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Title Page

Characterization of 14,15-Epoxyeicosatrienoyl-Sulfonamides as 14,15-Epoxyeicosatrienoic Acid Agonists: Use for Studies of Metabolism and Ligand Binding

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Running Title Page

Running Title: 14,15-EET-Sulfonamides: Stable 14,15-EET agonists

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Abbreviations: EET, Epoxyeicosatrienoic acids; 14,15-EET-PISA, 14,15-EET-phenyliodosulfonamide; DHET, dihydroxyeicosatrienoic acid; 14,15-EET-BSA, 14,15-EET-biotinsulfonamide; 14,15-EET-BZDC-SA, 14,15-EET-benzoyldihydrocinnamide-sulfonamide; 14,15-EET-mSA, 14,15-EET-methylsulfonamide; 14,15-EEZE-mSA, 14,15-epoxyeicosa-5(Z)-enoyl-methylsulfonamide; CYP, cytochrome P450; BK_{Ca}, large conductance, calcium-activated potassium channel; IBTX, iberiotoxin; sEH, soluble epoxide hydrolase; BCA, bovine coronary artery; HBSS, Hank's balanced salt solution; CDU, 1-cyclohexyl-3-dodecylurea; AUDA, adamantyl dodecanoic acid urea; HPLC, high performance liquid chromatography; MS, mass spectrometry; K_D, equilibrium dissociation constant; B_{max}, maximal binding site density; VCAM-1, vascular cell adhesion molecule-1; GPCR, G-protein coupled receptor.

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Abstract

Epoxyeicosatrienoic acids (EETs) are cytochrome P450 (CYP) epoxygenases metabolites of arachidonic acid. EETs mediate numerous biological functions. In coronary arteries, they regulate vascular tone by the activation of smooth muscle large conductance, calcium-activated potassium (BK_{Ca}) channels to cause hyperpolarization and relaxation. We developed a series of 14,15-EET agonists, 14,15-EET-phenyliodosulfonamide (14,15-EET-PISA), 14,15-EET-biotinsulfonamide (14,15-EET-BSA) and 14,15-EET-benzoyldihydrocinnamide-sulfonamide (14,15-EET-BZDC-SA) as tools to characterize 14,15-EET metabolism and binding. Agonist activities of these analogs were characterized in bovine coronary arterial rings precontracted with U46619. All three analogs induced concentration-dependent relaxation and were equipotent with 14,15-EET. Relaxations to these analogs were inhibited by the BK_{Ca} channel blocker iberiotoxin (IBTX, 100 nM), the 14,15-EET antagonist 14,15-epoxyeicosa-5(Z)-enoyl-methylsulfonamide (14,15-EEZE-mSA, 10 μM) and abolished by 20 mM extracellular K^+. 14,15-EET-PISA is metabolized to 14,15-dihydroxyeicosatrienoyl-PISA (14,15-DHET-PISA) by soluble epoxide hydrolase (sEH) in bovine coronary arteries and U937 cells but not U937 cell membrane fractions. 14,15-EET-P^{125}ISA binding to human U937 cell membranes was time-dependent, concentration-dependent and saturable. The specific binding reached equilibrium by 15 min at 4°C and remained unchanged up to 30 min. The estimated K_D and B_{max} were 148.3 ± 36.4 nM and 3.3 ± 0.5 pmol/mg protein, respectively. These data suggest that 14,15-EET-PISA, 14,15-EET-BSA and 14,15-EET-BZDC-SA are full 14,15-EET agonists. 14,15-EET-P^{125}ISA is a new radiolabeled tool to study EET metabolism and binding. Our results also provide preliminary evidence that EETs exert their biological effect through a membrane binding site/receptor.

EETs are CYP epoxygenases metabolites of arachidonic acid. There are four regioisomers of EET: 5,6-, 8,9-, 11,12- and 14,15-EET (Capdevila et al., 1981; Rosolowsky and Campbell, 1996; Nithipatikom et al., 2001). They play key roles in regulating vascular tone and homeostasis. Previous studies from our lab and other groups have characterized EETs as EDHFs in the coronary circulation (Rosolowsky and Campbell, 1993; Fulton et al., 1995; Campbell et al., 1996; Fisslthaler et al., 1999). Vascular endothelium synthesizes and releases EETs in response to bradykinin, acetylcholine, arachidonic acid, flow or cyclic stretch (Pinto et al., 1987; Campbell et al., 1996; Rosolowsky and Campbell, 1996; Fisslthaler et al., 1999; Gauthier et al., 2005; Huang et al., 2005). EETs then activate smooth muscle membrane large conductance, calcium-activated potassium (BK_{Ca}) channels to cause hyperpolarization and vascular relaxation (Campbell et al., 1996; Gauthier et al., 2005). In addition, EETs are involved in other biological functions including anti-inflammation (Node et al., 1999), antipyresis (Kozak et al., 2000), angiogenesis (Michaelis and Fleming, 2006) and fibrinolysis (Node et al., 2001). Mechanisms involved in EET bioactivities include activation of K^+ channels (Campbell et al., 1996; Yang et al., 2005), G-protein ADP-ribosylation (Li et al., 1999), PKA kinase and tyrosine kinase pathways (Node et al., 2001; Michaelis and Fleming, 2006). To maintain full agonist vascular activity of 14,15-EET in bovine coronary arteries, key structural components including the carbon-1 (C-1) carboxyl group, the 8,9-double bond, the 14(S),15(R)-cis epoxide group and a 14-carbon distance between the carboxyl and the epoxide group are required (Falck et al., 2003a; Gauthier et al., 2004). Similarly, specific structural components are required for 11,12-EET mediated inhibition of VCAM-1 expression (Falck et al., 2003b). Such structure-activity relationships indicate a possible lipid-protein binding event that requires precise conformation of the EET molecules. Numerous studies have indicated that the EET action site may be plasma

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membrane. 11,12-EET activates coronary smooth muscle BK_{Ca} channels in inside-out patches where a small portion of plasma membrane was excised (Li and Campbell, 1997). In aortic smooth muscle cells, a membrane-impermeable 14,15-EET derivative was equipotent as 14,15-EET in inhibiting aromatase activity indicating that the EET acts on the cell surface (Snyder et al., 2002). Wong et al reported protein binding sites in both intact guinea pig monocytes and mononuclear cell membranes by radioligand binding studies using [^{3}H] 14,15-EET (Wong et al., 1993; Wong et al., 2000). [^{3}H] 14,15-EET binding to guinea pig monocytes was sensitive to mild protease treatment, which suggests a protein identity of the 14,15-EET binding site. However, a membrane receptor / binding protein for EETs has not been purified or cloned. Tools to identify the putative EET binding protein/receptor are needed.

EETs are rapidly taken up into cells when applied exogenously (Snyder et al., 2002). Whether this uptake is through simple diffusion or a protein transporter is unclear. EETs are actively metabolized by multiple enzyme pathways. The major pathways are hydrolysis of the epoxide groups by soluble epoxide hydrolase (sEH) to the corresponding vicinal-dihydroxyeicosatrienoic acids (DHETs), β-oxidation that result in chain-shortened products and esterification into membrane phosphoglycerolipids (Spector et al., 2004). These studies were done with [^{14}C] or [^{3}H]-labeled EETs synthesized from radiolabeled arachidonic acid. At present, no commercial company markets radiolabeled EETs. The synthesis of radiolabeled EETs from radiolabeled arachidonic acid is inefficient and costly since 1) yields cannot exceed 50% or diepoxides are formed and 2) chemical methods produce all four EET regioisomers in varying amounts (Corey et al., 1979; Rosolowsky and Campbell, 1993). Thus, there is a need to produce radiolabeled EETs efficiently, cheaply and with high specific activity for studies of EET transport, metabolism and binding.

To serve these purposes, we developed and characterized a series of stable 14,15-EET agonists, 14,15-EET-PISA, 14,15-EET-BSA and 14,15-EET-BZDC-SA (Fig. 2). These analogs contain an N-acylsulfonamide group instead of a terminal carboxylic acid. The N-acylsulfonamide group has a similar pKa as a carboxyl group and obviates β-oxidation and phospholipid esterification (Backes and Ellman, 1994; Snyder et al., 2002). For 14,15-EET-PISA, a benzyl ring that may be iodinated with [^{125}I] or [^{127}I], is attached to the sulfonamide group. For 14,15-EET-BSA and 14,15-EET-BZDC-SA, a biotin or BZDC moiety is covalently attached to the sulfonamide group through a benzyl linker. We characterized 14,15-EET-P^{125}ISA as a radioactive tracer to study EET metabolism and as a radioligand for studying the binding kinetics of the putative EET receptor/binding site. It was used as a model for studying the metabolism of other EET-sulfonamides.

Materials and Methods

Preparation of 14,15-EET-PISA, 14,15-EET-BZDC-SA and 14,15-EET-BSA

Preparation of 14,15-EET-PISA (5) (Fig. 1, Scheme 1) **Synthesis of NHS-Ester 2**: N-Hydroxysuccinimide (NHS) (115 mg, 1 mmol), dried azeotropically using anhydrous toluene and then under high vacuum for 2 h, was added to a solution of 14,15-epoxyeicosatrienoic acid (14,15-EET, **1)** (132 mg, 0.412 mmol) in dry THF (8 ml) at room temperature (RT) under an argon atmosphere followed by N,N'-dicyclohexylcarbodiimide (DCC) (94 mg, 0.453 mmol). After stirring overnight the solvent was removed *in vacuo* and the residue was purified by SiO_2 column chromatography eluting with hexanes/EtOAc (70:30) to give NHS-ester **2** (151 mg, 88%). TLC: R_f ~ 0.30 (50% EtOAc/hexanes); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.92 (t, J = 7.0 Hz, 3H), 1.32-1.60 (m, 8H), 1.82 (quintet, J = 7.3 Hz, 2H), 2.17-2.22 (m, 3H), 2.37-2.45 (m, 1H), 2.62 (t, J = 7.3 Hz, 2H), 2.80-2.88 (m, 8H), 2.92-2.99 (m, 2H), 5.34-5.54 (m, 6H). **Synthesis of**

