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Biotransformation of prasugrel, a novel thienopyridine antiplatelet agent, to the pharmacologically active metabolite

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Running Title: Mechanism for prasugrel active metabolite formation

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Abbreviations: TNB, 2-nitro-5-thiobenzoic acid; DTNB, 5, 5'-dithio-bis(2-nitrobenzoic acid); TCEP, tris(carboxyethyl)phosphine; prasugrel, 2-acetoxy-5-(α-cyclopropylcarbonyl-2-fluorobenzyl)-4,5,6,7-tetrahydrothieno[3,2-c]pyridine

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ABSTRACT

Prasugrel, a novel thienopyridine antiplatelet agent, undergoes rapid hydrolysis *in vivo* to a thiolactone, R-95913, which is further converted to its a thiol-containing, pharmacologically active metabolite, R-138727, by oxidation *via* cytochromes P450. We trapped a sulfenic acid metabolite as a mixed disulfide with 2-nitro-5-thiobenzoic acid (TNB) in an incubation mixture containing the thiolactone R-95913, expressed CYP3A4 and NADPH. Further experiments investigated one possible mechanism for the conversion of the sulfenic acid to the active thiol metabolite *in vitro*. A mixed disulfide form of R-138727 with glutathione was found to be a possible precursor of R-138727 *in vitro* when glutathione was present. The rate constant for the reduction of the glutathione conjugate of R-138727 to R-138727 was increased by addition of human liver cytosol to the human liver microsomes. Thus, one possible mechanism for the ultimate formation of R-138727 *in vitro* can be through formation of a sulfenic acid mediated by CYPs followed possibly by a glutathione conjugation to a mixed disulfide and reduction of the disulfide to the active metabolite R-138727.

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INTRODUCTION

Prasugrel (Effient® in the US and Efient® in EU), clopidogrel (Plavix®/Iscover®) and ticlopidine (Ticlid™) are thienopyridine antiplatelet agents. Prasugrel is indicated to reduce the rate of thrombotic cardiovascular events and stent thrombosis in patients with acute coronary syndrome that are undergoing percutaneous coronary intervention (Wiviott et al., 2007; Effient package insert). The thienopyridines are prodrugs that are converted *in vivo* to their pharmacologically active metabolites possessing a thiol group *via* a corresponding thiolactone metabolite (Farid et al., 2009). However, except for an oxidation step catalyzed by cytochrome P450 (CYP) (Savi et al., 1994; Rehmel et al., 2006), the mechanism for the active metabolite formation from thienopyridines remained unknown until recently reported for ticlopidine and clopidogrel (Dansette et al., 2009). In the case of prasugrel, the active metabolite R-138727 was not detected when the thiolactone metabolite, R-95913, was incubated with liver homogenates or microsomes in the absence of cofactors, but was detected when NADPH and a reducing agent such as glutathione were added to the system (Kazui *et al*., 2000). A sulfenic acid, which is a likely intermediate for prasugrel's activation, reacts readily with a thiol compound, typically glutathione *in vivo*, to yield a disulfide metabolite (Decker *et al*., 1991; Kassahun *et al*., 2001; Reddy *et al*., 2005; Dansette et al., 2009). The formed

disulfide can be further reduced to provide a thiol-containing compound. The objective of this study is to investigate the involvement of a sulfenic acid and a glutathione conjugate of R-138727 in the *in vitro* production of prasugrel's active metabolite (R-138727) from the thiolactone intermediate (R-95913) (Figure 1).

Materials and Methods

Materials. R-138727 (prasugrel active metabolite), glutathione conjugate of R-138727, and R-95913 (prasugrel thiolactone metabolite) were synthesized by Ube Industries, Ltd. (Ube, Japan). DTNB and tris(carboxyethyl)phosphine (TCEP), used as a reagent to reduce DTNB to TNB, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Microsomes from insect cells expressing CYP3A4 (cDNA-expressed CYP3A4) were purchased from GENTEST Corporation (Woburn, MA, USA). All other chemicals and reagents were commercially available, and of the highest grade. Water was purified using a Milli-Q purification system (Millipore Co., Bedford, MA, USA).

Preparation of TNB-R-138727 disulfide. A mixed disulfide of R-138727 with TNB (TNB-R-138727 disulfide) was prepared by reacting R-138727 with equimolar DTNB in an aqueous solution (Figure 2) since the synthesis using the chemically unstable sulfenic acid-derivative of R-138727 and TNB was difficult to perform. After dilution of the reaction mixture with acetonitrile and following centrifugation, a 20-µl aliquot of the supernatant fraction was directly injected onto a YMC-Pack ODS-A (A-312) column, (150 mm x 6.0 mm i.d., 5 µm; YMC Co., Ltd., Kyoto, Japan), maintained at

ambient temperature. An HPLC analysis was performed using a low-pressure gradient elution system consisting of L-7100 Intelligent Pump, D-7500 Chromato-Integrator, and L-7400S UV Detector (Hitachi, Ltd., Tokyo, Japan). The mobile phase, 30% (v/v) acetonitrile in water containing 0.01% (v/v) trifluoroacetic acid was used at a flow rate of 1.0 ml/min. Profiling of the reaction products was performed with UV absorption detection at 220 nm. Two peaks in the chromatogram, corresponding to the two diastereoisomers of TNB-R-138727 disulfide [Retention times 19.18 (Peak A) and 21.57 (Peak B) minutes], were separately collected and lyophilized for structure elucidation by liquid chromatography with tandem mass (LC-MS/MS) spectrometry and NMR analysis, as described below.

