_2O. Gradient conditions were set up as follows—0 to 2 min: 5% mobile

phase B (v/v), a linear gradient of mobile phase B increasing over 20 min to 100%, held for 5 min, then returned back to the initial condition with column equilibration for an additional 5 min. MS spectra were obtained in the $APCI^+$ mode with the following tune settings: discharge voltage 3.9 kV, vaporizer temperature 450 ºC, sheath gas flow rate 50 arbitrary units, auxiliary gas flow rate 5 arbitrary units, sweep gas flow rate 5 arbitrary units, capillary voltage 22 V, capillary temperature 275 ºC.

Isotopic Labeling Experiments—A method for $^{16}O_2/^{18}O_2$ isotopic experiments was adapted from previous studies (13, 35). Thunberg tubes including reaction mixtures (as described under *LC-MS Metabolomics Experiments*) were charged (following 10 alternating cycles of vacuum and argon) separately with 100% $^{16}O_2$ and 97% $^{18}O_2$, and the two samples were mixed together before extraction. Reaction mixtures charged with 100% $^{16}O_2$ served as control samples. Samples were analyzed with the same LC-MS methods as in case of metabolomics experiments with tissue extracts.

Data Analysis—Data obtained from experiments with human liver tissue or bovine brain tissue extracts were evaluated with the metabolomics software XCMS working in R statistical language, which has been previously used for analysis of endogenous and exogenous metabolites in human serum (38). Other metabolomics data mining software was also used, e.g. MZmine (39) and an in-house Matlab program (14) for doublet searches (M, M+2) in the case of ^{18}O-based isotopic labeling experiments. MZmine2 (40) serves as a high performance tool in differential analysis of data from multiple LC-MS experiments, enabling noise filtering, peak detection, and retention time (t_R) alignment for corresponding peaks from different runs (40). After chromatogram construction and deconvolution in the MZmine2 program, the centroid data was displayed as a peak list, which included *m/z* values, retention time, duration, and peak height of individual peaks. The peak list was copied to an Excel file and exported as a CSV (Comma Separated Values) file. An in-house Matlab program was used to extract peak information from the CSV file and in searches for $^{18}O/^{16}O$ doublet patterns (14).

Incubation of P450s (2U1, 2W1, and 4A11) with the Substrate N-Arachidonoylserotonin—Reaction mixtures were prepared as described under *LC-MS Metabolomics* except that bovine superoxide dismutase (100 units per reaction mixture) was added together with bovine erythrocyte catalase (100 units per reaction mixture) to the mixture to remove reactive oxygen species formed during incubations. Samples were incubated for 50 min and then treated as described above. The concentration of *N*-arachidonoylserotonin in the reaction mixtures was 100 μM. In the case of experiments with P450 4A11, dithiothreitol (1 mM), and (*E. coli* recombinant) human cytochrome b_5 were added to individual samples to support enzymatic activity (41). Potential metabolites of *N*-arachidonoylserotonin were analyzed in the $APCI^+$ mode.

Purification and Identification of N-Arachidonoylserotonin Oxidation Product—Large Scale Incubation—To elucidate the structure of the *N*-arachidonoylserotonin oxidation product found in the isotope-labeling experiments, a larger reaction mixture was prepared to obtain more of the product for NMR analysis. Large scale incubation of purified P450 2U1 with *N*-arachidonoylserotonin was performed in 50 ml of 100 mM potassium phosphate buffer (pH 7.4) containing 100 nmol purified P450 2U1, 200 nmol NADPH-P450 reductase, 15 μM L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine, 150 μM *N*-arachidonoylserotonin, and 100 units each of catalase and superoxide dismutase (to prevent formation of reactive oxygen species). The enzymatic reaction was started by the addition of 15 ml of an NADPH-generating system including 10 ml of 100 mM glucose 6-phosphate, 5 ml of 10 mM $NADP^+$, and 100 μl of a 1 mg ml^{-1} solution of yeast glucose 6-phosphate. The reaction was run for 1.5 h. The reaction was terminated by the addition of a 2-fold volume of CH_2Cl_2 (100 ml), the products were extracted an additional three times with an equal volume of CH_2Cl_2 (50 ml), and the organic layers were combined and concentrated *in vacuo*. Samples were reconstituted

in a small volume of C_2H_5OH and fractions containing the main products formed from *N*-arachidonoylserotonin were collected from HPLC, monitored using UV detection (A_{254}). Fractions containing the *N*-arachidonoylserotonin product were combined, concentrated under a stream of nitrogen, and stored under argon at -80 °C. Samples were analyzed with use of LC-MS (t_R, mass spectra, fragmentation patterns). Frozen samples were redissolved in CD_2Cl_2 for NMR experiments (Bruker NMR 600 MHz spectrometer).

Targeted Search for N-Arachidonoylserotonin in Human Brain Tissue—Human benign brain tumor tissues were homogenized and followed by Folch extraction ($CHCl_3/CH_3OH$, 2:1, v/v) and the resulting extracts were combined, concentrated under a stream of nitrogen, reconstituted in C_2H_5OH, and analyzed for the presence of *N*-arachidonoylserotonin using LC-MS ($APCI^+$) under the same conditions described (*vide supra*).

Inhibition of Fatty Acid Amide Hydrolase—A fatty acid amide hydrolase inhibitor screening assay kit was used with the protocol described by the manufacturer (Cayman). The data were analyzed using GraphPad Prism (version 5.0a), with the IC_{50} value calculated using the equation:

Y=Minimum + (Maximum-Minimum)/(1+10^((X-LogIC50)))

RESULTS

Heterologous Expression of E. coli Recombinant P450 2U1 and Purification—An optimal expression level for P450 2U1 (400 nmol $liter^{-1}$) was achieved with *E. coli* Top 10 cells co-expressed with a chaperone protein. Purified P450 2U1 gave a typical 450 nm band in the usual ferrous-CO vs. ferrous difference spectrum (Fig. 2).

SDS-polyacrylamide gel electrophoresis consistently showed two protein bands in the expected M_r migration region (Fig. 3*A*). Both bands were cut from a gel, subjected to trypsin digestion, and analyzed by LC-MS in the Vanderbilt Proteomics Facility (Fig. 4). The upper band showed 72% coverage of the expected peptides, and the lower band showed 80% (the first residue identified was 36). The upper band showed additional peptides in the *N*-terminus (residues 20-44 of expected protein), which were not present in the lower band. However, the upper band did not show a peptide including the entire N-terminus.

In order to resolve the issue of which of the protein bands contained P450 2U1, both electrophoretic bands were excised and submitted for Edman N-terminal degradation (amino acid sequencing) (Table 1, Fig. 3*B*). The upper band clearly confirmed residues 2-10 of the predicted sequence (Table 1, yields varying form 9.6-19.3 pmol). The removal of the N-terminal Met is expected in light of the known specificity of bacterial N-terminal processing (42). The lower band was characterized as a mixture of three polypeptides (Fig. 3*C*) (in all three cases 11 residues could be identified, with yields varying from 7.4-15.6 pmol). The proteolytic cleavage occurred even in the presence of protease inhibitors, under conditions in which other mammalian P450s have generally not shown any cleavage (43).

Oxidation of Arachidonic Acid by P450 2U1—P450 2U1 oxidized arachidonic acid to a roughly equimolar mixture of the 19- and 20-hydroxy products (Fig. 5). The rate was 19 (± 5) pmol (total) product formed min^{-1} (nmol P450 $2U1)^{-1}$. The rate was not changed in the presence of either an equimolar concentration of or 3.3-fold excess of cytochrome b_5.

Metabolomic Searches for P450 2U1 Reactions in Bovine Brain—To search for novel endogenous substrates for the P450 2U1 enzyme, the recombinant enzyme was incubated with a tissue extract of bovine brain due to its higher expression in certain parts (cortex, limbic system, and cerebellum) (17, 18). Untargeted analysis using the program XCMS (44, 45) to compare enzymatic and non-enzymatic incubations only identified fatty acids that had been already reported to be substrates for P450 2U1, including arachidonic acid (mono- and dihydroxylation, *m/z* 319 and 336) and docosahexaenoic acid (mono-

and dihydroxylation, *m/z* 343 and 359). The same results were obtained with human liver extracts.

On the other hand, the incubation of bovinebrain extract and P450 2U1 with $^{16}O_2$ and $^{18}O_2/^{16}O_2$ (doublet search) (14) resulted in formation of a doublet, with *m/z* 479/481 (Fig. 6). HRMS analysis for the sample from the doublet search (*m/z* 479.3221, tentatively $C_{30}H_{42}N_2O_2$, calcd 479.3268) followed by a lipidomics database search (LIPIDMAPS, http://www.lipidmaps.org) showed that the doublet could possibly be assigned to an oxygenated product of *N*-arachidonoylserotonin.

In a separate experiment, we identified *N*-arachidonoylserotonin in bovine brain extract, using LC-MS selective ion monitoring (*m/z* 463) and coincidence with a commercial sample (t_R 9.78 min, data not shown). Incubation of commercial *N*-arachidonylserotonin with P450 2U1 yielded the same product detected in the brain extract incubation (Fig. 7, t_R 16.6 min), and product formation was not attenuated in the presence of catalase and superoxide dismutase (data not presented). In addition, *N*-arachidonylserotonin was found in human brain samples, utilizing LC-MS selective ion monitoring (data not presented).

P450 2U1, as isolated, appeared to be largely in the low-spin iron configuration (Soret λ_{max} at 418 nm, data not presented). The addition of *N*-arachidonoylserotonin resulted in a "Reverse Type I" (46) difference spectrum (Fig. 8).