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Stannane 3: A mixture of 3-bromobenzenesulfonamide (Sigma-Aldrich Co., St. Louis, MO, 1 gm, 4.23 mmol), tetrakis(triphenylphosphine)palladium(0**)** (0.24 g, 0.209 mmol), and bis(tributyditin) (4.3 gm, 7.42 mmol) was stirred at rt in a sealed pressure tube under an argon atmosphere in anhydrous CH_3CN (25 ml) and toluene (5 ml) for 10 min, then warmed to 95°C. After 48 h, the reaction mixture was cooled to rt, concentrated in *vacuo*, and the residue was purified by silica gel column chromatography to yield **3** (870 mg, 51%). TLC: R_f ~ 0.32 (30% EtOAc/hexanes); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88 (t, J = 7.4 Hz, 9H), 1.11 (app t, J = 7.4 Hz, 6H), 1.25-1.36 (m, 6H), 1.42-1.58 (m, 6H), 5.08 (s, 2H), 7.42-7.47 (m, 1H), 7.66 (dd, $J_{H,H}$ = 8.3 Hz, $J_{Sn,H}$ = 34.5 Hz, 1H), 7.85 (dd, J = 1.8, 8.4 Hz, 1H), 8.02 (dd, $J_{H,H}$ = 1.8 Hz, $J_{Sn,H}$ = 34.5 Hz, 1H). **Synthesis of Sulfonimide 4**: A solution of 3-(tri-*n*-butylstannanyl)benzenesulfonamide (**3**) (80 mg, 0.179 mmol) and DMAP (20 mg, 0.157 mmol) in dry HMPA (1.5 ml) was cannulated into a solution of NHS-ester **2** (60 mg, 0.143 mmol) in HMPA (2.5 ml) under an argon atmosphere. After stirring for 10 min at room temperature, the reaction mixture was warmed to 65ºC and allowed to stir for 16 h, then quenched by the addition of water (10 ml). The mixture was extracted with EtOAc (3 × 15 ml) and the combined extracts were washed with water, brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by SiO_2 column chromatography to give stannane **4** (38 mg, 36%). TLC: R_f ~ 0.52 (30% EtOAc/hexanes); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.86-0.93 (m, 12H), 1.11 (app t, J = 7.4 Hz, 6H), 1.26-1.39 (m, 10H), 1.47-1.68 (m, 12H), 2.04 (dd, J = 7.0, 7.0 Hz, 2H), 2.17-2.26 (m, 3H), 2.43-2.51 (m, 1H), 2.69-2.91 (m, 4H), 3.02-3.12 (m, 2H), 5.20-5.61 (m, 6H), 7.40-7.48 (m, 1H), 7.70 (dd, $J_{H,H}$ = 8.4, $J_{Sn,H}$ = 34.5 Hz,1H), 7.96 (dd, J = 1.8, 8.4 Hz,1H), 8.08 (dd, $J_{H,H}$ = 1.8 Hz, $J_{Sn,H}$ = 34.5 Hz, 1H), 8.99 (br s,1H). **Synthesis of Iodide 5**: A solution of sodium iodide (40 mg, 0.027 mmol) in water (1.0 ml) was added to a 0°C solution of stannane **4** (20 mg, 0.027 mmol) in 95% EtOH (4 ml) under

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an argon atmosphere followed by H_2O_2 (30% aq. soln, 24 µL, 0.213 mmol) and peracetic acid (32% in AcOH, 26 µL, 0.213 mmol). The reaction mixture was warmed to rt. After 2 h, the reaction mixture was quenched with sat. aq. sodium thiosulfate (1 ml) and extracted with EtOAc (3 × 15 ml). The combined organic extracts were washed with water, brine, dried over Na_2SO_4, and concentrated *in vacuo*. The residue was purified by silica gel column chromatography to give **5** (9 mg, 58 %). TLC: R_f ~ 0.32 (30% EtOH/hexanes); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.92 (t, J = 7 Hz, 3H), 1.32-1.69 (m, 1H), 2.04 (dd, J = 7.0, 7.0 Hz, 2H), 2.18-2.26 (m, 3H), 2.46-2.54 (m, 1H), 2.70-2.94 (m, 4H), 3.06-3.17 (m, 2H), 5.20-5.60 (m, 6H), 7.27 (dd, J = 8.4, 8.4 Hz, 1H), 7.95 (dd, J = 1.8, 8.4 Hz, 1H), 7.96 (dd, J = 1.8, 8.4 Hz, 1H), 8.05 (dd, J = 1.8, 8.4 Hz, 1H), 8.33 (dd, J = 1.8, 1.8 Hz, 1H), 9.37 (br s, 1H).

Preparation of 14,15-EET-BZDC-SA (8) (Fig. 1, Scheme 2) **Synthesis of Aniline 6**: *n*-BuLi (0.21 ml of a 2.5 M solution in hexanes, 0.539 mmol) was added slowly to a -78ºC solution of 4-aminobenzenesulfonamide (93 mg, 0.539 mmol) in anhydrous THF (8 ml) and HMPA (2 ml) under an argon atmosphere. After 5 min, a solution of NHS-ester **2** (150 mg, 0.359 mmol) in THF (4 ml) was added at -78ºC and then the mixture was slowly warmed to room temperature. After stirring overnight, the reaction mixture was quenched with saturated aq. NH_4Cl (5 ml) and extracted with EtOAc (3 × 20 ml). The combined organic extracts were washed with water, brine, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by SiO_2 column chromatography to give **6** (120 mg, 71%). TLC: R_f ~ 0.34 (50% EtOAc/hexanes); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.92 (t, J = 7.0 Hz, 3H), 1.32-1.60 (m, 8H), 1.63 (quintet, J = 7.3 Hz, 2H), 2.03 (dd, J = 7.3, 7.3 Hz, 2H), 2.18-2.26 (m, 2H), 2.42-2.49 (m, 1H), 2.71-2.89 (m, 4H), 3.01-3.07 (m, 2H), 4.31 (br s, 2H), 5.25-5.54 (m, 6H), 6.65 (d, J = 8.6 Hz, 2H), 7.80 (d, J = 8.6 Hz, 2H), 9.01 (br s, 1H). **Synthesis of BZDC 8**: A solution of 4-(benzoyl)benzenepropanoic acid

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NHS ester (Kort et al., 2000) (BZDC-NHS) (**7**) (22.3 mg, 0.063 mmol) and Et_3N (13 µL, 0.126 mmol) in DMF (2 ml) was added dropwise to a solution of **6** (20 mg, 0.042 mmol) in anhydrous DMF (2 ml) followed by DMAP (7.7 mg, 0.063 mmol) under an argon atmosphere. After 48 h, the reaction mixture was quenched with water (3 ml), extracted with EtOAc (3 × 15 ml), and the combined organic extracts were washed with water, brine, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by silica gel column chromatography to yield **8** (13 mg, 30%). TLC: R_f ~ 0.42 (20% EtOAc/CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.90 (t, J = 7.0 Hz, 3H), 1.31-1.62 (m, 8H), 1.63 (quintet, J = 7.3 Hz, 2H), 2.41-2.53 (m, 1H), 2.71-2.92 (m, 6H), 3.02-3.20 (m, 4H), 5.20-5.60 (m, 6H), 7.34 (d, J = 8.2 Hz, 2H), 7.48 (t, J = 7.6 Hz, 2H), 7.56-7.68 (m, 3H), 7.73 (d, J = 7.9 Hz, 2H), 7.78 (d, J = 7.9 Hz, 2H), 7.88 (d, J = 8.8 Hz, 2H), 8.21 (br s, 1H), 9.35 (br s, 1H).

Preparation of 14,15-EET-BSA (10) (Fig. 1, Scheme 3) **Synthesis of Acid 9**: 4-Carboxybenzenesulfonamide (Aldrich, 130 mg, 0.358 mmol), dried azeotropically with anhydrous benzene and then dried under high vacuum for 2 h, was dissolved in anhydrous THF (6 ml) and HMPA (1.5 ml) and cooled to -78°C under an argon atmosphere. To this was added *n*-BuLi (0.57 ml of a 2.5 M solution in hexanes, 0.862 mmol) and after 10 min a solution of **2** (180 mg, 0.431 mmol) in THF (4 ml) was slowly added. The reaction mixture was then slowly warmed to room temperature. After stirring overnight, the reaction mixture was quenched with saturated aq. NH_4Cl solution (15 ml), extracted with EtOAc (3 × 30 ml), and the combined organic extracts were washed with water, brine, dried over Na_2SO_4 and concentrated *in vacuo*. The residue was purified by silica gel column chromatography to give acid **9** (85 mg, 71%). TLC: R_f ~ 0.23 (50% EtOAc/hexanes); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.91 (t, J = 7.0 Hz, 3H), 1.34-1.60 (m, 8H), 2.02-2.12 (m, 2H), 2.26 (t, J = 8.5 Hz, 2H), 2.42-2.49 (m, 1H), 2.71-2.89 (m,

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4H), 3.07-3.16 (m, 2H), 5.23-5.57 (m, 6H), 8.14 (d, J = 8.4 Hz, 2H), 8.23 (d, J = 8.6 Hz, 2H).

Synthesis of Biotin 10: To a -78°C solution of **9** (50 mg, 0.099 mmol) in dry CH_2Cl_2 (3 ml) under an argon atmosphere were added 1-methylpiperdine (Aldrich, 12 µL, 0.099 mmol) followed by isobutyl chloroformate (Aldrich, 13 µL, 0.099 mmol). After stirring for 45 minutes at the same temperature, the reaction mixture was warmed to -25°C, stirred for another 30 min, and then a solution of biotin hydrazide (Sigma-Aldrich Co., 100 mg, 0.39 mmol) in DMF (1 ml, prewarmed to ~80°C to dissolve) was added. The reaction mixture was slowly warmed to RT over 1 h. After stirring at rt overnight, the reaction mixture was diluted with CH_2Cl_2 (10 ml), filtered, washed with water, and concentrated *in vacuo*. The residue was purified by SiO_2 PTLC to give **10** (15 mg, 38%) and recovered starting material (30 mg). TLC: R_f ~ 0.45 (10% MeOH/CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.90 (t, J = 6.7 Hz, 3H), 1.28-1.42 (m, 6H), 1.43-1.84 (m, 10H), 1.99-2.10 (m, 2H), 2.25 (apparent t, J = 7.0 Hz, 2H), 2.28-2.33 (m, 2H), 2.36 (apparent t, J = 6.8 Hz, 2H), 2.68-2.78 (m, 4H), 2.84 (apparent t, J = 5.8 Hz, 2H), 2.91-3.01 (m, 2H), 3.18-3.28 (m, 1H), 3.32 (br s, 1H, D_2O exchangeable), 3.90 (d, J = 6.4 Hz, 1H, D_2O exchangeable), 4.30-4.65 (m, 1H), 4.48-4.54 (m, 1H), 5.26-5.40 (m, 4H), 5.42-5.60 (m, 2H), 8.04 (d, J = 8.5 Hz, 2H), 8.10 (d, J = 8.5 Hz, 2H).