Identification of TNB-R-138727 disulfide. Electrospray ionization (ESI) analysis was performed using a Q-TOF hybrid-type MS/MS spectrometer (Micromass UK., Ltd., Manchester, UK). An LC-MS analysis in the ESI mode was performed using a low-pressure gradient system consisting of L-6000, L-6300 Intelligent Pump, D-2500 Chromato-Integrator, and L-4000 UV-Detector (Hitachi, Ltd.) equipped with a YMC Pack ODS-A column (50 mm x 1.5 mm i.d., 5 μm). The other conditions for HPLC were the same as those described above. The flow rate was set at 1 ml/min, and after passage through the column, the eluent was introduced to the ESI source at a flow rate

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of about 100 µl/min. The acceleration voltage of 3.3 kV and cone voltage of 45-50 V were used in this ionization mode. The source block was maintained at 120°C. The desolvation gas was heated to 300°C. Xenon was used as the collision gas and the laboratory frame collision energy was 20 eV when acquiring the product ion spectra.

After purification of Peaks A and B by HPLC, NMR analysis was performed using a Varian (INOVA-500) 500 MHz spectrometer and were reported as δ in ppm relative to Me_4Si as the internal standard. Abbreviations of the 1H-NMR peak splitting patterns are as follows: bs = broad singlet; s = singlet; m = multiplet.

Metabolism of R-95913 by cDNA-expressed CYP3A4 supplemented with NADPH in the presence of TNB or DTNB. Duplicate mixtures (total volume of 390 µl each) containing potassium phosphate buffer (final concentration: 11.25 mM, pH 7.4), NADP (final concentration: 2.5 mM), D-glucose-6-phosphate (final concentration: 25 mM), glucose-6-phosphate dehydrogenase (final concentration: 0.5 units/ml), magnesium chloride (final concentration: 10 mM) and cDNA-expressed CYP3A4 (final concentration: 800 pmol/ml) was preincubated at 37°C for 5 min in the presence of 1 mM of TNB or DTNB as final concentration. TNB was prepared immediately before use by mixing equimolar DTNB with TCEP, dissolved in buffer (Han *et al*., 1994). Then, 10 µl of R-95913 (final concentration: 250 µM) was added to the mixture, which

was further incubated at 37°C for 0, 15, 30 and 60 min. At each time point, 80 µl of the incubation mixture was collected, added to 160 µl of acetonitrile to stop the reaction, and centrifuged (15,000 × g, 3 min, 4°C). For determination of R-95913, the supernatant samples were diluted with the supernatant fraction obtained by adding acetonitrile to cDNA-expressed CYP3A4 without any other components and following centrifugation. A 10 µl-aliquot of each sample was subjected to an LC/MS analysis as described in the following section.

Quantification of R-95913, R-138727 and TNB-R-138727 disulfide by LC/MS. Quantification of R-95913, R-138727 and TNB-R-138727 in the incubation mixture of R-95913 was carried out on an Alliance HPLC system consisting of a 2795 Separations Module (Waters Corporation, Milford, MA, USA) coupled to a ZQ-2000 (Waters Corporation) with the ESI source in positive ion mode. Each sample (10 µl) was injected onto an XTerra MS C18 column (50 mm x 2.1 mm i.d., 3.5 µm; Waters Corporation), which was maintained at 40°C. A two-component mobile phase, pumped at 0.5 ml/min, contained solvent A, which is a mixture of formic acid, 5 mM ammonium acetate and acetonitrile (0.2/95/5, v/v/v) and solvent B, which is a mixture of formic acid, 5 mM ammonium acetate and acetonitrile (0.2/5/95, v/v/v). The mobile phase initially consisted of 100% solvent A for 0-1 min, then solvent B was

increased from 50% to 100% linearly from 1 to 3.75 min, and was maintained at 100% B from 3.75 to 4.5 min, before recycling back to the initial condition.

The operating parameters of the MS detector were set as follows: capillary voltage, 3.5 kV; ion source temperature, 100°C; capillary temperature, 350°C; cone gas (N_2) flow and desolvation gas (N_2) flow, 50 L/h and 400 L/h, respectively; multiplier voltage, 650 V. Detection was performed in the single ion monitoring (SIM) mode. The cone voltages were 33, 33 and 27 V with selected reaction monitoring (SRM) of *m/z* 332, 350 and 547 for R-95913, R-138727 and TNB-R-138727 disulfide, respectively. Phenacetin (Sigma Chemical Co.) was used as the internal standard. The concentrations of each analyte in the samples were calculated using the computer software MassLynx (Version 3.5, Micromass UK Ltd.) and are expressed to three significant figures.

Standard stock solution containing 1 mM of TNB-R-138727 disulfide in 50% ethanol solution was prepared by adding a solution of DTNB in potassium phosphate buffer to that of R-138727 in ethanol at a final concentration of 1 mM. This standard stock solution was diluted two-fold sequentially with acetonitrile to prepare standard solutions of a TNB-R-138727 disulfide at concentrations from 15.6 nM to 1000 nM. In the same manner as described above, standard solutions of R-95913 or R-138727 were

prepared by diluting the standard stock solution containing 1 mM R-95913 or 1 mM R-138727 sequentially two-fold with acetonitrile. The standard curves were generated using each analyte's peak area ratio to that of the internal standard versus the nominal concentration of the analytes.