P450 2U1 Oxygenation Occurs at the Indole 2-Position—Based on the fragmentation in mass spectral analysis (MS/MS), oxygen addition was suggested to occur on the indole ring (Fig. 9). Fragmentation spectra of the product formed in the presence P450 2U1 showed formation of a unique fragment with *m/z* 193, which was absent in the fragmentation spectra of the substrate and the product formed in the presence of P450 2W1 or 4A11. (The major products formed with these latter two P450s also showed shorter t_R values, distinct from each other, data not presented. We have not elucidated their identities yet.) More detailed analysis of the MS/MS spectrum of the parent compound and the P450 2W1 and 4A11 products revealed a fragment with *m/z* 177, which was assigned to the intact serotonin moiety; thus the *m/z* 193 (177 + 16 (O)) fragment indicated oxidation in the serotonin part of the product.

Although the isolated major product purified from the incubation by HPLC did not yield a completely pure compound (due to decomposition, *vide infra*), a tentative structure was deduced from 1-D and 2-D proton ^{1}H NMR spectra (Fig. 10). Preliminary ^{1}H NMR data obtained with the isolated major product of *N*-arachidonoylserotonin suggested oxidation at the 2-position to form 2-oxo-*N*-arachidonoylserotonin. Because the oxidation had been shown to occur in the indole ring based on the MS fragmentation (*vide supra*), the focus of the NMR data analysis of the product was in the aromatic region (δ 6-8 ppm) of the ^{1}H NMR spectrum (CD_2Cl_2, referenced at 5.32 ppm, was used as the NMR solvent). The splitting pattern in this region indicated a doublet (d) at δ_H 6.60 ppm (J = 8 Hz), a d at δ_H 7.28 ppm; J = 2 Hz), and a dd pattern at δ_H 7.06 ppm (J = 8 and 2 Hz). Another set of distinct protons was observed with splitting patterns of d (δ_H 7.48 ppm; J = 9 Hz) and a dd (δ_H 7.16 ppm; J = 9 and 2 Hz) (Fig. 8*A*). This splitting pattern was indicative of oxygenation at the 2-position. Although there were two sets of these protons in the 1-D ^{1}H NMR, the 2-D NMR data (^{1}H-^{1}H COSY) confirmed the coupling partners of the d at δ_H 6.60 ppm and dd at δ_H 7.06 ppm (the other set of d at δ_H 7.48 ppm and dd at δ_H 7.16 ppm showed a COSY correlation as well) (Fig. 10*B*). The duplicate sets of the protons suggested possible dimerization of the product, presumably due to decomposition during the extraction, purification, or concentration processes used with the compound.

2-Oxo-*N*-arachidonoylserotonin was synthesized in a 6-step chemical reaction sequence and characterized by its NMR and mass spectra (Supplemental Data, Figs. S1, S2, S3). This synthetic standard and the product from an enzymatic incubation of P450 2U1 with *N*-arachidonoylserotonin both eluted at same t_R, 16.49 and 16.44 min respectively (within experimental error), under same conditions. The CID fragmentation patterns were compared in MS/MS analysis (Fig. 11), and the product from the enzymatic reaction gave the same fragmentation pattern as the synthetic standard. Further, HRMS showed the synthetic standard

with the parent ion at *m/z* 479.3257 which matched *m/z* 479.3256 for the enzymatic product (both Δ 2.3 ppm from 479.3268 calculated for $C_{30}H_{42}N_2O_2$). These results clearly showed that P450 2U1 oxidized *N*-arachidonoylserotonin to 2-oxo-*N*-arachidonoylserotonin.

Rate of P450 2U1 Oxidation of N-Arachidonoylserotonin—P450 2U1 oxidized *N*-arachidonoylserotonin to the 2-oxo product, and the kinetic parameters were determined using LC-MS with the synthetic material as an external standard (Fig. 12). The k_{cat} was 32 ± 9 pmol product formed min^{-1} (nmol P450 2U1)$^{-1}$ and the K_m was 82 ± 2 μM.

Inhibition of Fatty Acid Amide Hydrolase—An IC_{50} value of 14 ± 1 μM was measured with *N*-arachidonoylserotonin (Fig. 13), nearly identical to that reported earlier (21). The IC_{50} of 2-oxo-*N*-arachidonoylserotonin in the same system was 47 ± 5 μM.

DISCUSSION

P450 2U1 was first identified in 2004 through searches of the human genome (17). The closest human relatives are P450s 2R1 (37% identity) and 2J2 (36% identity). mRNA studies in humans, mice and rats consistently show highest expression in thymus followed by brain and heart (17-19). In rats, the protein is preferentially expressed in the brain (17). In rats, the highest level of immunodetectable P450 2U1 was found in the brain, followed by the thymus (17). However, the level of mRNA was similar in rat thymus and brain (17). In humans, the level of P450 2U1 mRNA was > 10-fold higher in thymus compared to brain (18, 19) but protein levels have not been reported. In rat brain, P450 2U1 protein expression was highest in limbic structures and cortex (and also found in cerebellum, olfactory bulbs, and pons and medulla) (17). In human brain a high level of P450 2U1 mRNA has been reported in cerebellum (18). On the basis of work in zebrafish, Goldstone *et al*. (47) have proposed that P450 2U1 has a pre-vertebrate origin and that P450 2U is the oldest P450 subfamily. Relatively little is known about the regulation of P450 2U1, but in mouse liver mRNA levels are attenuated by Nrf2 activation (48).

Recombinant P450 2U1 has been expressed in human embryonic kidney (HEK 293) cells (17) and in baculovirus-infected Sf9 insect cells (18) but the only substrates identified were long-chain fatty acids (18), hydroxylated at the ω- and ω-1 positions. However, no rates were reported in that work. We also found that *E. coli* recombinant human P450 2U1 catalyzed these reactions with arachidonic acid, at relatively low but significant rates, yielding these two products (Fig. 5). Indirect evidence has recently suggested that P450 2U1 catalyzes these reactions with arachidonic acid in platelets (20).

Because of the conservation of P450 2U1 in numerous species and expression of the enzyme in brain, we utilized bovine brain extracts in a metabolomic search for substrates and products of human P450 2U1. As expected, fatty acids were found as substrates of P450 2U1 with use of an untargeted metabolomics approach, confirming the results of Chuang *et al*. (18). Some other candidates were also identified, and one met several criteria, i.e. reproducibility, correct isotopic signature, and absence in controls (Fig. 6). The substrate was identified as *N*-arachidonoylserotonin by HRMS of the product and co-chromatography of the product with that generated from a commercial standard. Although ω- and ω-1 hydroxylation might be have been suggested, based on the fatty acid work (18) (Fig. 5), the oxidation site was judged not to be on the fatty acid chain as judged by MS fragmentation analysis. Subsequent ^{1}H NMR analysis and chemical synthesis confirmed the site of oxidation as C-2 of the indole ring (Figs. 10, 11). This is a reaction analogous to that of P450 2E1 and some other P450s with indole (49) and can be envisioned as hydroxylation followed by tautomerization. Because of the nature of the reaction, we considered melatonin as a possible substrate (50). However, we did not see consistent oxidation and when products were observed in some reactions, they were sensitive to the inclusion of catalase in the reaction, supporting the view that they were formed by the action of H_2O_2 adventitiously produced in the P450 2U1 reaction.

Fatty acids are known to be hydroxylated by P450s at the ω-carbon and the ω-1, -2, and -3 positions (3, 51, 52). However, mid-chain hydroxylations (14) and α- and β-hydroxylations

(53, 54) are also known. We found that P450 2U1 yielded a "Reverse Type I" difference spectrum (46), generally considered to indicate a change in the iron spin state (from high- to low-spin), due to an increase in the occupancy of H_2O as the distal ligand. With P450 2U1 the iron was largely in the low-spin state in the isolated protein (λ_{max} 418 nm), so the transition must result from a small population of the protein. The difference spectrum (Fig. 8) is not a typical "Type II" spectrum (46) indicative of nitrogen atom binding to iron. We did not observe a Type I change when arachidonic acid (50 μM) was added to P450 2U1 (data not presented). The difference between the binding spectra of arachidonic acid and *N*-arachidonoylserotonin may reflect the different regioselectivity of oxidation of a fatty acid and its amide conjugate.

N-Arachidonoylserotonin was first reported in 1998 as a synthetic inhibitor of fatty acid amide hydrolase, an enzyme that hydrolyzes anandamide agonists of cannabinoid receptors (21), being the most active of a series of compounds (IC_{50} 12 μM). Subsequently *N*-arachidonoylserotonin has been characterized in terms of it analgesic actions, with antagonistic activity at vanilloid TRPV1 receptors, with an IC_{50} of 37-40 nM (55). *N*-Arachidonoylserotonin also inhibited T-type calcium channels with IC_{50} values between 50 and 250 nM (56). Of a series of arachidonoyl endocannabinoids tested, *N*-arachidonoylserotonin preferentially inhibited the release of β-hexosaminidase (a marker of degranulation) and tumor necrosis factor-α (a pro-inflammatory cytokine) in immunoglobulin E-activated RBL-243 cells (57). In addition, *N*-arachidonoylserotonin suppressed the formation of prostaglandin D_2 (IC_{50} 1.3 μM) and leukotriene B_4 (IC_{50} 1.2 μM). The anti-allergic action of *N*-arachidonoylserotonin was attributed to suppression of the activation of several factors, including Syk, LAT, p38, JNK, PI3K, and Akt (57). The K_m value of P450 2U1 for *N*-arachidonoylserotonin 2-oxidation is high (Fig. 12), and the contribution to the 2-oxygenation *in vivo* is unknown. However, many enzymes with high K_m values are functional, e.g. catalase (58).