Synthesis of 14,15-EET-P^{125}ISA and 14,15-DHET- P^{125}ISA

To synthesize 14,15-EET-P^{125}ISA, carrier-free Na^{125}I (2 µl, pH 9-10, 200 µCi, 17.4 Ci/mg) (GE Healthcare, Arlington Heights, IL) was mixed with NaI (1 µl, 1.08 mg in 10 ml H_2O). 14,15-EET-stannane (**4**) (10 µg in 43 µl ethanol) was added to the NaI solution followed by H_2O_2 (10.67 nmol in 1.25 µl H_2O) and peracetic acid (2.69 nmol in 1.68 µl acetic acid) at 4°C (Fig.1, Scheme 1). The reaction mixture was warmed to room temperature and allowed to react for 45 min. On quenching with saturated aqueous $Na_2S_2O_3$ (250 µl), the aqueous layer was

extracted with ethyl acetate (2 x 300 µl). The combined organic extracts were washed with H_2O (300 µl) and 0.9% NaCl (300 µl), and dried under N_2. The residues were purified by reverse-phase high performance liquid chromatography (HPLC) as previously described (Campbell et al., 1996). The extracts were redissolved in 100 µl acetonitrile/glacial acetic acid (999:1) and 100 µl distilled H_2O and resolved on a Nucleosil C18 reverse-phase column (5 µm, 4.6 x 250 mm, Phenomenex, Torrance, CA). Solvent A was distilled H_2O, and solvent B was acetonitrile/glacial acetic acid (999:1). A linear gradient from 50% solvent B in solvent A to 94% solvent B within 40 min was used at a flow rate of 1 ml/min. Column effluent corresponding to synthetic 14,15-EET-PISA was collected, extracted and dried under N_2.

To synthesize 14,15-DHET-P^{125}ISA, 14,15-EET-P^{125}ISA was dissolved in HEPES buffer containing (in mM) 10 HEPES, 150 NaCl, 5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5.5 glucose and pH 7.4. Recombinant human sEH (35 µg) was added and incubated for 30 min at 37°C. The residues were extracted with ethyl acetate and purified by HPLC as described above. Specific activities of 14,15-EET-P^{125}ISA and 14,15-DHET-P^{125}ISA were 257.5 Ci/mmol.

Vascular reactivity of bovine coronary arteries. Bovine hearts were purchased from a local slaughterhouse. The left anterior descending coronary artery was dissected and cleaned of connective tissue. Arteries of 2 mm diameter were cut into rings (3 mm width) and suspended on a pair of stainless hooks in a 6-ml water-jacketed organ chamber in Kreb's buffer consisting of (in mM) 119 NaCl, 4.8 KCl, 24 $NaHCO_3$, 0.2 KH_2PO_4, 0.2 $MgSO_4$, 11 glucose, 0.02 EDTA, and 3.2 $CaCl_2$. The buffer was equilibrated with 95% O_2-5%CO_2 and maintained at 37°C. Tension was continuously recorded as previously described (Campbell et al., 1996). Briefly, submaximal concentrations of the thromboxane-mimetic U46619 (10-20 nM, Caymen Chemical Co. Ann Arbor, MI) were administered to contract the vessels to 50-75% of KCl-induced contraction.

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Cumulative concentrations of 14,15-EET, 14,15-EET-PISA, 14,15-EET-BSA or 14,15-EET-BZDC-SA were added to the chamber. In some studies, the arteries were incubated with iberiotoxin (IBTX, 100 nM, Sigma-Aldrich Co.), 14,15-epoxyeicosa-5(Z)-enoyl-methylsulfonamide (14,15-EEZE-mSA, 10 μM) or vehicle for 10 min before U46619 contraction (Gauthier et al., 2003). In the high extracellular potassium ($[K^+]_o$) studies, K^+ was increased to 20 mM by substitution of Na^+. Results are expressed as percent relaxation with 100% relaxation representing basal tension.

Culture of U937 cells and membrane preparation. U937 cells were cultured in suspension in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT), 25 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. The cultures were maintained at a density of 5-10 $x10^5$ cells/ml at 37°C in a humidified atmosphere of 5% CO_2 in air. Membrane extraction was modified from previously reported protocol (Wong et al., 1993). Cells were collected by centrifugation at 1000 xg for 2 min and washed twice with Hank's balanced salt solution (HBSS) containing 5 mM $CaCl_2$, 5mM $MgCl_2$ and protease inhibitor cocktail (Roche, Germany). The pellet was resuspended with HBSS (without $CaCl_2$ or $MgCl_2$) containing protease inhibitors and sonicated for 20 sec bursts on ice 5 times at setting 4. The homogenate was centrifuged at 1000 x g for 10 min at 4°C to remove unbroken cells. The supernatant was then centrifuged at 150,000 x g for 45 min at 4°C. The pellet represents the membrane fraction and was resuspended in binding buffer consisting of (in mM) 10 HEPES, 5 $CaCl_2$, 5 $MgCl_2$, 5 EDTA and pH 7.4.

Bovine coronary artery homogenate preparation. Bovine coronary arteries (BCAs) were dissected as described above, cut into small pieces and homogenized with a glass

homogenizer in binding buffer containing miconazole (10 μM, Sigma-Aldrich Co.) and protease inhibitor cocktail (Roche) at 4°C. Unbroken tissue and cell debris were removed by centrifugation at 6000 x g for 5 min at 4°C. The supernatant represents the BCA homogenate which contains membrane and cytosolic fractions.

Tissue incubation of 14,15-EET-P^{125}ISA. 14,15-EET-P^{125}ISA was incubated and extracted using following protocols. 1). 14,15-EET-P^{125}ISA (30-33 nM) was incubated with 500 μg BCA homogenate in binding buffer for 1 h at 30°C. The metabolites were extracted by ethyl acetate. In some incubations, 1 μM 1-cyclohexyl-3-dodecylurea (CDU), a sEH inhibitor (Morisseau et al., 2002), was included. 2). 14,15-EET-PISA (10 μM) with tracer amounts of 14,15-EET-P^{125}ISA was incubated with BCA rings (1 g) in Kreb's buffer bubbled with 95% O_2-5%CO_2 for 15 min at 37°C. The metabolites were harvested by solid phase extraction using BondElut C_{18} octadecylsilica column (Varian Inc., Harbor City, CA) as previously described (Nithipatikom et al., 2001). 3). 14,15-EET-P^{125}ISA (35 nM) was incubated with U937 cells (800,000 cells) in 200 μl HBSS for 15 min at 37 °C. The metabolites were extracted by liquid-liquid extraction using 500 μl chloroform/methanol (1:2) followed by addition of 167 μl chloroform and 150 μl H_2O. The organic phase was collected and dried under N_2. 4). 14,15-EET-P^{125}ISA (1 nM) was incubated with 50 μg U937 cell membrane proteins in 200 μl binding buffer for 15 min at 4°C. In some studies, incubations were performed with buffer alone or boiled membranes. The metabolites were extracted by the same liquid-liquid extraction protocol as for intact U937 cell incubations. The extracts were then analyzed by reverse-phase HPLC as described above. The column effluent was collected in 0.5 ml fractions and radioactivity was measured by a Packard Cobra-II auto-gamma counter.

Mass spectrometry (MS) analysis. To identify the 14,15-EET-PISA metabolite, 14,15-

EET-PISA was incubated with BCA homogenate and extracted as described above. The HPLC effluent (21 min) containing the 14,15-EET-PISA metabolite was collected, extracted with cyclohexane/ethyl acetate (50:50) and further analyzed by MS. The MS analysis used an Agilent 1100 MSD mass spectrometer with electrospray ionization. The residues were introduced into the electrospray chamber by flow injection. Solvent A was water and solvent B was acetonitrile with both solvents containing 0.005% glacial acetic acid. 50% of each solvent was used at a flow rate of 0.2 ml/min and data were collected for 3 min. Detection was in the negative ion mode. Results are expressed as relative abundance with the most abundant ion set as 100%.

Radioligand binding. U937 membranes (50 μg) were incubated with 14,15-EET-P^{125}ISA in the presence of 20 μM 14,15-EEZE-mSA (non-specific binding) or equivalent amount of ethanol (total binding) at 4°C with shaking. For the time course study, 1 nM 14,15-EET-P^{125}ISA was incubated at various times (1-30 min). For the concentration-dependent study, increasing concentrations of 14,15-EET-P^{125}ISA (0.1-150 nM) were incubated for 15 min. Incubations (total volume 200 μl) were performed in binding buffer and were terminated by filtration through GF/A glass filter paper on a Brandel harvester (Brandel Inc., Gaithersburg, MD). Filters were washed four times with 5 ml of ice cold binding buffer and counted by a Packard Cobra-II auto-gamma counter. Specific binding was defined as total binding minus non-specific binding. The K_D (equilibrium dissociation constant) and B_{max} (maximal binding site density) values are determined from saturation studies using non-linear regression to fit the data to the single-site binding equation using Prism software (GraphPad Inc., San Diego, LA).

Statistical Analysis. Data are expressed as means ± SEM. Statistical evaluation was performed by a one-way analysis of variance followed by the Student-Newman-Keuls multiple

comparison test when significant differences were present. $P < 0.05$ was considered statistically significant.

Results

Previously, we characterized 14,15-EET-methylsulfonamide (14,15-EET-mSA, structure shown in figure 2) as a full agonist of 14,15-EET (Falck et al., 2003a). The N-acylmethylsulfonamide group has a similar pKa as the carboxyl group (Backes and Ellman, 1994) and is a commonly used substitute for a carboxyl group (Imig et al., 2001; Snyder et al., 2002). Based on this knowledge, we developed 14,15-EET-PISA, 14,15-EET-BSA and 14,15-EET-BZDC-SA, which contain an iodine, biotin and BZDC group, respectively, attaching to the sulfonamide group through a benzyl linker (Fig. 2).