Formation of the glutathione conjugate and R-138727 by cDNA-expressed CYP3A4. Duplicate mixtures (total volume of 990 µl each) containing potassium phosphate buffer (final concentration: 29-34 mM, pH 7.4), NADP (final concentration: 2.5 mM), D-glucose-6-phosphate (final concentration: 25 mM), glucose-6-phosphate dehydrogenase (final concentration: 0.5 units/ml), magnesium chloride (final concentration: 10 mM), cDNA-expressed CYP3A4 (final concentration: 108 pmol/ml) with or without glutathione (final concentration: 5 mM) were preincubated at 37°C for 5 min. Then, 10 µl of R-95913, glutathione conjugate of R-138727 or R-138727 (final concentration: 1, 0.4 or 1 µM, respectively) were added to the mixture, which was further incubated at 37°C for 5, 15, 30, 45 and 60 min. At each time point, 160 µl of the incubation mixture were collected, added to the solution containing 320 µl of methanol, 3.2 µl of 500 mM *m*-methoxyphenacybromide (MPBr solution in acetonitrile) and 160 µl of the internal standard solution (100 ng/ml R-135766) to terminate the reaction. The mixture was stored for 10 min at room temperature and centrifuged

(15,000 × g, 3 min, 4°C). A 10 µl-aliquot of the supernatant fraction was subjected to the LC-MS/MS system as described below.

Formation of the glutathione conjugate metabolite and R-138727 from R-95913 in human liver microsomes and cytosol. Triplicate mixtures (total volume of 990 µl each) contained potassium phosphate buffer (36-39 mM, pH 7.4), an NADPH generating system ($NADP^+$ 1.55 mM), glucose-6-phosphate (3.3 mM), glucose-6-phosphate dehydrogenase (0.4 units/ml), $MgCl_2$ (3.3 mM)), glutathione (5 mM) and human liver microsome (1 mg/ml) with or without human liver cytosol (1 mg/ml). Each mixture was preincubated at 37°C for 5 min, and 10 µl of R-95913 (10 µM as a final concentration) were added to each respective mixture, which was incubated at 37°C for 1, 2, 5, 10, 15 and 30 min. At each time point, 100 µl of the incubation mixture were collected and mixed with a double amount of 5 mM MPBr acetonitrile solution and an equivalent amount of the internal standard solution to stop the reaction, and left for 10 min at room temperature to derivatize the thiol moiety of R-138727. After derivatization, the mixture was centrifuged (15,000 × g, 3 min, 4°C). A 10 µl-aliquot of the supernatant fraction was subjected to LC-MS/MS analysis.

Calculation of rate constants for formation of the glutathione conjugate metabolite and R-138727. As one possible pathway, the kinetic model for the formation of the

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glutathione conjugate of R-138727 and R-138727 from R-95913 in a closed incubation system is shown in Figure 3. The corresponding differential equations for this model are:

$$\frac{dZ(1)}{dt} = -(k1 + k4) \times Z(1)$$

$$\frac{dZ(2)}{dt} = k1 \times Z(1) - k2 \times Z(2) + k3 \times Z(3)$$

$$\frac{dZ(3)}{dt} = k2 \times Z(2) - k3 \times Z(3)$$

where Z(1), Z(2) and Z(3) represent the concentrations of R-95913, the glutathione conjugate of R-138727 and R-138727, respectively, and k1, k2, k3 and k4 represent the rate constants of the formation of the glutathione conjugate of R-138727 from R-95913 (reflecting the rate limiting, likely the oxidation, step in two consecutive steps), the formation of R-138727 from the glutathione conjugate of R-138727, the formation of the glutathione conjugate of R-138727 from R-138727, and conversion of R-95913 to metabolites other than the glutathione conjugate of R-138727 and R-138727, respectively. The concentrations of the glutathione conjugate of R-138727 and R-138727 were simultaneously fitted to the equations above by the computer software WinNonlin Professional (ver.4.0.1, Pharsight Corp.).

Quantification of R-95913, R-138727 and glutathione conjugate by LC-MS/MS. The assays were performed based on the methods described in the preceding section and previously reported (Farid *et al.*, 2007b). Quantification of R-95913,

MPBr-derivatized R-138727 and glutathione conjugate was carried out with an Alliance HPLC system consisting of 2690 Separations Module (Waters Corporation) coupled to a Quattro LC MS/MS system (Waters Corporation) with the ESI source in positive ion mode. The mobile phase containing methanol, purified water and trifluoroacetic acid (570/430/0.5, v/v/v) was applied on an Inertsil ODS-3 column (150 mm × 2.1 mm i.d., 5 μm: GL Sciences Inc.).

The concentrations of each analyte in the samples were calculated using the computer software MassLynx (Version 4.0, Waters Corporation). The standard stock solution was diluted five-fold sequentially with acetonitrile to prepare the standard solutions at concentrations from 1.6 nM to 1000 nM. The standard curves were generated using each analyte's peak area ratio to that of the internal standard versus the nominal concentration of the analytes.