In 2011 *N*-arachidonoylserotonin was identified in biological systems, i.e. hog and mouse intestine, in the nM range (23). We have now identified this compound in bovine and human brain tissue. The IC_{50} value for inhibiting fatty acid amide hydrolase increased 4-fold following 2-oxygenation (Fig. 11); we have not determined the effect on the other reported biological activities but suggest that they are probably also attenuated. Thus, a function of P450 2U1 might be to down-regulate the biological activities of endogenous *N*-arachidonoylserotonin. We are not aware of any information about how this molecule is synthesized; presumably it is formed from the enzymatic reaction of arachidonoyl CoA with serotonin (anandamide (*N*-arachidonoylethanolamine) arises from degradation of a phosphatidylethanolamine (59)).

We have not determined if the proteolytic fragments derived from P450 2U1 are catalytically active. The ferrous-CO vs. ferrous difference spectra (e.g., Fig. 2) consistently showed little evidence of (inactive) cytochrome P420, so the fragments (Figs. 3, 4) may well contain spectrally defined P450. We suspect that they are not active but have based all calculations of activity on the total amount of spectrally detectable P450. As indicated earlier, the repeated proteolytic cleavage of a mammalian P450 expressed in *E. coli* was unexpected (60, 61).

As indicated earlier, we used several metabolomic search modes with P450 2U1 and pursued what was found to be *N*-arachidonoylserotonin. However, further searches may be of interest, especially using thymus as a source (18).

In conclusion, we have utilized a metabolomics approach to identifying substrates for the orphan P450 2U1. The substrate *N*-arachidonoylserotonin was found in brain and shown to be converted to the C-2 oxidation product, which was shown to have attenuated biological activity. The significance of these findings remains to be determined in *vivo*.

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Abbreviations: APCI, atmospheric pressure chemical ionization; BHT, butylated hydroxytolene; CID, collision-induced dissociation; COSY, correlated NMR spectroscopy; d, doublet (^{1}H NMR); ESI, electrospray ionization; HRMS, high resolution mass spectrometry; LC-MS, coupled liquid chromatography-mass spectrometry; mCPBA, *m*-chlorperbenzoic acid; MS/MS, tandem mass spectrometry; P450, cytochrome P450; TBDMS, *tert*-butyldimethylsilyl chloride; THF, tetrahydrofuran.

FOOTNOTE

[1] Co-first authors.

FIGURE LEGENDS

FIGURE 1. Structures of *N*-arachidonoylserotonin and 2-oxo-*N*-arachidonoylserotonin.

FIGURE 2. Reduced ferrous-CO v. ferrous difference spectrum of purified recombinant P450 2U1. The calculated P450 concentration was 3.0 μM (32).

FIGURE 3. P450 2U1 N-terminal peptides identified by Edman degradation. *A*, preparative SDS-polyacrylamide gel of P450 2U1 protein. Electrophoresis consistently showed two protein bands, marked with arrows on the right side (direction of migration top to bottom). Colloidal Coomassie Blue staining (1

μg of protein applied to gel) was used and the indicated bands were excised and submitted to proteomic analysis. *B*, repetitive yields of amino acids recovered in Edman degradation cycles. The yields were fit to semi-logarithmic plots. *C*, assignments. From the upper SDS gel band: residues 2-10. From the lower SDS gel band: peptides 34-44, 36-46, and 43-53.

FIGURE 4. Proteomic analysis of upper and lower protein bands of the P450 2U1. The yellow shading shows the peptides that were identified in each protein band (Fig. 3*A*). The Cys (C) and Met (M) residues are shaded turquoise to indicate that these residues were purposely oxidized in the protein workup. *A*, from upper band; *B*, from lower band.

FIGURE 5. HPLC-radiochromatogram for oxidation of [1-^{14}C]-arachidonic acid by P450 2U1. The ω- and ω-1 hydroxylation products and the residual substrate are labeled. The nature of the radioactivity eluting at the void volume is unknown.

FIGURE 6. LC-MS chromatograms from a bovine brain doublet search experiment. *A*, sample derived from brain tissue extract, NADPH-generating system, and inactivated P450 2U1 enzyme. *B*, sample generated with P450 2U1 incubated in an $^{16}O_2$ atmosphere. *C*, sample from two reaction mixtures, incubated either with $^{16}O_2$ or $^{18}O_2$, combined together. *m/z* 479 (1-O atom addition to *N*-arachidonoylserotonin) was monitored in each case.

FIGURE 7. 2-Oxygenation of *N*-arachidonoylserotonin by P450 2U1. *A*, minus P450 2U1; *B*, plus P450 2U1. The scales are identical, and the t_R of 2-oxo-*N*-arachidonoylserotonin is marked with the double-headed red arrow. The *m/z* 479 peak in the blank reaction (part *A*) appeared at the position of *N*-arachidonoylserotonin and is attributed to a solvent adduct (+16), as revealed by fragmentation. The peak eluted at t_R 16.8 min in part *B* was not identified.

FIGURE 8. Binding of *N*-arachidonoylserotonin to P450 2U1. Each of two 1-ml cuvettes contained 3.0 μM P450 2U1. A baseline was recorded and 6 μM *N*-arachidonoylserotonin was added to the sample cuvette, prior to rescanning.

FIGURE 9. LC-MS (APCI$^+$) fragmentation patterns of *N*-arachidonoylserotonin products formed in the presence of either P450 2U1 or P450 2W1 and comparison with the fragmentation spectrum of the substrate. The fragment with *m/z* 193 shows the presence of an additional oxygen moiety in the indole moiety of *N*-arachidonoylserotonin. *A*, P450 2U1 reaction product; *B*, P450 2W1 reaction product; *C*, substrate.

FIGURE 10: 1-D ^{1}H NMR spectrum of the aromatic region (δ 6-8 ppm) of the HPLC-purified product from a P450 2U1 incubation with *N*-arachidonoylserotonin. Proton numbering of the indole ring is indicated. *A*, 1-D spectrum; *B*, 2-D COSY spectrum.

FIGURE 11. Mass fragmentation of synthetic 2-oxo-*N*-arachidonoylserotonin with the product of oxidation of *N*-arachidonoylserotonin by P450 2U1. An APCI$^+$ source was used.

FIGURE 12. Steady-state kinetic analysis of oxidation of *N*-arachidonoylserotonin to 2-oxo-*N*-arachidonoylserotonin by P450 2U1. k_{cat} 32 (± 9) pmol product formed min^{-1} (nmol P450 2U1)$^{-1}$; K_m 82 ± 2 μM.

FIGURE 13. Inhibition of fatty acid amide hydrolase by synthetic *N*-arachidonoylserotonin and 2-oxo-*N*-arachidonoylserotonin. *N*-Arachidonoylserotonin: IC_{50} 14 ± 1 μM; 2-oxo-*N*-arachidonoylserotonin: IC_{50} 47 ± 5 μM.

TABLE 1. **Edman degradation of the upper and lower bands of a P450 2U1 preparation** (Fig. 3). The values in parentheses indicate that a residue was assigned to two peptides. For the upper band, the expected N-terminal Met is shown but was not observed in cycle 1; Ala was.

	pmol recovered			pmol recovered		
	Upper band			Lower band (three peptides)		
cycle			cycle	**1**	**2**	**3**
	(M)	—	1	R 13.7	A 11.3	S 9.8
1	A	14.5	2	R 15.6	A 11.7	W 7.4
2	K	19.3	3	S 11.4	G 10.7	L 10.9
3	K	19.3	4	W 9.4	I 13.0	S 9.5
4	T	12.2	5	L 14.5	D 6.7	S 9.6
5	S	9.1	6	S 10.7	P 12.2	R 10.4
6	S	9.1	7	S (11.9)	S (11.9)	T 9.8
7	K	10.6	8	R (11.6)	V 10.3	R (11.6)
8	G	10.3	9	T 11.4	I 12.2	A 9.1
9	K	9.6	10	R 11.1	G 10.5	A 9.8
			11	A 11.2	P 11.3	G 10.3