In bovine coronary arteries precontracted with U46619, 14,15-EET causes concentration-dependent relaxation with an EC_{50} of 1 μM and a maximum relaxation averaging 80-94% at 10 μM. Similarly, 14,15-EET-PISA, 14,15-EET-BSA, 14,15-EET-BZDC-SA relaxed these arteries with an EC_{50} of 1 μM and maximal relaxation of 84.5 ± 7.5 %, 89.6 ± 3.9 % and 92.9 ± 5.0% at 10 μM, respectively (Fig. 3A). We tested the effect of K^+ channel inhibition on these analogs mediated relaxations. Increasing extracellular K^+ from 4 to 20 mM eliminated relaxations to 14,15-EET-PISA, 14,15-EET-BSA and 14,15-EET-BZDC-SA (maximum relaxation = 7.8 ± 12.8 %, 8.6 ± 6.3% and 4.5 ± 3.2%, respectively, Fig. 3B-D). BK_{Ca} channel blocker, IBTX (100 nM) markedly attenuated the 14,15-EET-PISA, 14,15-EET-BSA and 14,15-EET-BZDC-SA-induced relaxations (maximum relaxation = 39.3 ± 12.8%, 20.8 ± 2.6% and 17.3 ± 6.1 and respectively, Fig. 3B-D). We next examined the effect of 14,15-EEZE-mSA, a 14,15-EET antagonist (Gauthier et al., 2003), on these anlogs mediated relaxation. Pretreatment with 14,15-EEZE-mSA (10 μM) inhibited 14,15-EET-PISA, 14,15-EET-BSA and 14,15-EET-BZDC-SA

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relaxations (maximum relaxation = 23.5 ± 9.1%, 21.4 ± 13.1% and 34.7 ± 8.6%, Fig. 3B-D). These results demonstrate that 14,15-EET-PISA, 14,15-EET-BSA and 14,15-EET-BZDC-SA caused vascular relaxation by mechanisms similar to 14,15-EET, i.e. they cause relaxation by activating BK_{Ca} channels and the relaxations are inhibited by the 14,15-EET antagonist.

14,15-EET is metabolized through three major pathways including β-oxidation, epoxide hydration and phospholipid esterification. 14,15-EET-mSA, the parent compound of 14,15-EET-PISA, does not undergo β-oxidation or incorporation into membrane lipids (Snyder et al., 2002). It is reasonable to predict that 14,15-EET-mSA derived analogs are likewise not metabolized by these pathways. However, the 14,15-epoxide group is susceptible to hydration. We used 14,15-EET-P^{125}ISA as a radioactive tracer to study its metabolism in bovine coronary artery (BCA) homogenates. 14,15-EET-P^{125}ISA was incubated with buffer or BCA homogenate in the presence and absence of 1 μM CDU for 1 h at 30°C. The metabolites were extracted and resolved on reverse-phase HPLC. With buffer, 94.8% of radioactivity was extracted into the organic phase. The HPLC chromatogram showed one single peak at 29 min (peak 29), which comigrates with the synthetic 14,15-EET-PISA standard (Fig. 4A). After 30 min incubation with BCA homogenate at 30°C, an additional more polar metabolite migrating at 21 min (peak 21) was produced. The radioactivity ratio of peak 21 to peak 29 was 1.27 (Fig. 4B). In the presence of 1 μM CDU, a sEH inhibitor, the production of peak 21 was inhibited. The peak 21/peak 29 ratio was reduced to 0.22 (Fig. 4C). Similar metabolism was observed with 90 min incubation and similar inhibition was obtained using another sEH inhibitor, adamantyl dodecanoic acid urea (AUDA, 1 μM) (data not shown). This suggests that peak 21 is likely the sEH metabolite of 14,15-EET-PISA, 14,15-DHET-PISA. To identify this metabolite, peak 21 was collected, reextracted and analyzed by negative electrospray ionization MS. The most abundant ion in the

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mass spectrum of this peak was at m/z 602.1 (M-H) (Fig. 4E). This indicated a molecular weight of 603 which is consistent with the molecular weight of 14,15-DHET-PISA. Thus, peak 21 was identified as 14,15-DHET-PISA. We then tested 14,15-EET-P^{125}ISA metabolism by bovine coronary arterial rings using conditions under which the vascular activities were tested. After a 15 min incubation at 37°C, the majority of radioactivity eluted at 29 min and a small portion of radioactivity was converted into peak 21. The peak 21/peak 29 ratio was 0.18 (Fig. 4D). These data indicate that 14,15-EET-PISA is hydrolyzed to 14,15-DHET-PISA by sEH in both BCA homogenate and arterial rings. The arterial rings showed less conversion. There was no evidence of the metabolism of 14,15-EET-PISA by β-oxidation.

Similar results were obtained when 14,15-EET-P^{125}ISA was incubated with U937 cells for 15 min at 37°C (Fig. 5A). The major metabolite eluted at 29 min and a small radioactive product eluted at 21 min. Only the 29 min radioactive metabolite was observed when the cells were incubated at 4°C (data not shown). Thus, U937 cells metabolized 14,15-EET-PISA to 14,15-DHET-PISA as did coronary arteries. Next, we investigated the stability of 14,15-EET-P^{125}ISA under the experimental conditions of radioligand binding. After incubation with U937 membranes at 4°C for 15 min, the majority of the radioactivity was extracted with an organic solvent and the extraction efficiency was 93.6 ± 1.0% (n=3). When 14,15-EET-P^{125}ISA was incubated with boiled and control U937 membranes, the percentage of radioactivity in the organic phase were 95.8 ± 0.1% and 95.6 ± 0.2% respectively (n=3). The HPLC chromatograms showed a single peak eluting at 29 min (Fig. 5B, C & D). These results indicate that 14,15-EET-P^{125}ISA is metabolically stable under the conditions for radioligand binding.

To determine whether 14,15-EET-P^{125}ISA is a radioligand useful for the characterization of 14,15-EET binding, we used membrane fractions from U937 cells, a cell line that was

reported to contain an EET binding site (Wong et al., 1997). To test whether 14,15-EET-P^{125}ISA binds to U937 membrane in a time-dependent manner, 1 nM 14,15-EET-P^{125}ISA was incubated with U937 membranes (50 μg protein) at 4°C for 1-30 min. The binding of 14,15-EET-P^{125}ISA to U937 membranes was rapid and specific. Equilibrium was reached in 15 min and remained stable until 30 min (Fig. 6A). Then, we tested the saturability of the specific 14,15-EET-P^{125}ISA binding. Various concentrations of 14,15-EET-P^{125}ISA (0.1 -150 nM) were incubated with U937 membrane (50 μg protein) at 4°C for 15 min. 14,15-EET-P^{125}ISA specific binding was concentration-dependent and reached an apparent plateau at 100 nM (Fig. 6B). The data were fit to a one-site saturation model, and the predicted K_D and B_{max} were 148.3 ± 36.4 nM and 3.3 ± 0.5 pmol/mg protein, respectively. To confirm the specificity of this binding site to 14,15-EET-PISA, the binding of 14,15-DHET- P^{125}ISA to U937 membranes was determined. Specific binding of 14,15-DHET-P^{125}ISA to U937 membranes was very low with a maximum binding of 0.3 pmol/mg protein (Fig. 6C). The data poorly fit with saturation models. This suggests that 14,15-DHET-PISA does not specifically bind to U937 membranes. Therefore, the 14,15-epoxide group is required for 14,15-EET-P^{125}ISA binding to U937 membranes. Taken together, binding of 14,15-EET-P^{125}ISA to U937 membranes is specific, time-dependent, concentration-dependent and saturable.

Discussion

Our present study developed and characterized a series of stable 14,15-EET-mSA derivatives, 14,15-EET-PISA, 14,15-EET-BSA and 14,15-EET-BZDC-SA, as tools to study EET transport, metabolism and binding. The analogs caused concentration-dependent relaxation of bovine coronary arteries and retained full agonist potency and activity as compared to 14,15-EET. Attachment of a bulky group at the C1 of 14,15-EET did not alter its vascular activity. The

relaxations to the analogs were blocked by inhibition of K^+ efflux by 20 mM extracellular K^+ and BK_{Ca} channel blockade by iberiotoxin. These data suggest that these agonists, like 14,15-EET, mediate vascular relaxation by activation of BK_{Ca} channels and membrane hyperpolarization. 14,15-EEZE-mSA, a known 14,15-EET antagonist, inhibited the relaxations (Gauthier et al., 2003). It is likely that these agonists bind to the same binding site or receptor as 14,15-EET and act through the similar cellular mechanisms as 14,15-EET.

EET-mediated activation of vascular smooth muscle BK_{Ca} channels require active Gsα protein and may involve ADP-ribosylation of Gsα (Li and Campbell, 1997; Li et al., 1999). Similarly, Gsα is required for 11,12-EET-mediated activation of BK_{Ca} channels expressed in human embryonic kidney 293 cells (Fukao et al., 2001). Since G proteins characteristically couple to membrane receptors, it is likely that EETs initiate their signaling events by binding to a G protein coupled receptor (GPCR). Node et al showed that 11,12-EET stimulated GTP-binding activity of Gsα in human saphenous veins endothelial cells and activated endothelial t-PA gene expression through a cAMP-dependent mechanism (Node et al., 2001). Taken together, this suggests that EET may mediate their cellular events through a GPCR signaling pathway. However, characterization and purification of such a membrane receptor/binding site have been limited due to lack of proper tools. Additionally, the mechanisms of EETs uptake and secretion by cells are unclear. Many eicosanoids such as prostaglandins (Kanai et al., 1995), leukotrienes (Leier et al., 1994) and arachidonic acid (Krischer et al., 1998) have been described to enter and leave cells through transporter facilitated diffusion. It is unknown whether a similar transporter is present for EETs.

We designed 14,15-EET-mSA derivatives as tools to study EET transport, metabolism and binding. Each agonist contains a function group, i.e. a biotin, a photo-linking reagent or a

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radioiodinated group. With aid of convenient avidin conjugates such as agarose beads and fluorophore conjugates, 14,15-EET-BSA can be used to pull down an EET binding protein or image 14,15-EET binding sites. Upon ultraviolet radiation, 14,15-EET-BZDC-SA cross links with molecules in close proximity. This reaction may add a tag to the target molecule, which may be detected by mass spectrometric analysis. 14,15-EET-PISA can be radiolabeled with [^{125}I] to provide a new radioactive 14,15-EET agonist. Radioactive EET analogs are valuable tools to study their metabolism and binding.