Results

TNB-R-138727 disulfide. The thiol-exchange reaction of R-138727 with DTNB (Figure 2) and trapping the sulfenic acid intermediate of R-138727 with TNB (Figure 1) generate a mixed disulfide between TNB and R-138727 (TNB-R-138727 disulfide). We synthesized TNB-R-138727 disulfide as the reference standard for analysis by mixing DTNB and R-138727 (Figure 2) since TNB and sulfenic acid are unstable and unavailable. Major reaction products appeared as two chromatographic peaks, Peaks A and B. The MS and MS/MS spectra of Peaks A and B had identical fragmentation patterns as well as exact mass and relative intensity of the fragment ions, demonstrating that Peaks A and B are diastereoisomers. The presence of the diastereoisomers is consistent with the chemical structure of TNB-R-138727 disulfide which possesses two asymmetric carbons adjacent to the sulfur and nitrogen atoms. The $(M+H)^+$ ions observed in MS spectra of Peaks A and B were detected with *m/z* 547. Accordingly, the molecular mass of the compounds appeared as Peaks A and B were determined to be 546, 197 Da higher than that of R-138727, consistent with the chemical structures of the compounds as Peaks A and B being TNB-R-138727 disulfide. The MS/MS spectrum of the $(M+H)^+$ ion with *m/z* 547 gave a series of fragment ions with *m/z* 206, 248 and

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317, and the proposed assignments of the fragment ions are shown in Figure 4. The ion with *m/z* 317 was considered to be a fragment ion of $(M+H)^+$ produced by the elimination of the disulfanyl nitrobenzoic acid moiety as shown in proposed fragmentation in Figure 4.

We determined the NMR spectra of Peaks A and B, which were mixed due to rapid epimerization to each other. 1H-NMR (Pyridine-d_5) of the compounds were as follows: 0.82 ppm (2H, *m*), 1.09 ppm (2H, *m*), 2.07 ppm (1H, *m*), 2.38 ppm (2H, *m*), 2.81 ppm (2H, *m*), 3.36 ppm (2H, *m*), 4.96 ppm and 4.98 ppm (total 1H, each *s*), 6.00 ppm (1H, *bs*), 6.26 ppm and 6.32 ppm (total 1H, each *s*), 7.26 ppm (2H, *m*), 7.37 ppm (1H, *m*), 7.65 ppm (1H, *m*), 7.93 ppm (2H, *m*), 8.37 ppm (1H, s), clearly demonstrating that Peaks A and B are the mixed disulfide of R-138727 with TNB (Figure 5).

Metabolism of R-95913 by cDNA-expressed CYP3A4 supplemented with NADPH in the presence of DTNB or TNB. The thiolactone R-95913 was incubated with cDNA-expressed CYP3A4 supplemented with NADPH-generating system. DTNB or TNB was added to the incubation mixture in order to trap the thiol-metabolite R-138727 (Figure 2) or the sulfenic acid metabolite of R-138727 (Figure 1), respectively, in the form of TNB-R-138727 disulfide. The concentrations of R-95913, R-138727 and TNB-R-138727 disulfide in the incubation mixture at various times as determined by

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LC/MS are shown in Figure 6. The concentration of R-95913 gradually decreased along with increasing the incubation time in the presence of DTNB or TNB (Figure 6a or 6b, respectively). Upon incubating R-95913 in the presence of DTNB, the rate of the substrate decrease was slower than in the presence of TNB (1.7±1.4 and 5.6±1.8 μM/min with DTNB and TNB, respectively, as mean±SD values) and this incubation produced neither TNB-R-138727 disulfide nor free R-138727, which indicates no production of the thiol-metabolite R-138727 (Figure 6a). On the other hand, incubation in the presence of TNB led to a production of TNB-R-138727 disulfide, indicating the formation of the sulfenic acid intermediate (Figure 6b). TCEP seemed to have no significant effect apparently with an exception that a very low amount of R-138727 was detected (data not shown).

Formation of the glutathione conjugate of R-138727 and R-138727 by cDNA-expressed CYP3A4. R-95913, glutathione conjugate of R-138727 or R-138727 was incubated with cDNA-expressed CYP3A4 in the presence of NADPH with or without glutathione. Incubation of R-95913 with glutathione (Figure 7a), but not without glutathione (Figure 7b), showed immediate formation of the glutathione conjugate of R-138727, which was gradually decreased and was replaced by the formation of R-138727. Although the thiolactone decreased rapidly in the absence of

glutathione (Figure 7b), no active metabolite was detected. These data may indicate that other metabolic pathways may have been occurring simultaneously in the *in vitro* system. The glutathione conjugate of R-138727 when added to the incubation mixture rapidly decreased and converted to R-138727 in the presence of glutathione, but very little in the absence of glutathione (Figure 7c). R-138727 when added to the incubation mixture was minimally converted to the glutathione conjugate in the incubation mixture even in the presence of glutathione (Figure 7d), and remained stable in the presence/absence of glutathione (data not shown).

Effect of human liver cytosol on the formation of the glutathione conjugate of R-138727 and R-138727. R-95913 (10 μM) was incubated in human liver microsomes (1 mg/ml protein) with or without human liver cytosol fraction (1 mg/ml protein) in the presence of 5 mM glutathione and an NADPH generating system. The apparent formation of the glutathione conjugate of R-138727 was remarkably decreased when the human liver cytosol fraction was added to human liver microsomes with the formation rates of the glutathione conjugate of R-138727 and R-138727 of 54.2±6.5 and 4.49±1.77 pmol/min/mg protein, respectively, in human liver microsomes and 9.28±2.05 and 31.5±7.1 pmol/min/mg protein, respectively, in a mixture of human liver micorosmes and cytosols (expressed as mean±SD values, Figure 8). The simultaneous

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fitting exihibited a good fit to the observed data. The rate constant of the formation of R-138727 from the glutathione conjugate of R138727 (k2) was substantially increased by the addition of human liver cytosols (0.143 and 2.82 s^{-1} without and with human liver cytosols, respectively) as shown in Figure 8.