Fig. 1

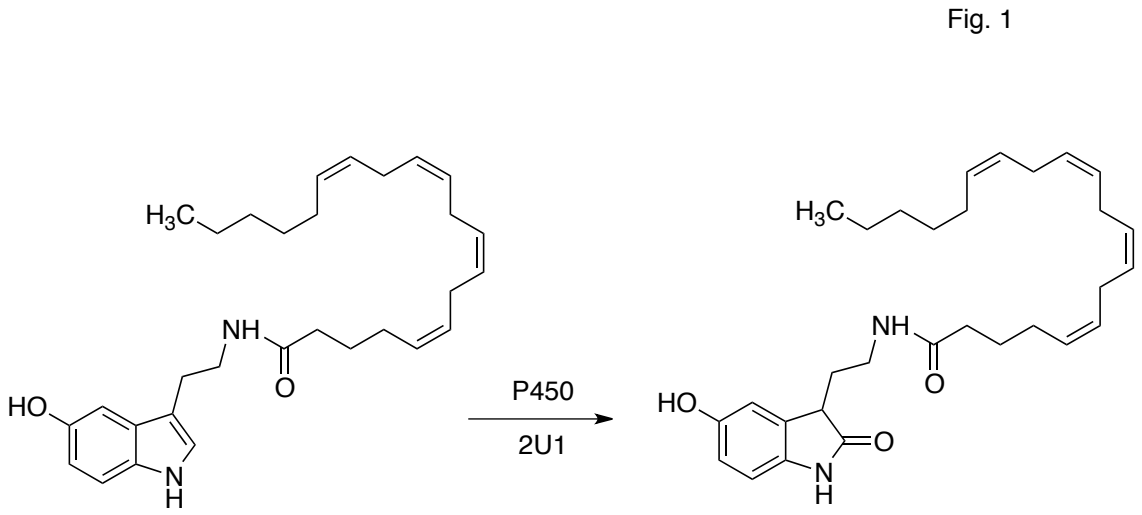


Fig. 2

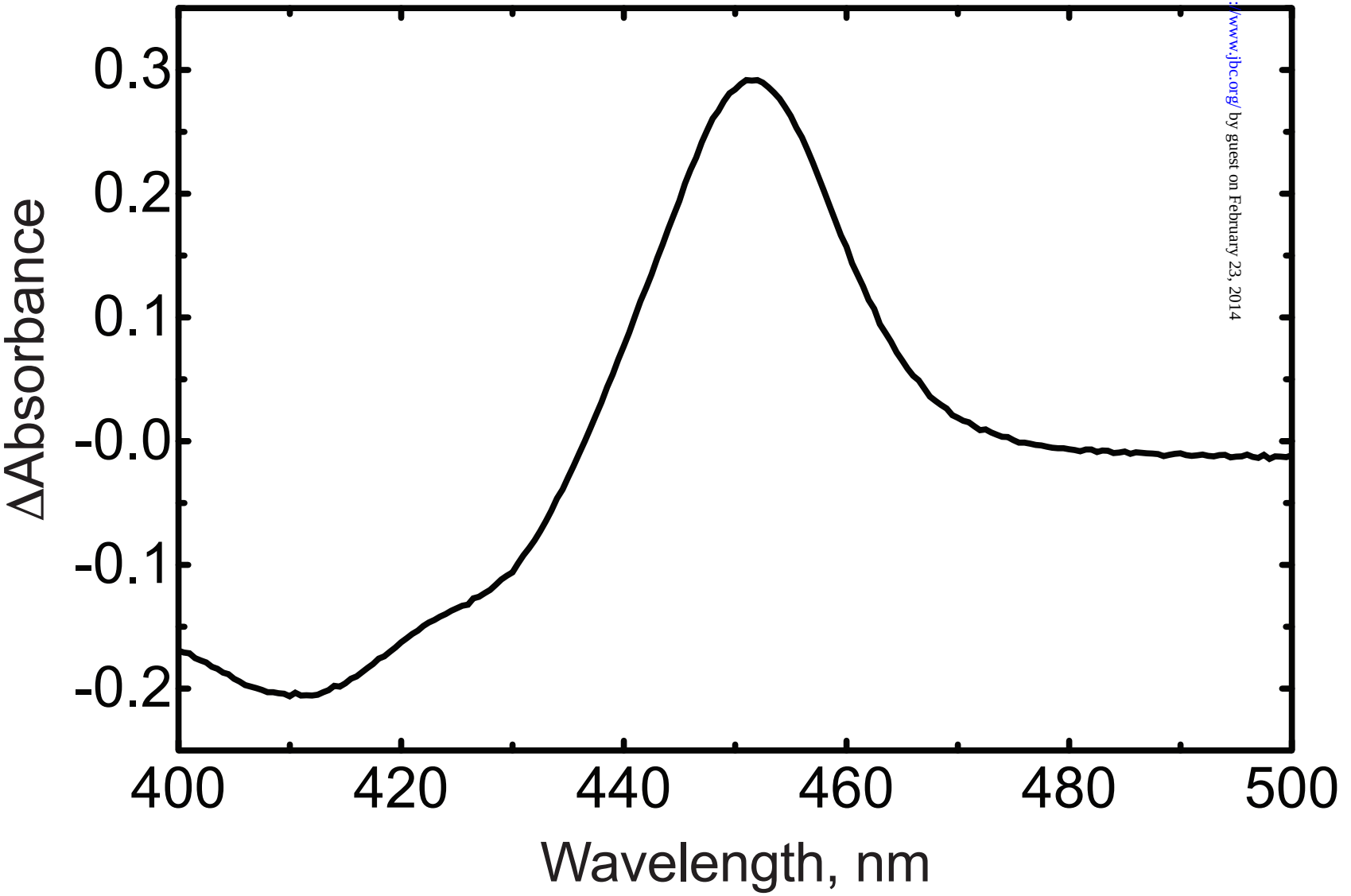
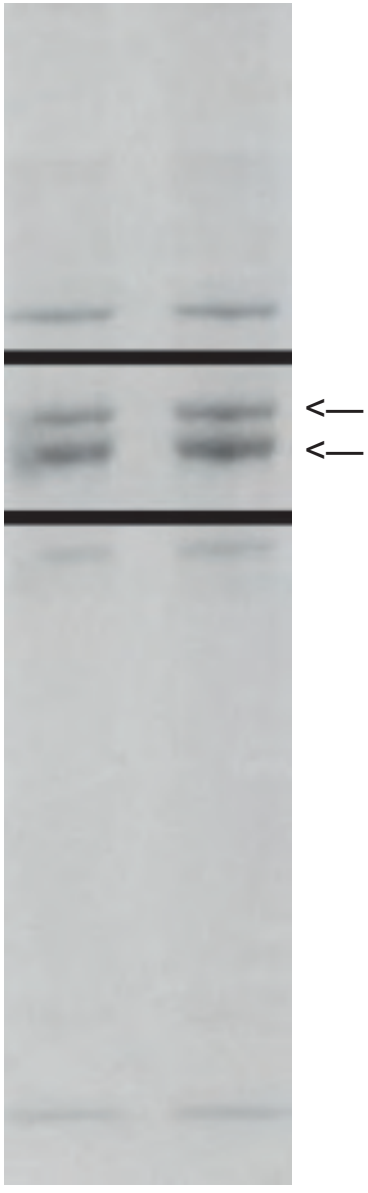
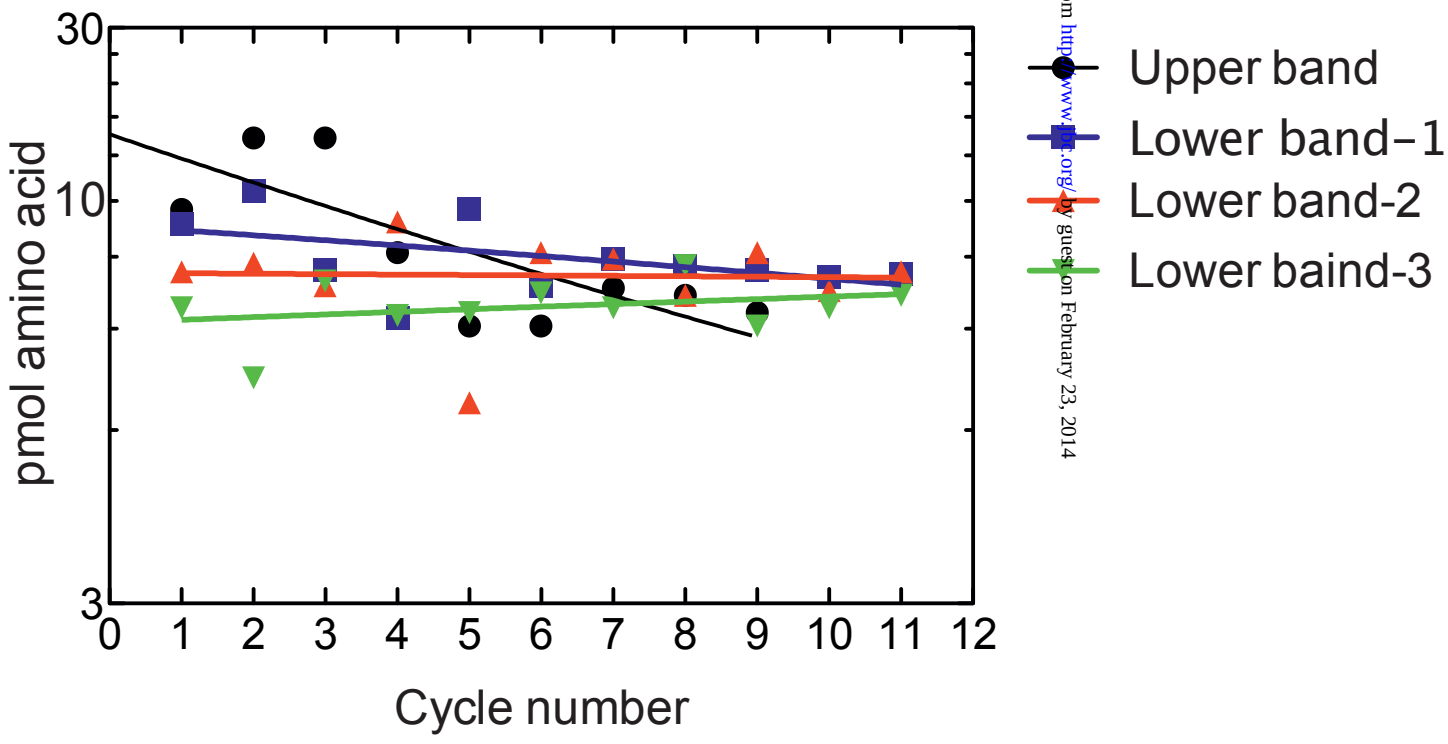