Currently, radiolabeled 14,15-EET is made by epoxidation of [^{14}C] or [^{3}H] labeled arachidonic acid which results in all four EET regioisomers and less than 25% production of 14,15-EET (Corey et al., 1979; Rosolowsky and Campbell, 1993). In contrast, radioiodination of 14,15-EET-P^{125}ISA from 14,15-EET-stannane is specific and efficient. In addition, [^{125}I] has higher specific activity than [^{3}H], which results in increased sensitivity for radioligand binding assays. Therefore, 14,15-EET-P^{125}ISA is a radioactive 14,15-EET agonist with high specific activity, low production cost and efficient yields.

β-Oxidation and phospholipid esterification of 14,15-EET require the C-1 carboxyl group. Other routes of 14,15-EET metabolism include sEH hydration, CYP ω-hydroxylation (Cowart et al., 2002) and glutathione conjugation (Spearman et al., 1985) and are independent of the C-1 carboxyl group. Therefore, 14,15-EET-P^{125}ISA is a useful tool to study C1-carboxyl-independent metabolism of 14,15-EET. Like 14,15-EET, 14,15-EET-PISA is hydrolyzed into its corresponding 14,15-DHET-PISA. This metabolism is effectively inhibited by sEH inhibitors. Among the tested tissues, the hydration was most active in BCA homogenate where sEH is exposed, markedly less active in BCA rings and U937 cells where sEH is intracellular and less accessible, and absent in U937 membrane fractions where sEH is not localized. These data

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indicate that 14,15-EET-PISA is actively metabolized into 14,15-DHET-PISA by sEH.

Previously in the human leukemic monocyte lymphoma cell line U937 cells, a 14,15-EET binding site was characterized by whole cell radioligand binding using [^{3}H] 14,15-EET. The K_D and B_{max} were 13.84 ± 2.58 nM and 3.54 ± 0.28 pmol/10^6 cells, respectively (Wong et al., 1997). We used U937 cells membrane fraction to test 14,15-EET-P^{125}ISA binding. 14,15-EET-P^{125}ISA remained stable and unchanged when it was incubated with U937 membrane proteins at 4°C for 15 min. In agreement with the [^{3}H] 14,15-EET whole cell binding data, 14,15-EET-P^{125}ISA bound to U937 membranes in a time-dependent, concentration-dependent and saturable manner. Interestingly, the binding of 14,15-EET-P^{125}ISA to U937 membranes was with a lower affinity and lower receptor density. 14,15-EET-P^{125}ISA binding to U937 membranes reached equilibrium by 15 min and remained unchanged to 30 min at 4°C. The K_D was 148.3 ± 36.4 nM which was 10 fold higher than that observed with [^{3}H] 14,15-EET in whole cells. The B_{max} of 14,15-EET-P^{125}ISA binding to U937 membranes was 3.3 ± 0.5 pmol/mg protein which was lower than that observed in whole cells. The reason for these discrepant data could be due to esterification of [^{3}H] 14,15-EET into phospholipids or conjugation to cellular components. This could contribute to the observed binding in whole cells whereas these processes are absent or less active in membrane fractions. It is also possible that intracellular components maintain the binding sites in a conformation that favors binding to the ligand. This intracellular factor is missing in extracted membranes, which may change the receptor conformation to a ligand-unfavorable form. In other words, a smaller portion of the receptors in the membrane fractions than whole cell is available for the ligand, which results in a decreased receptor density. Also, ligands differ in affinity. The addition of the sulfonamide group to C-1 may reduce the affinity for the binding site. The variability between U937 cell lines could also account for the discrepancies. The 14,15-EET-

P^{125}ISA binding requires the 14,15-epoxide because its hydrolyzed product, 14,15-DHET-P^{125}ISA did not bind to U937 membranes. Taken together, our binding data validate the use of 14,15-EET-P^{125}ISA as a radiolabeled tool to study 14,15-EET binding.

EETs are key regulators of vascular tone and active mediators in pathophysiologic conditions such as anti-inflammation, fibrinolysis, angiogenesis and ischemic reperfusion protection (Seubert et al., 2004; Nithipatikom et al., 2006). However, whether they initiate their signaling through binding to specific protein(s)/receptor(s) is unclear. 14,15-EET-PISA, 14,15-EET-BSA and 14,15-EET-BZDC-SA are 14,15-EET agonists that serve as tools to identify and purify EET binding protein(s). 14,15-EET-P^{125}ISA, in particular, is a radioactive tracer to study EET metabolism and a radioligand to characterize EET binding kinetics.

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Footnotes

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Legends for Figures

Figure 1. Synthesis of 14,15-EET-sulfonamides.

Figure 2. Structures of 14,15-EET and 14,15-EET-sulfonamides.

Figure 3. Effects of 14,15-EET and 14,15-EET-sulfonamides on vascular tone of U46619 preconstricted bovine coronary arteries. A. Relaxations to 14,15-EET, 14,15-EET-PISA, 14,15-EET-BSA and 14,15-EET-BZDC-SA. B-D. Effects of the BK_{Ca} channel inhibitor, iberiotoxin (IBTX, 100 nM); the EET antagonist, 14,15-EEZE-mSA (10 μM) or increased extracellular K^+(20 mM) on 14,15-EET-PISA (B), 14,15-EET-BSA (C) and 14,15-EET-BZDC-SA (D). *Significantly different from control, $P < 0.05$.

Figure 4. Metabolism of 14,15-EET-P^{125}ISA by BCA homogenate and arterial rings. A-C. HPLC chromatographs of extracted 14,15-EET-P^{125}ISA metabolites after incubation with buffer, BCA homogenate (500 μg) in the presence or absence of CDU (1 μM) for 30 min at 30°C, respectively. D. HPLC chromatograph of extracted 14,15-EET-P^{125}ISA metabolites after incubation with arterial rings for 15 min at 37°C. E. Negative electrospray ionization MS spectrum of 14,15-EET-PISA metabolite (peak 21).

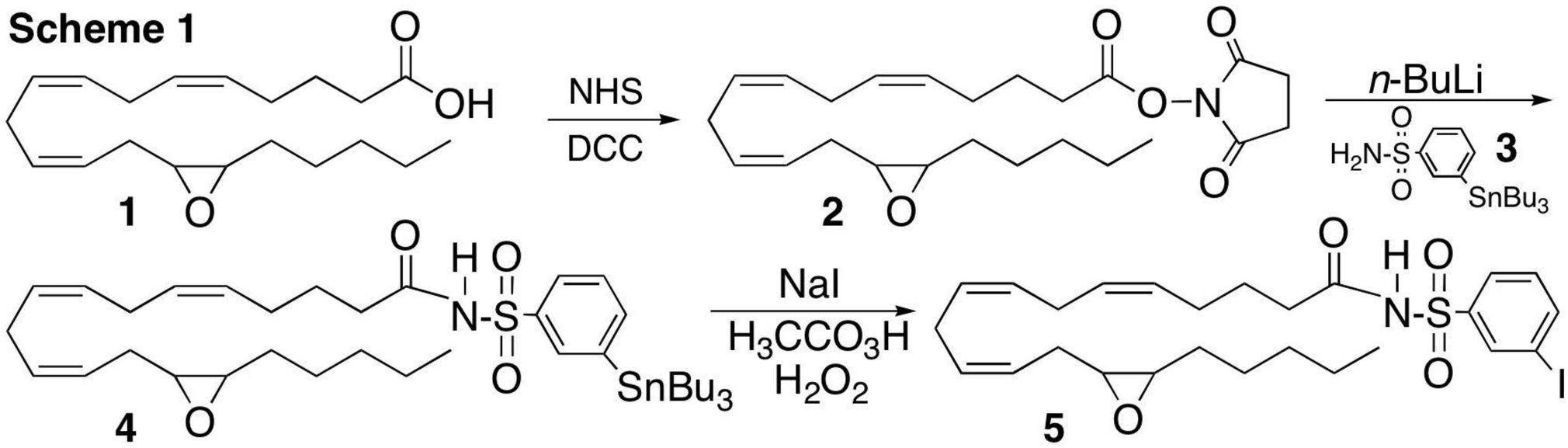
Figure 5. Metabolism of 14,15-EET-P^{125}ISA by U937 cells and U937 membrane protein. A. 14,15-EET- P^{125}ISA (35 nM) was incubated with intact U937 cells (800,000 cells) for 15 min at 37°C. B-D. 14,15-EET-P^{125}ISA (1 nM) was incubated with buffer (B), boiled U937 membrane protein (C), or U937 membrane protein (50 μg, D) for 15 min at 4°C. HPLC chromatographs of extracted 14,15-EET- P^{125}ISA metabolites from the incubations were shown.

Figure 6. 14,15-EET-P^{125}ISA binding to U937 membrane protein. A. Time course of 14,15-EET-P^{125}ISA binding. 14,15-EET-P^{125}ISA (1 nM) was incubated with 50 μg protein at 4°C for various time (1-30 min). n=2. B. Concentration-dependent 14,15-EET-P^{125}ISA binding.

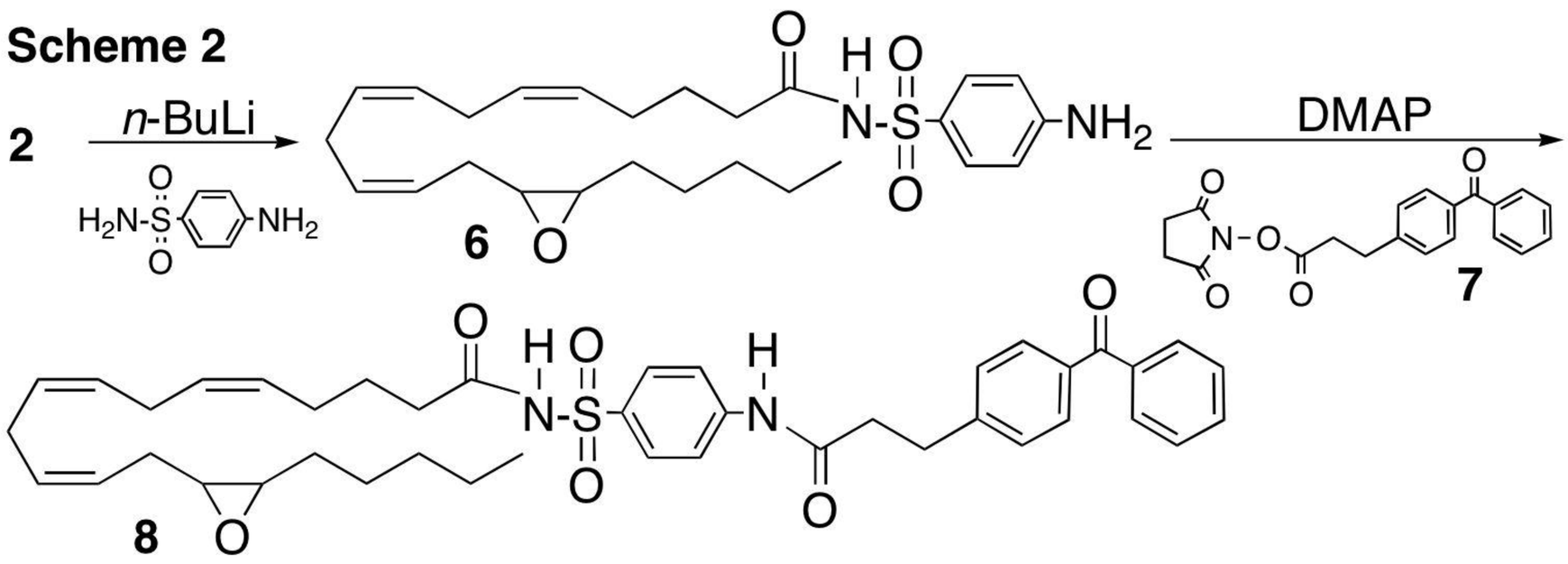
Increasing concentrations of 14,15-EET-P^{125}ISA (0.1-150 nM) were incubated with 50 μg protein at 4°C for 15 min. n = 2-7.