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Discussion

We hypothesized that a sulfenic acid intermediate can be produced from the thiolactone intermediate of prasugrel, R-95913, and converted to a disulfide with glutathione in the process of producing the pharmacologically active metabolite R-138727 (Figure 1). The basis for this hypothesis is that R-138727, the thiol metabolite, is not the immediate metabolite of R-95913 but a sulfenic acid of R-138727 produced initially by the action of CYP enzymes could possibly be an intermediate. In the present study, as a tool for trapping and detecting the sulfenic acid intermediate, we used 2-nitro-5-thiobenzoic acid (TNB), which forms a disulfide with the sulfenic acid groups (Lin *et al.*, 1975; Boschi-Muller *et al*., 2000; Poole and Ellis, 2002; Poole *et al*, 2004). We also used dithio-bis-2-nitrobenzoic acid (DTNB), which forms a disulfide with thiol groups by a thiol exchange reaction (Riddles *et al*., 1979; Boschi-Muller *et al*., 2000), for R-138727 detection. A mixed disulfide of R-138727 with TNB (TNB-R-138727 disulfide) is a key substance in the present study since TNB-R-138727 disulfide could be produced either by a thiol exchange reaction between R-138727 and DTNB (Figure 2), or by a coupling reaction between the sulfenic acid derivative of R-138727 and TNB (Figure 1). The sulfenic acid intermediate of R-138727 was detected as TNB-R-138727 disulfide after incubation of R-95913 with CYP3A4 and NADPH without glutathione (Figure 6b)

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while R-138727 was not observed under this condition (Figure 6a), indicating that the sulfenic acid of R-138727 is likely produced as an intermediate in the metabolism of R-95913 prior to the formation of R-138727. Additionally, we showed the formation of a glutathione conjugate from R-95913 by CYP3A4 in the presence of glutathione (Figure 7a), but not in the absence of glutathione (Figure 7b). As the sulfenic acid intermediate was formed even in the absence of glutathione (Figure 6b), R-95913 is likely first oxidized to the sulfenic acid intermediate in a glutathione independent manner, and then possibly converted to a glutathione conjugate. Another glutathione molecule could then reduce the R-138727 glutathione conjugate to form R-138727. This concept is supported by the data showing that the glutathione conjugate is rapidly converted to R-138727 in the presence of glutathione (Figure 7c) while R-138727 is minimally converted to its glutathione conjugate (Figure 7d). Addition of cytosolic fraction to liver microsomes resulted in much lower formation of the glutathione conjugate with increased formation of R-138727 (Figure 8), indicating that the glutathione conjugate generated can be immediately reduced to R-138727 in the presence of cytosol.

Reddy et al determined that the formation of a thiol metabolite from sulfenic acid could proceed *via* a reduction or disproportionation step which would not be

glutathione-dependent (Reddy et al. 2005). This is another possible metabolic pathway for prasugrel's active metabolite formation *in vivo*, and would support the reported *in vivo* metabolic data in animals and humans (Farid et al 2007(a) and Smith et al 2007). The conjugation with glutathione does not fully explain the observed stereoselectivity in prasugrel's active metabolite that was observed *in vivo*. In humans, greater concentrations of the R-configuration at the 4-position of R-138727 (Wickremsinhe *et al*., 2007) were observed in plasma. This *in vivo* stereoselectivity may be explained in part by the observed *in vitro* stereoselectivity in the CYP mediated oxidation of the thiolactone to R-138727 by cDNA-expressed CYP isoforms (Rehmel et al., 2005, Baker et al., 2008). Additionally, stereoselective S-methylation of R-138727 was observed *in vitro* where the S-methylated metabolite of R-138727 was generated only from the S-configuration of R-138727 in human liver microsomes (Kazui et al., 2008).

Very recently, Dansette et al. proposed a scheme including sulfenic acid and glutathione conjugation after the formation of thiolactones from clopidogrel and ticlopidine, which is similar to the one proposed here for prasugrel (Dansette et al., 2009). Differences in the active metabolite formation from thienopyridines do exist especially in the initial step to form the respective thiolactones. For both clopidogrel and ticlopidine, the first step in the formation of the thiolactone, is an oxidative step in which reactive

intermediates are likely to be formed as evidenced by their mechanism-based inhibitory effects on the activities of CYP2B6 and CYP2C19 (Ha-Duong *et al*., 2001; Nishiya *et al*., 2009a and 2009b). However, prasugrel is converted to the thiolactone by hydrolysis in the intestine during the absorption process through the action of carboxylesterases (Williams *et al*., 2008), and the key prasugrel metabolites are not mechanism-based inhibitors (Rehmel *et al.*, 2006; Hagihara *et al*., 2008; Nishiya *et al*., 2009a and 2009b). The active metabolite of clopidogrel is thought to be formed primarily in the liver (Savi *et al*., 1992; Farid *et al*., 2009), whereas the active metabolite of prasugrel is thought to be partly formed by intestinal CYP3A prior to the thiolactone reaching the liver (Farid *et al*., 2007, Farid *et al*., 2009).