Fig. 3

A



B



C

Fig. 4

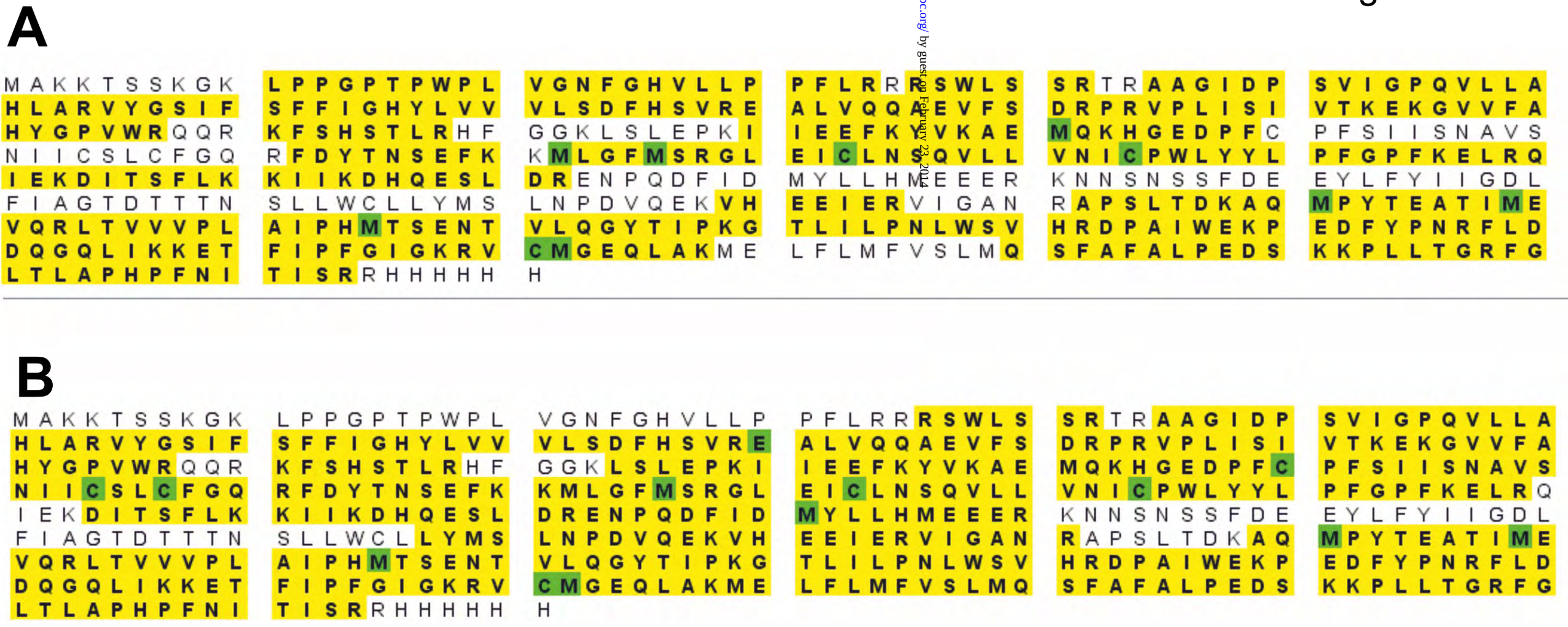


Fig. 5

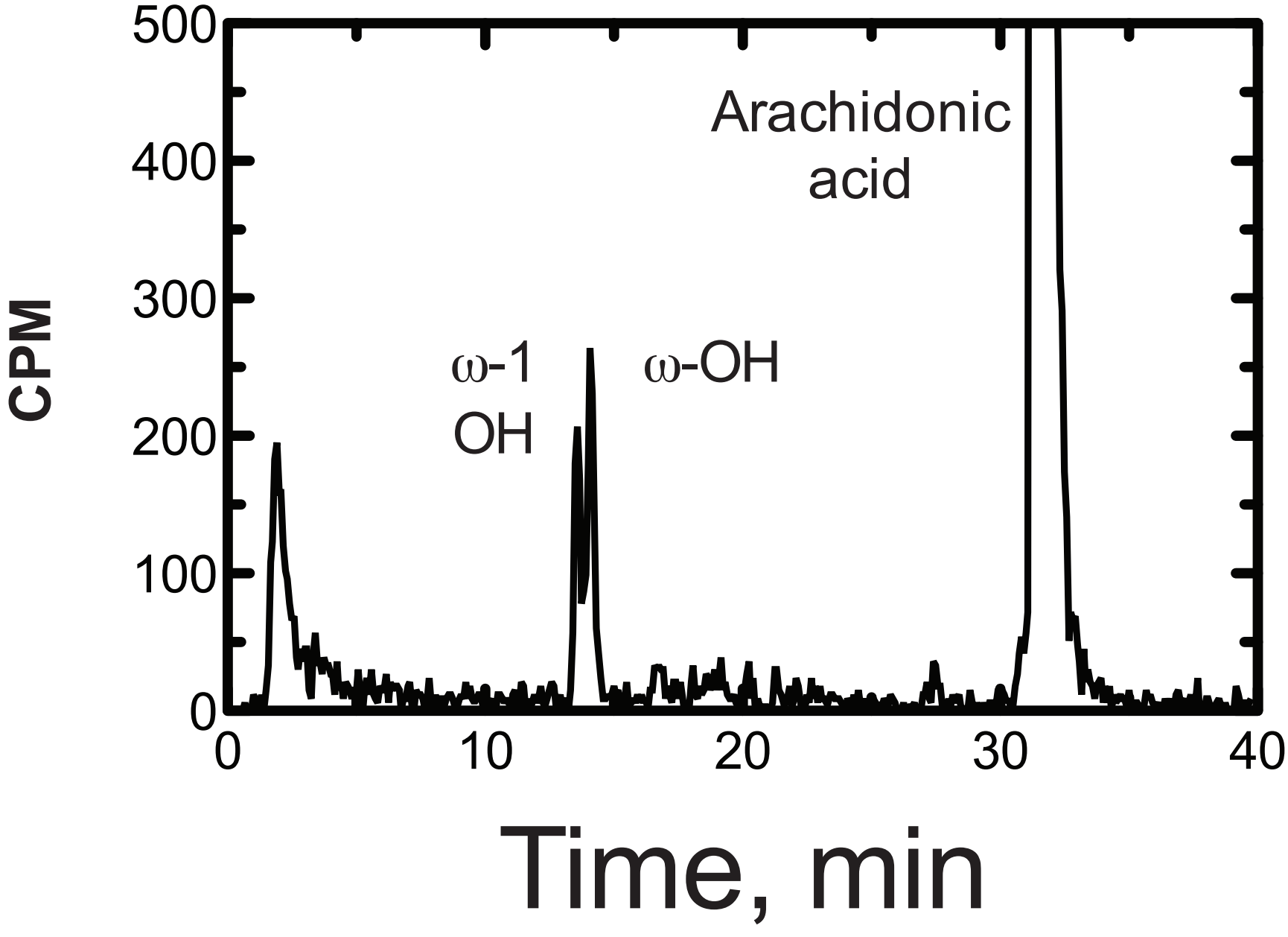

500
400
300
200
100
0
CPM
Arachidonic
acid
ω-1
OH
ω-OH
0
10
20
30
40
Time, min

Fig. 6

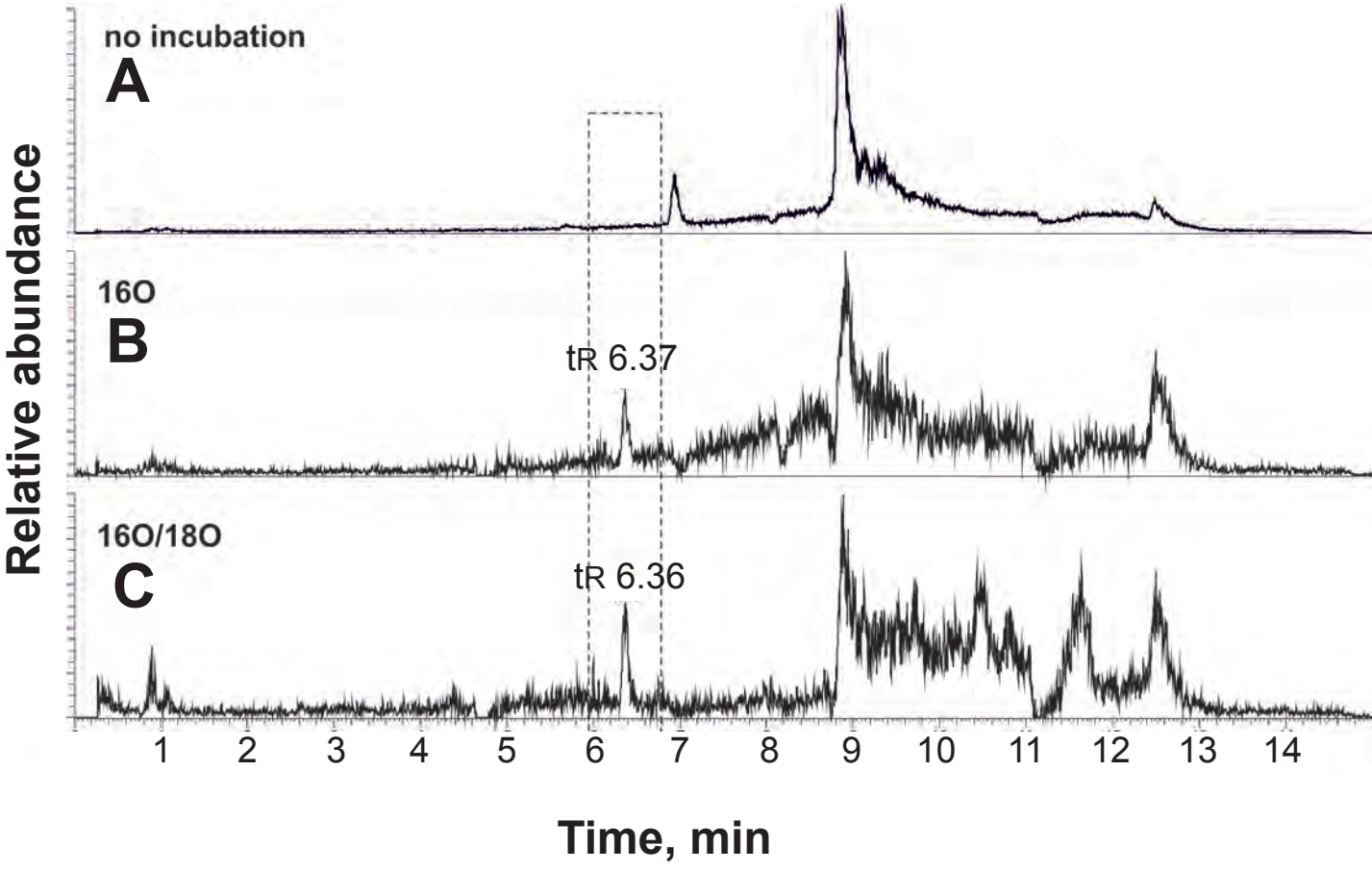

no incubation
A
16O
B
tR 6.37
16O/18O
C
tR 6.36
Relative abundance
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Time, min