Fig. 1

Scheme 1



Scheme 2



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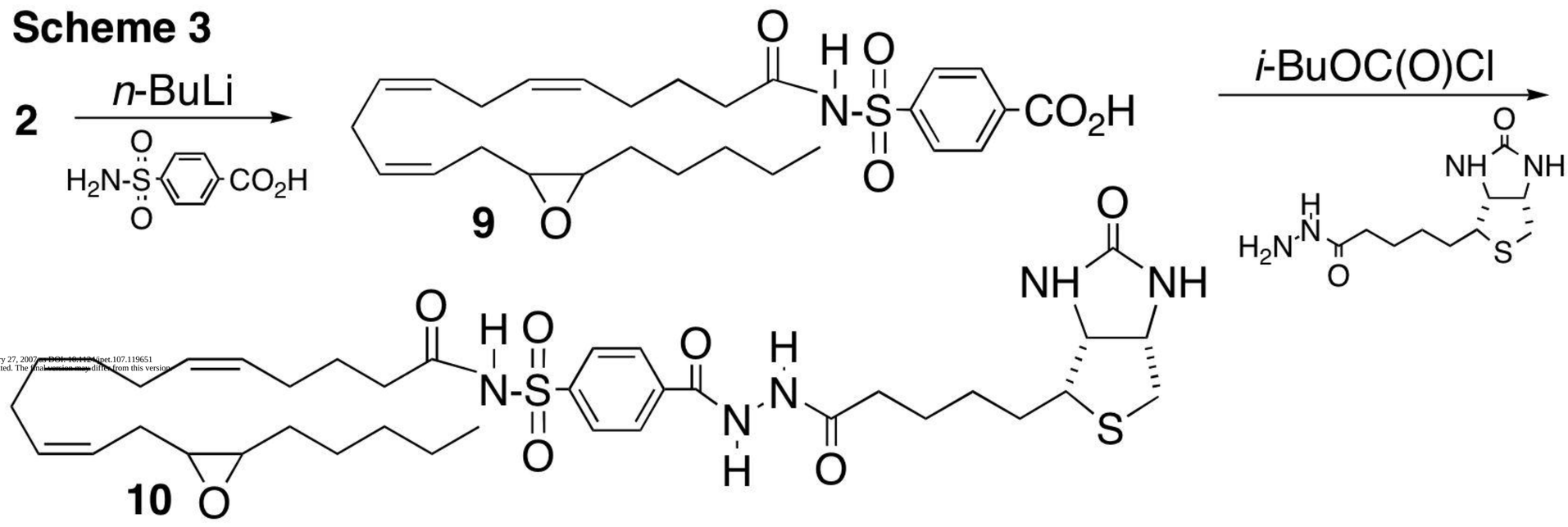

Fig. 2

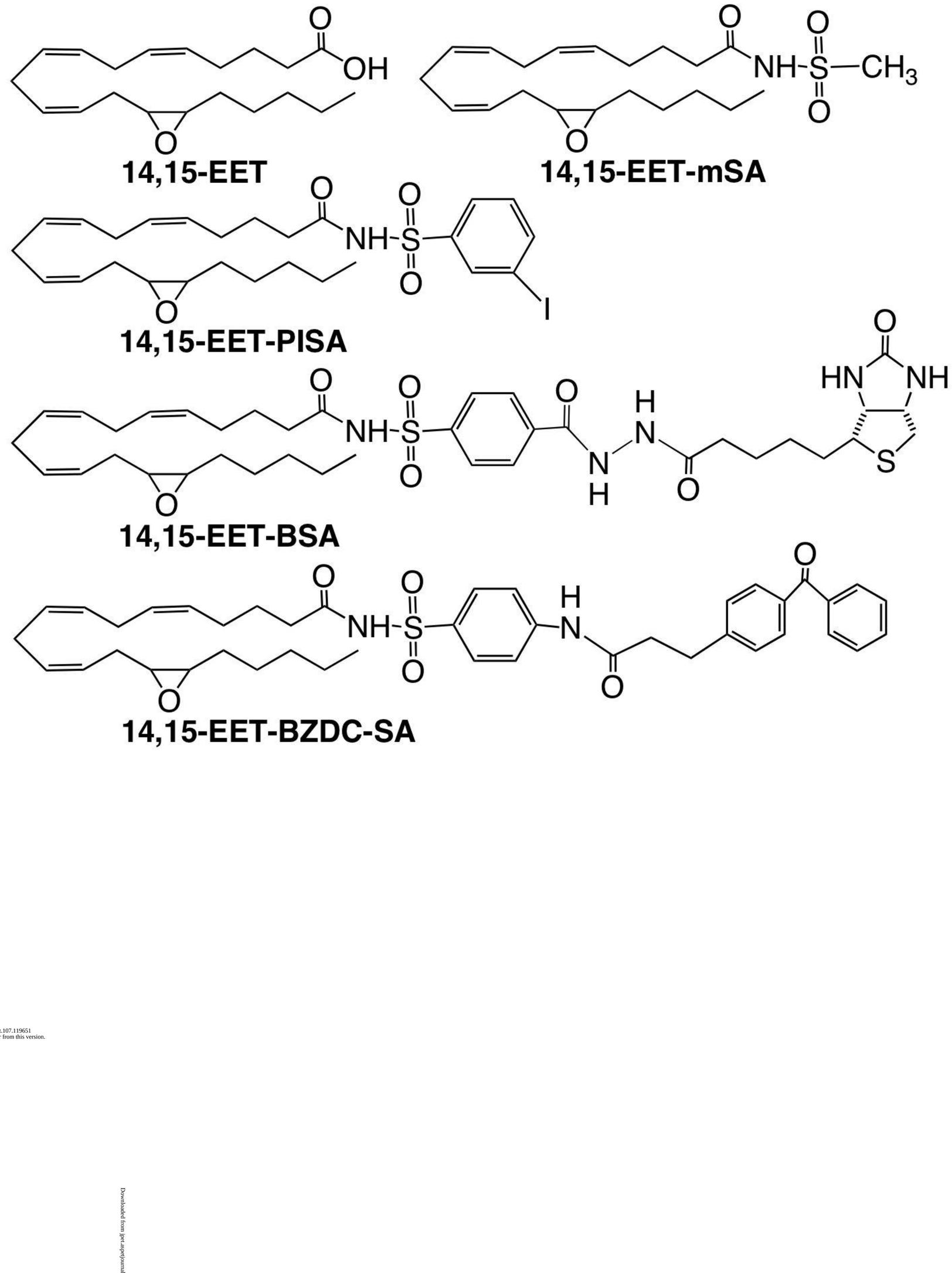

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Fig 3.