In conclusion, the results of the present *in vitro* study have provided an insight into prasugrel bioactivation mechanism including a sulfenic acid intermediate and possibly a glutathione conjugate although there are marked differences in thiolactone formation and site of active metabolite formation among thienopyridines.

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FOOTNOTES

The co-author Nagy A. Farid has retired from Eli Lilly and Company. Current email address: nagyfarid@gmail.com

FIGURE LEGENDS

Figure 1. Proposed metabolic pathway from the thiolactone of prasugrel to the pharmacologically active metabolite *via* a sulfenic acid intermediate.

TNB was used to trap a sulfenic acid intermediate forming TNB-R-138727 disulfide.

GSSG: Oxidized form of glutathione.

TNB: 2-nitro-5-thiobenzoic acid.

Figure 2. Thiol-exchange reaction of R-138727 with DTNB forming TNB-R-138727 disulfide.

DTNB: 5, 5'-dithio-bis(2-nitrobenzoic acid).

Figure 3. Schematic diagram of the model for the formation of the glutathione conjugate of R-138727 and R-138727 from R-95913 in a closed incubation system.

k1: rate constant of the formation of the glutathione conjugate of R-138727 from R-95913

k2: rate constant of the formation of R-138727 from the glutathione conjugate of R-138727

k3: rate constant of the formation of the glutathione conjugate of R-138727 from R138727

k4: rate constant of conversion of R-95913 to the metabolites other than the glutathione conjugate of R-138727 and R-138727

Figure 4. MS/MS spectra of Peak A (a) and Peak B (b) and proposed fragmentation in positive ion LC-ESI/MS.

Figure 5. 1H NMR spectra of Peaks A and B obtained by NMR analysis.

Figure 6. Concentrations of R-95913 (●), R-138727 (◊) and TNB-R-138727 disulfide (▲) *vs* time during metabolism of R-95913 by cDNA-expressed CYP3A4 supplemented with NADPH-generation system in the presence of DTNB (a) or TNB (b).

Figure 7. Formation of R-138727 and the glutathione conjugate of R-138727 by cDNA-expressed CYP3A4 in the presence or absence of glutathione.

A 1 μM R-95913 with (a) or without (b) 5 mM glutathione, or 400 nM of the

glutathione conjugate of R-138727 with/without 5 mM glutathione (expressed as solid/dashed line, respectively) (c) or 1 μM R-138727 with 5 mM glutathione (d) was incubated with cDNA-expressed CYP3A4 and NAPDH for 5, 15, 30, 45 and 60 min. The concentrations of R-95913 (●), R-138727 (◊) and glutathione conjugate of R-138727 (×) were determined by LC-MS/MS.

The graphs show the mean values of duplicated data.

Figure 8. Formation of the glutathione conjugate of R-138727 and R-138727 from R-95913 in human liver microsomes and cytosols.

R-95913 (10 μM) was incubated in 1 mg/ml human liver microsomes (a) or 1 mg/ml human liver microsomes and cytosols (b) supplemented with 5 mM glutathione and an NADPH generating system. The concentrations of R-138727 (◊) and glutathione conjugate of R-138727 (×) were determined over time (mean+SD, n = 3). The solid lines represent the simultaneous fitting curves of the concentration-time profiles of R-138727 and the glutathione conjugate of R-138727.