Fig. 7

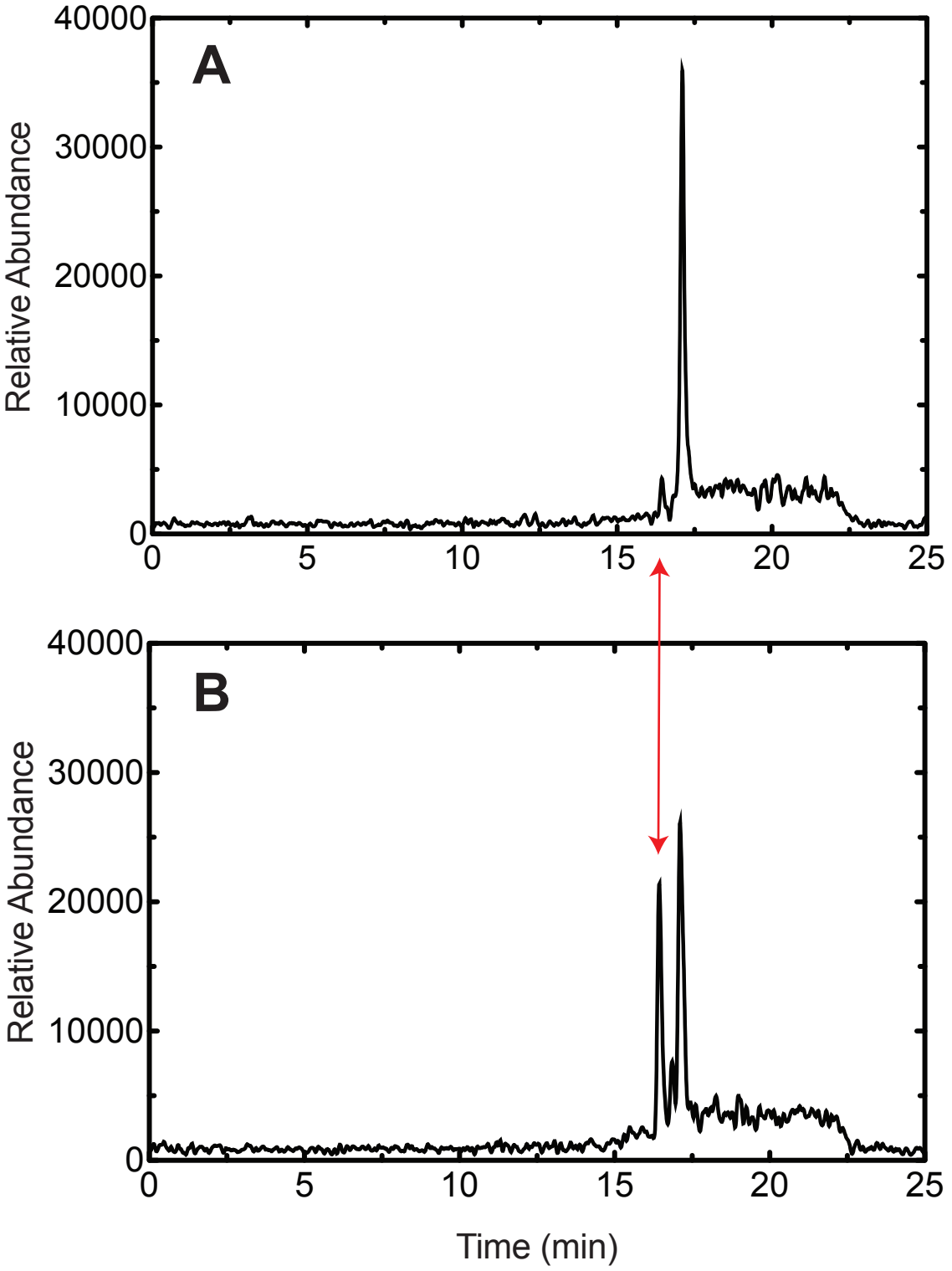

A
B
Relative Abundance
Relative Abundance
40000
30000
20000
10000
0
0
5
10
15
20
25
Time (min)