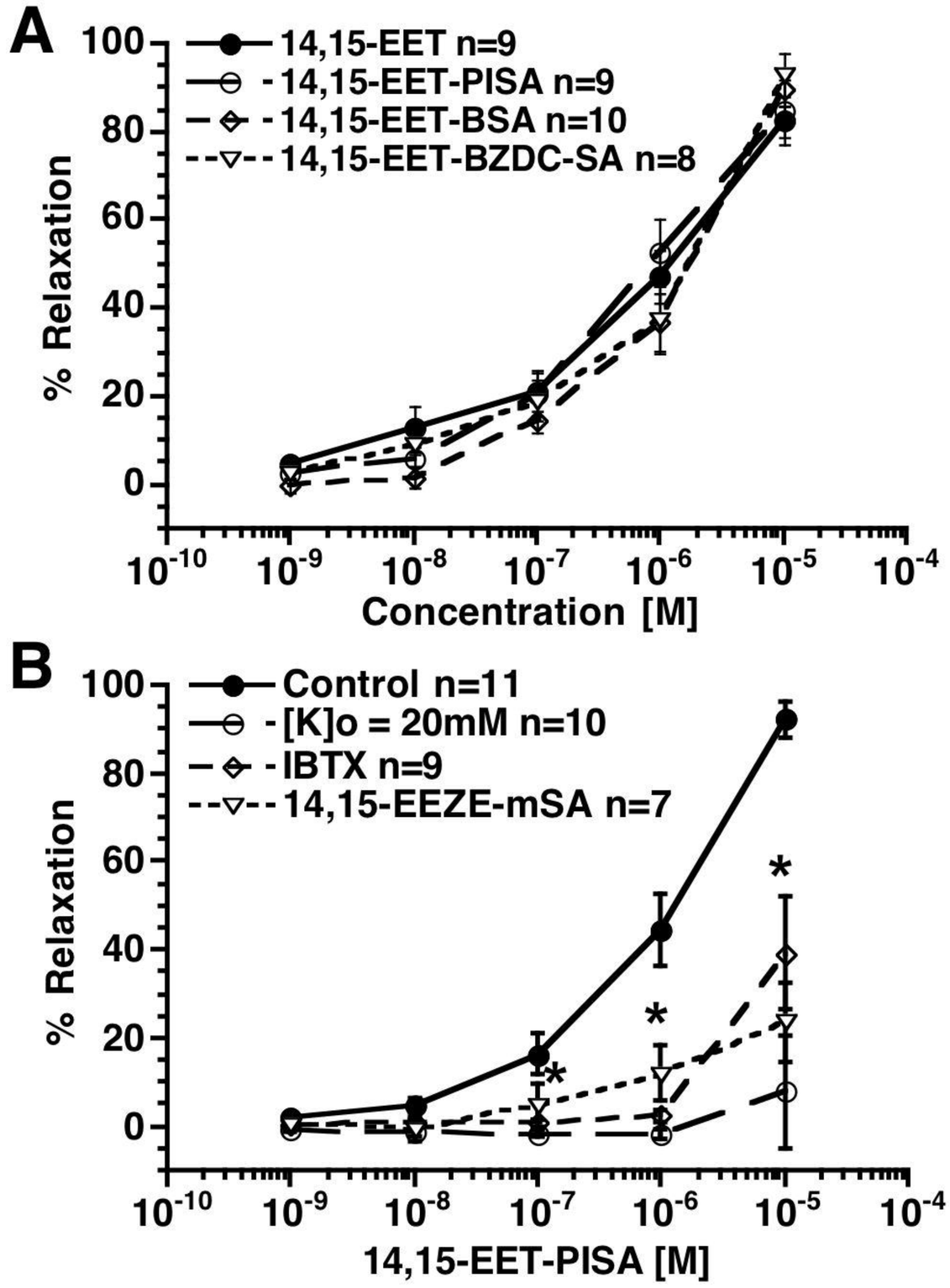

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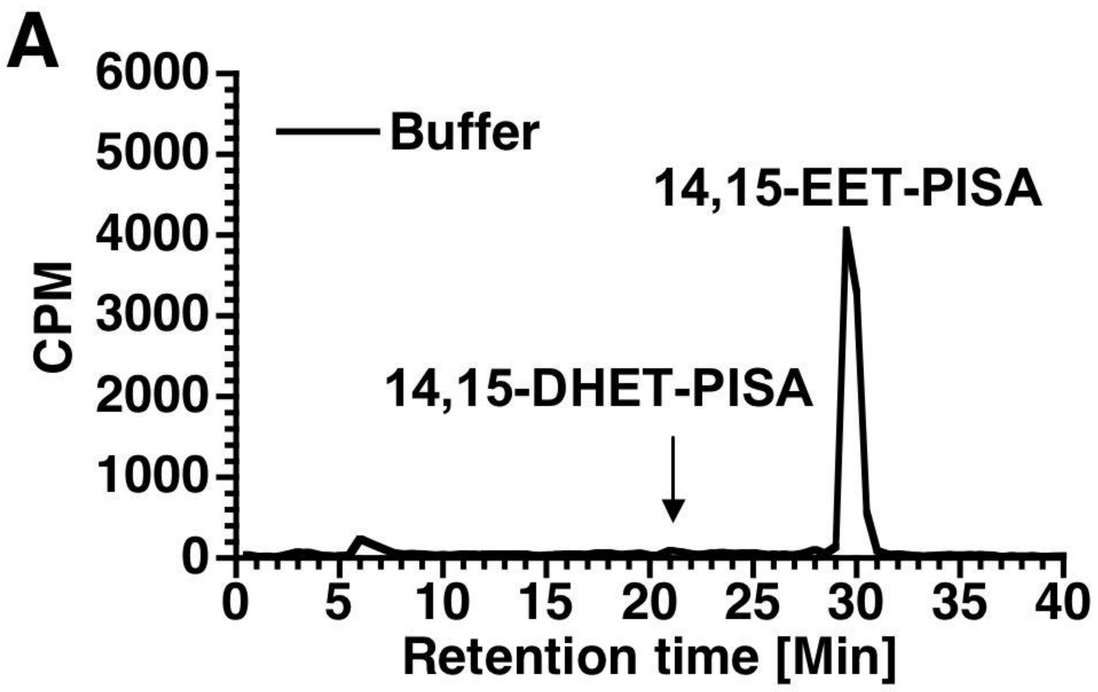
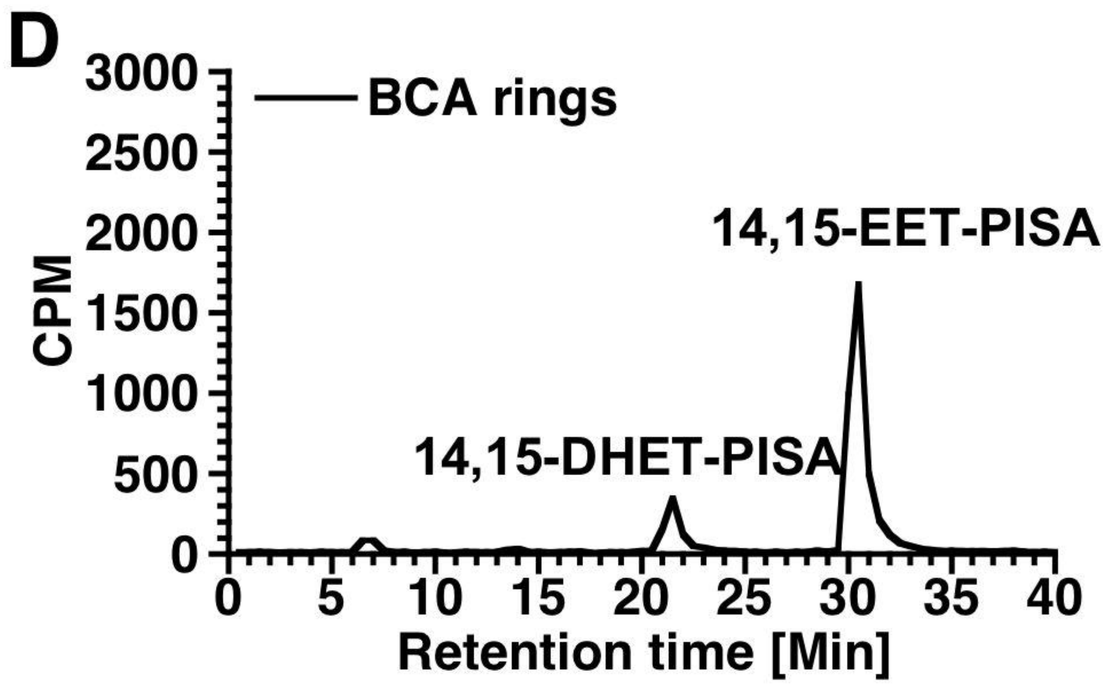
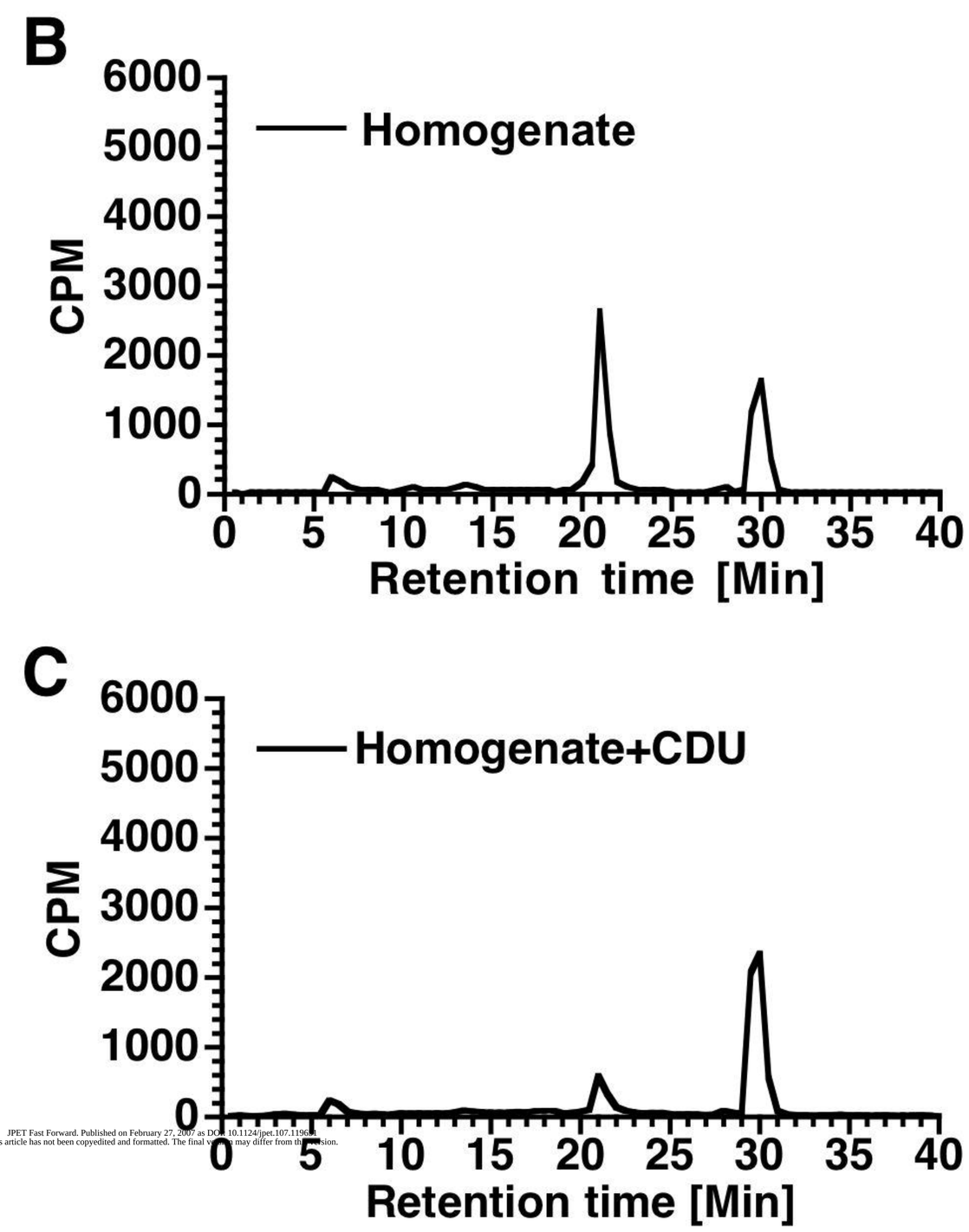
Fig. 4