Figure 1

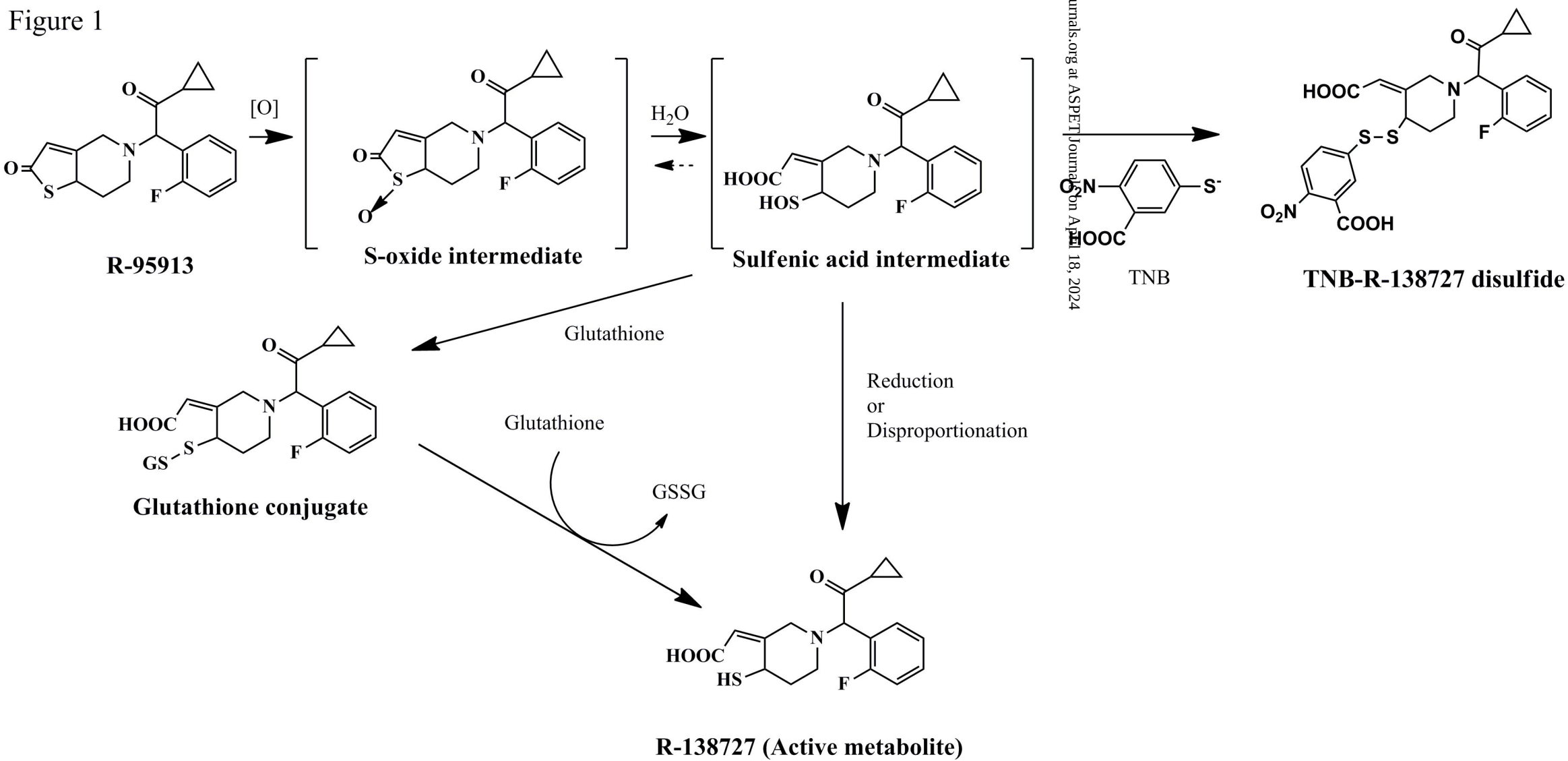
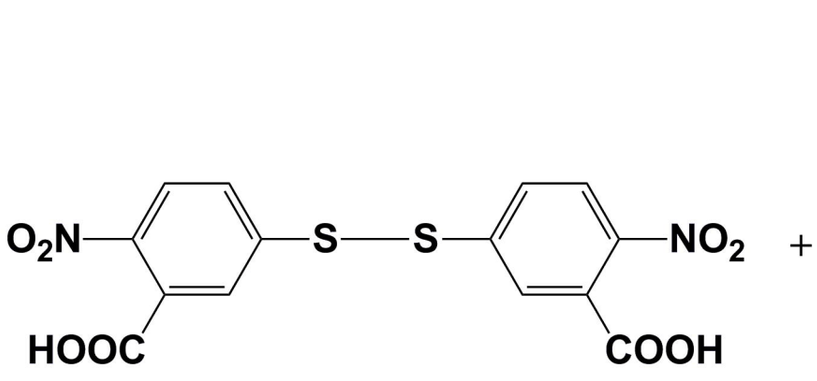
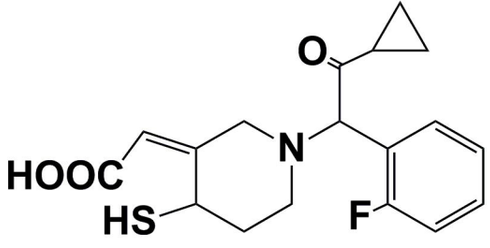