Fig. 8

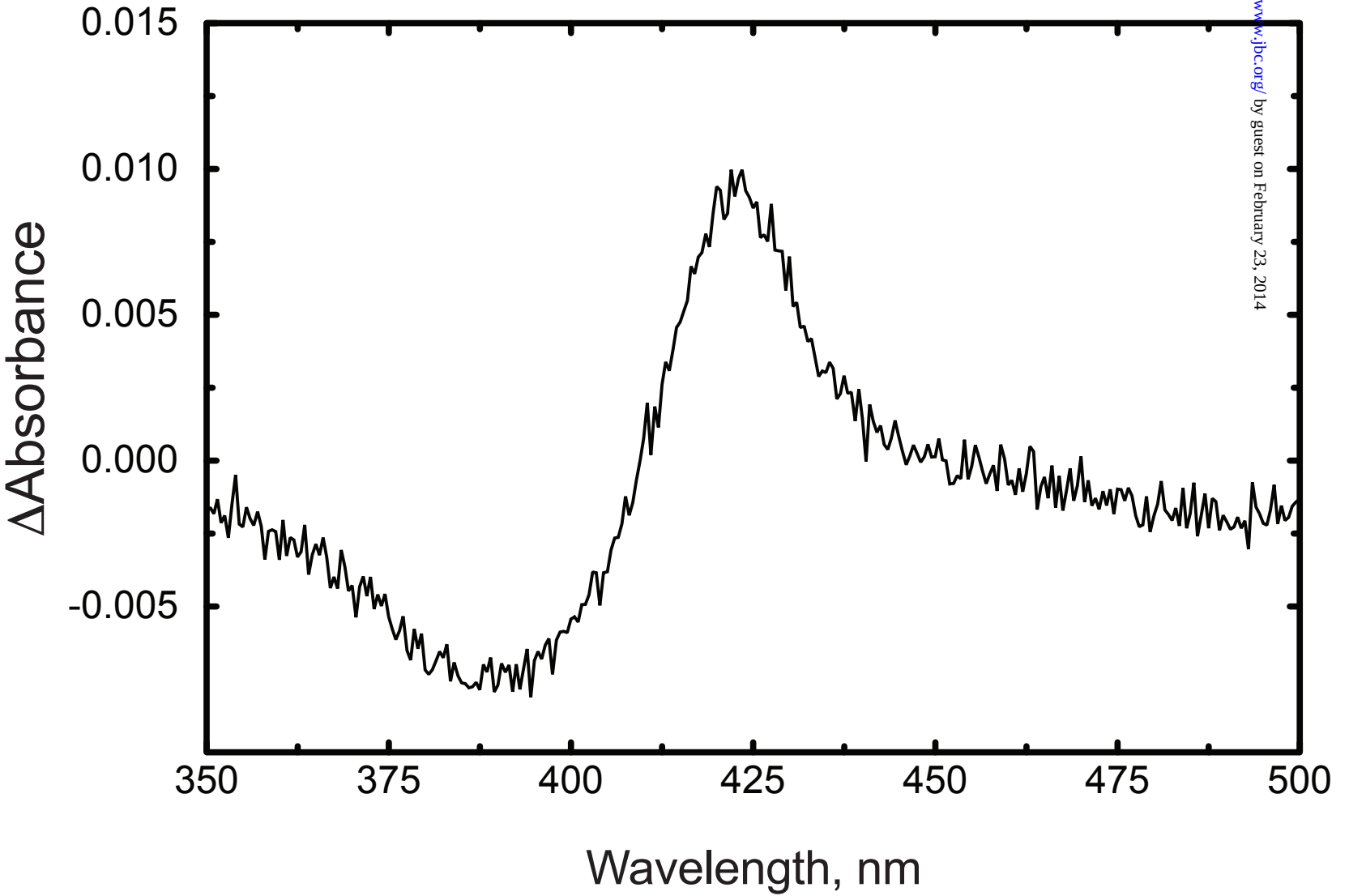


Fig. 9

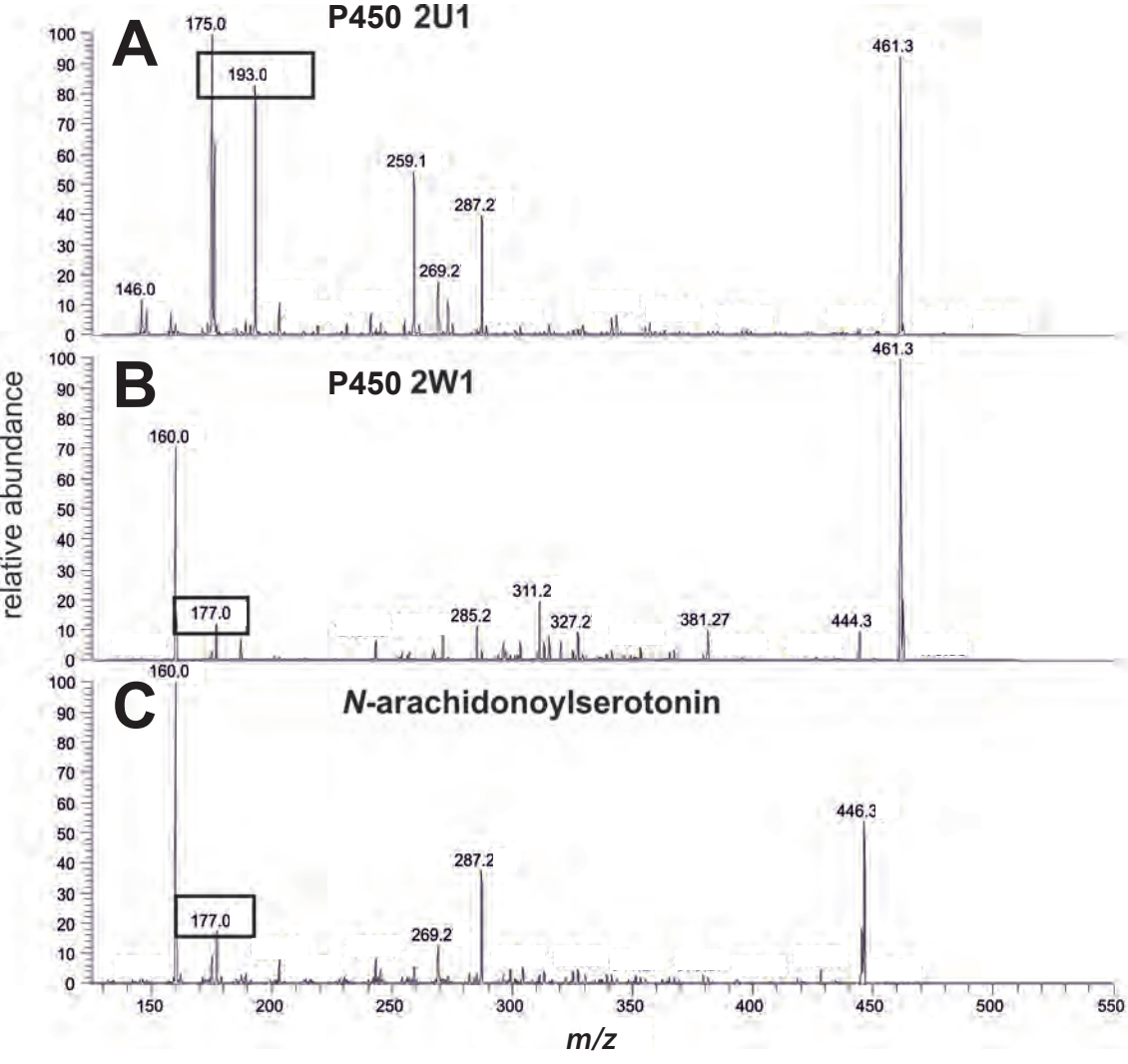
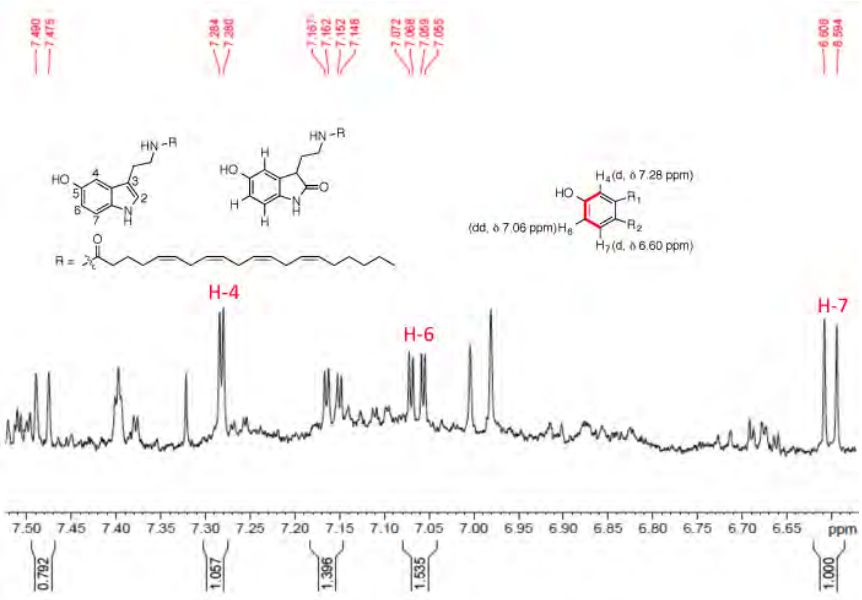

A
P450 2U1
175.0
193.0
461.3
259.1
287.2
269.2
146.0
B
P450 2W1
461.3
160.0
177.0
311.2
285.2
327.2
381.27
444.3
C
N-arachidonoylserotonin
160.0
446.3
287.2
177.0
269.2
relative abundance
m/z
100
90
80
70
60
50
40
30
20
10
0
150
200
250
300
350
400
450
500
550