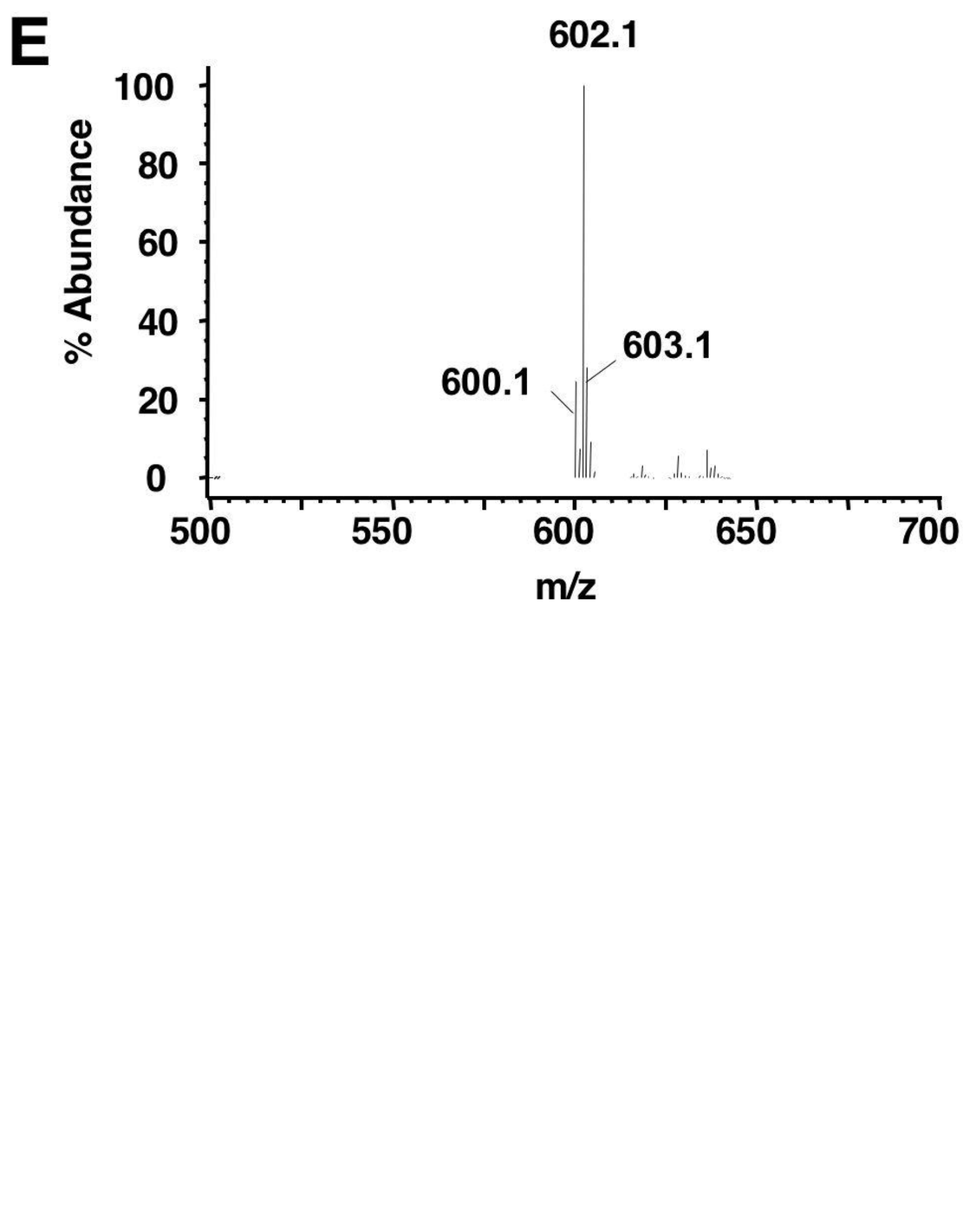


Fig. 5

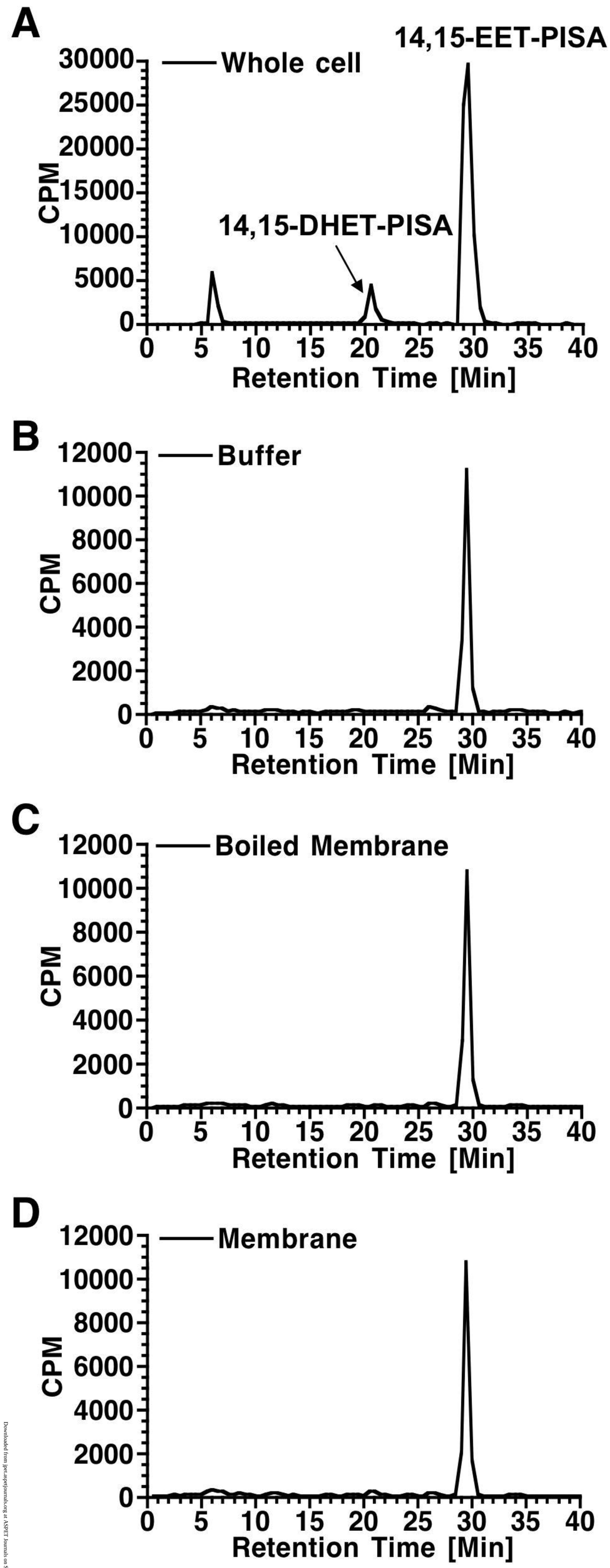


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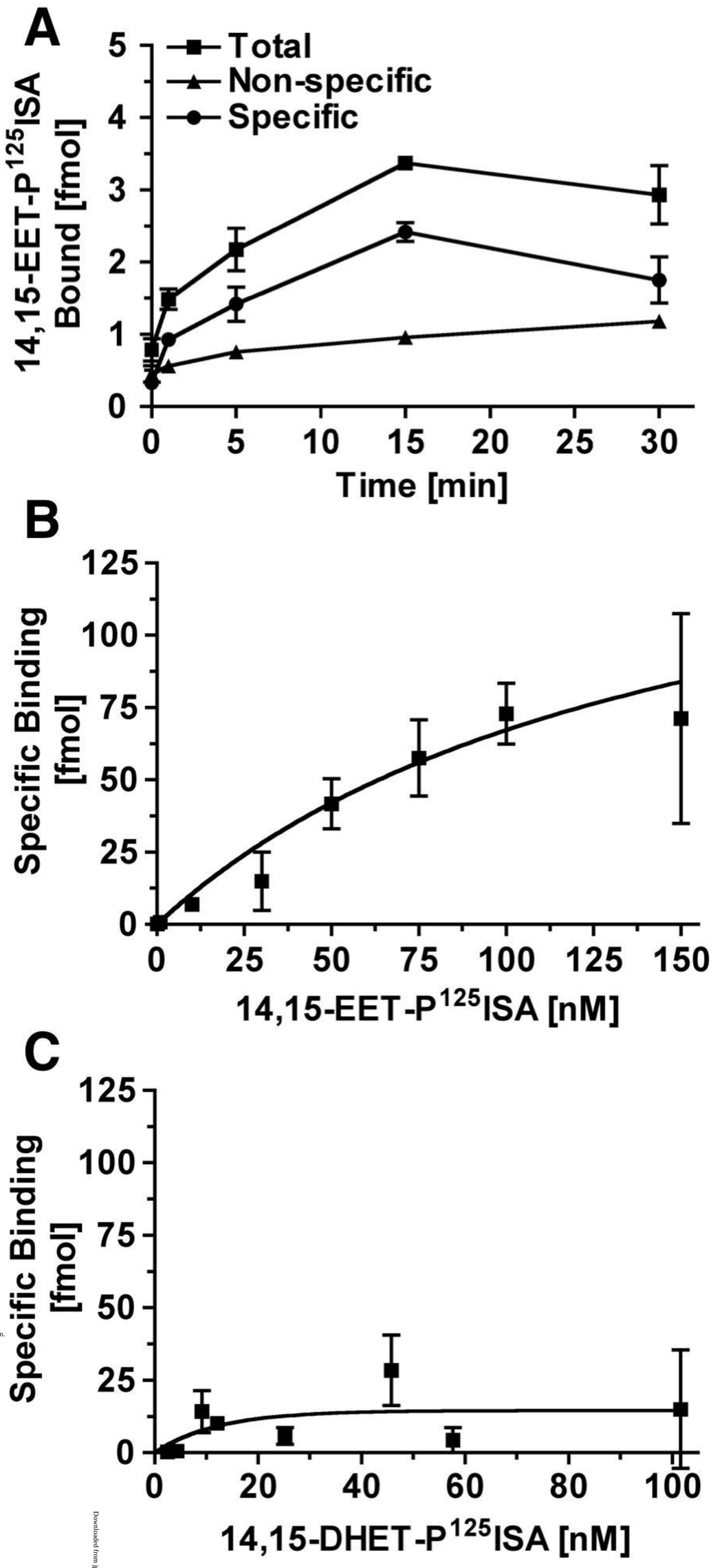
Fig. 6



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