Fig. 10

A



B

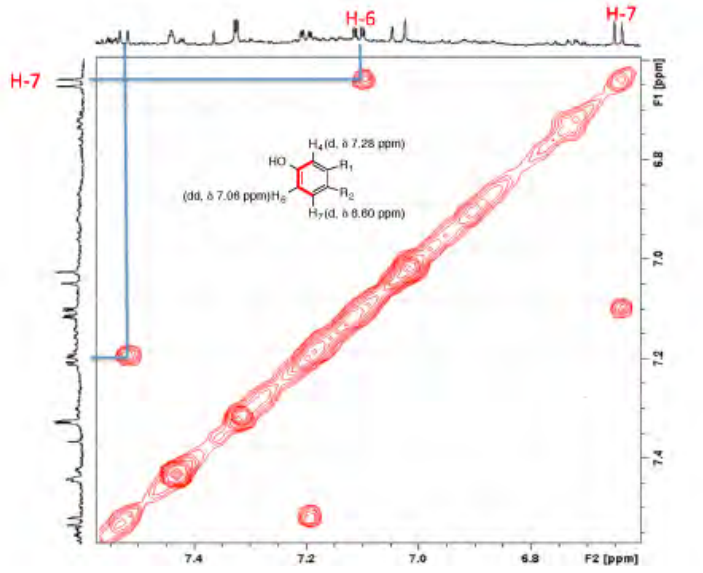


Fig. 11

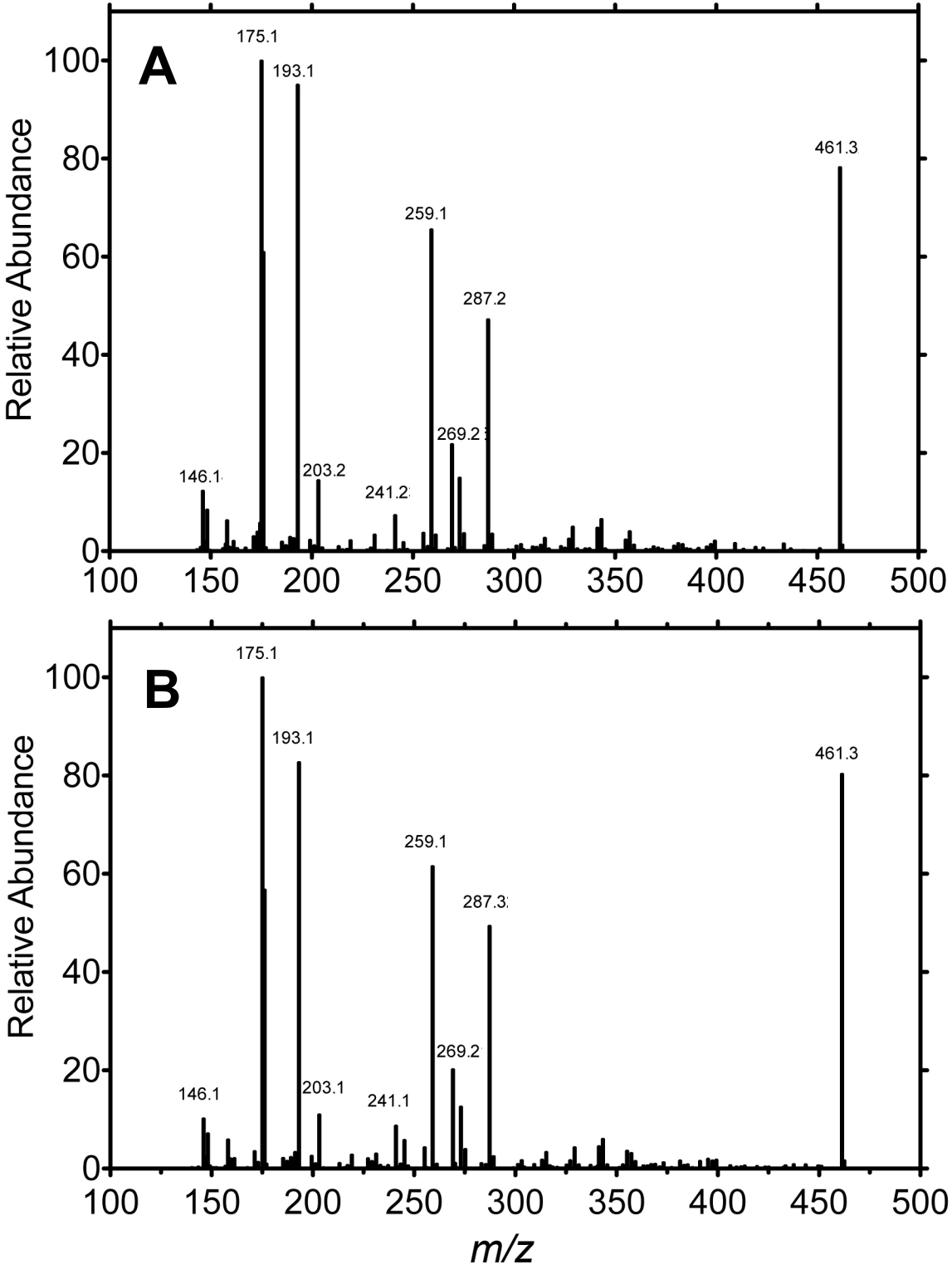

A
175.1
193.1
461.3
259.1
287.2
269.2
146.1
203.2
241.2
B
175.1
193.1
461.3
259.1
287.3
269.2
146.1
203.1
241.1
Relative Abundance
m/z
0
20
40
60
80
100
100
150
200
250
300
350
400
450
500

Fig. 12

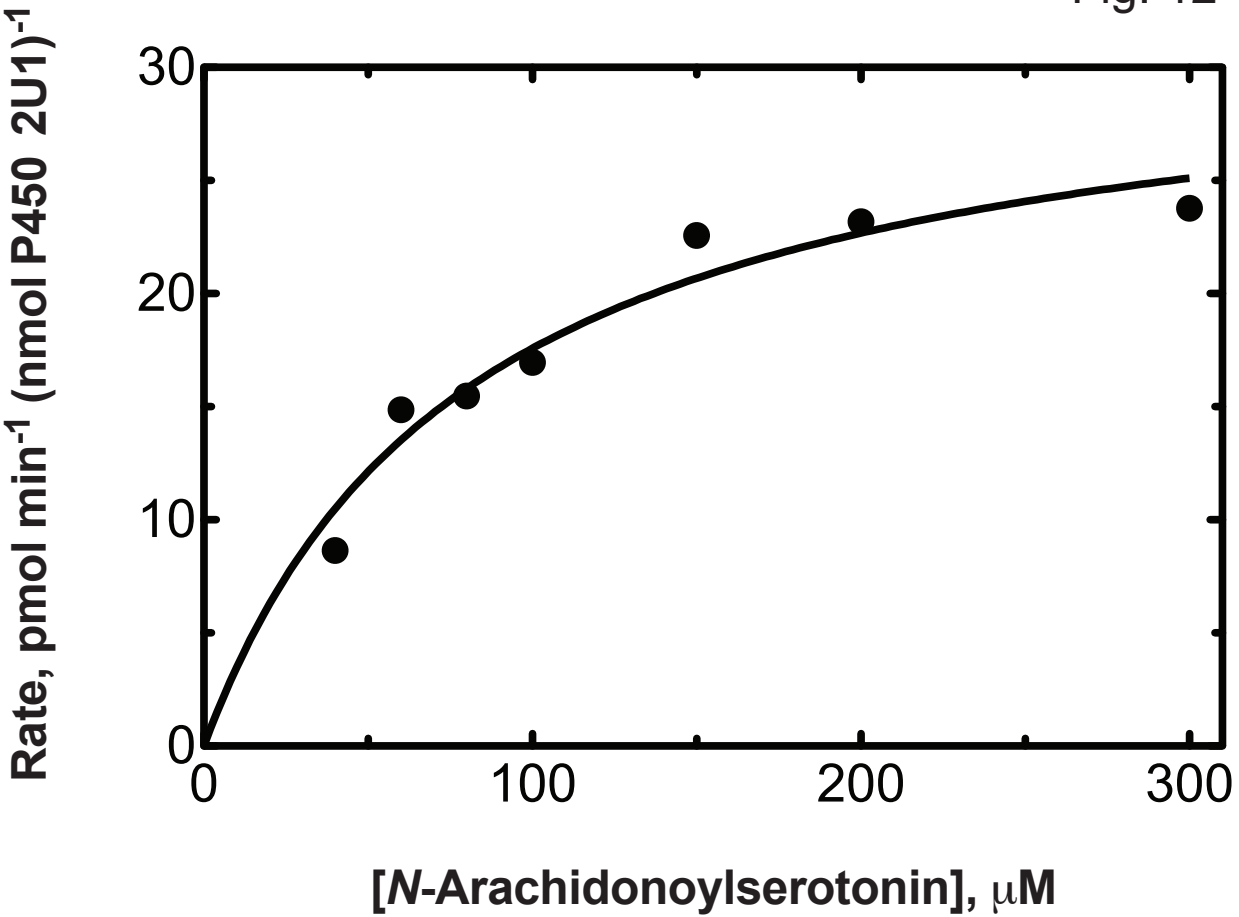
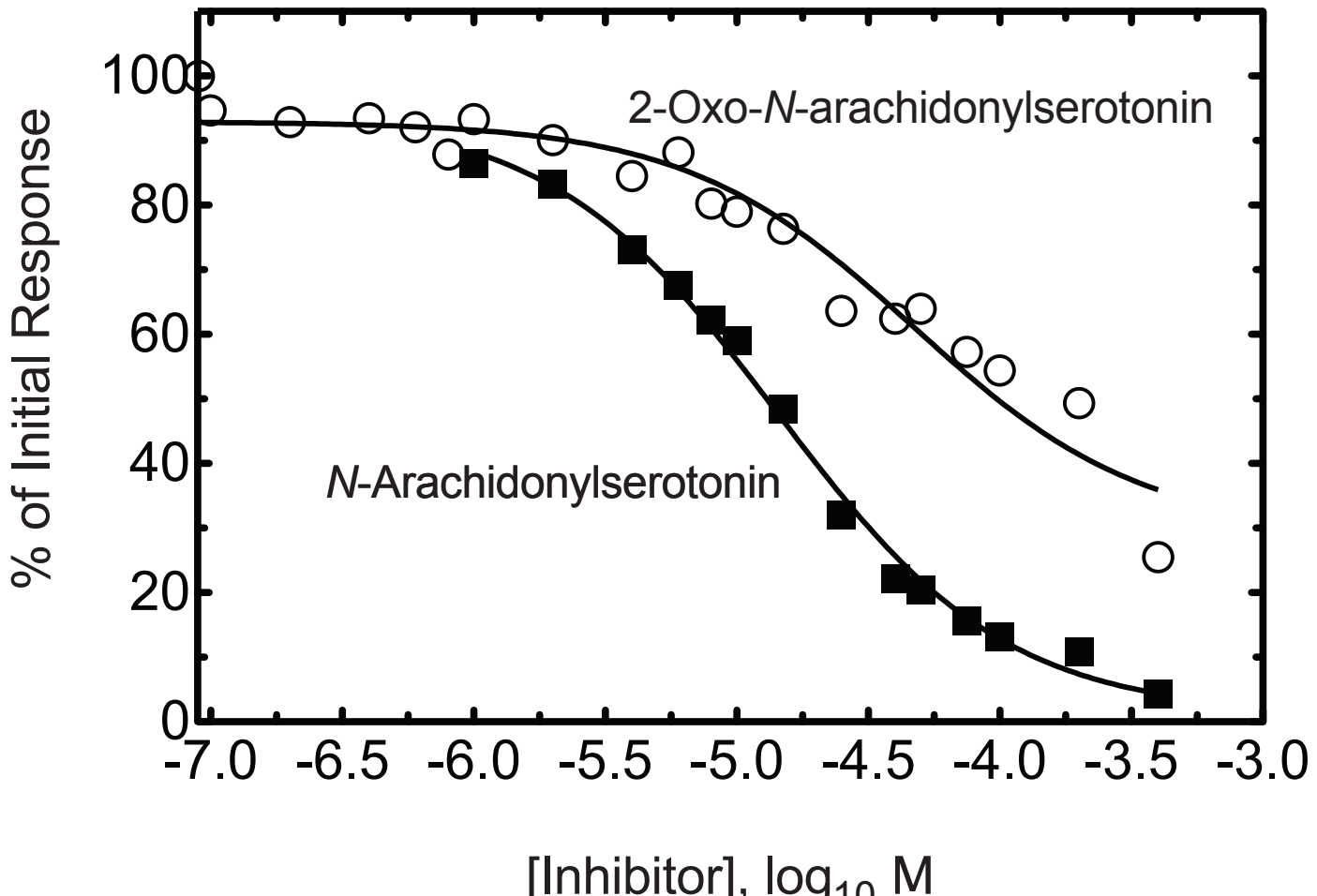

Rate, pmol min-1 (nmol P450 2U1)-1
30
20
10
0
0
100
200
300
[N-Arachidonoylserotonin], μM

Fig. 13


% of Initial Response
100
80
60
40
20
0
2-Oxo-*N*-arachidonylserotonin
N-Arachidonylserotonin
-7.0
-6.5
-6.0
-5.5
-5.0
-4.5
-4.0
-3.5
-3.0
[Inhibitor], log10 M