Figure 2

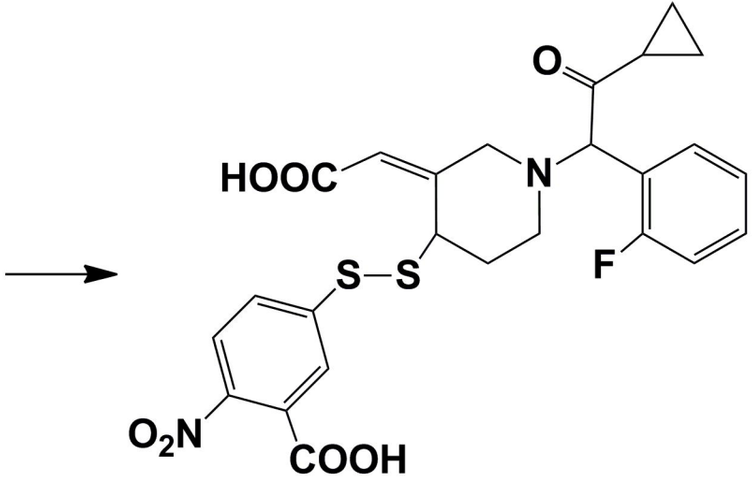


DTNB

+



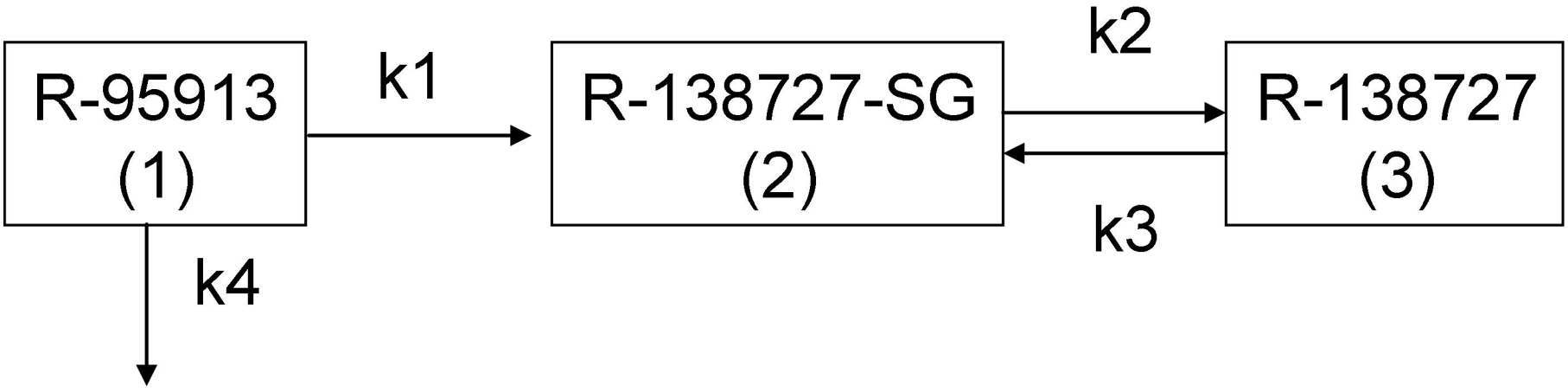
R-138727



TNB-R-138727 disulfide

+ TNB + H^+

Figure 3



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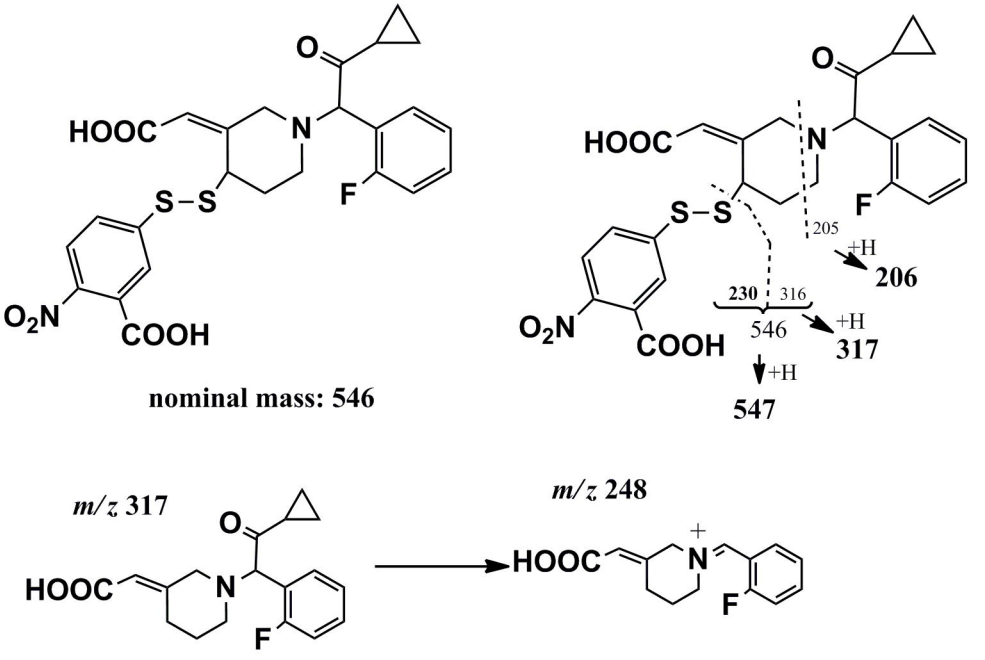
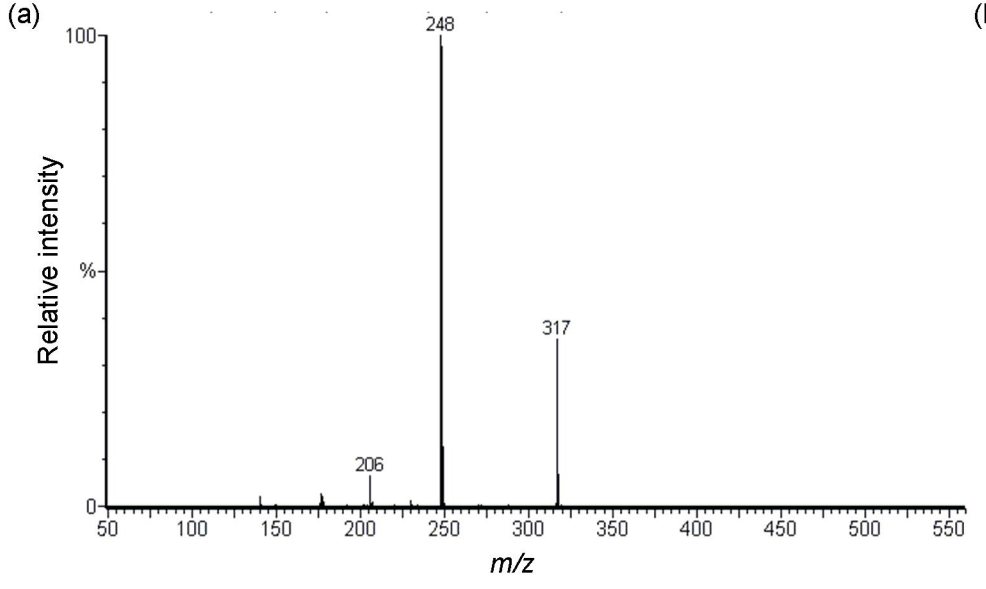




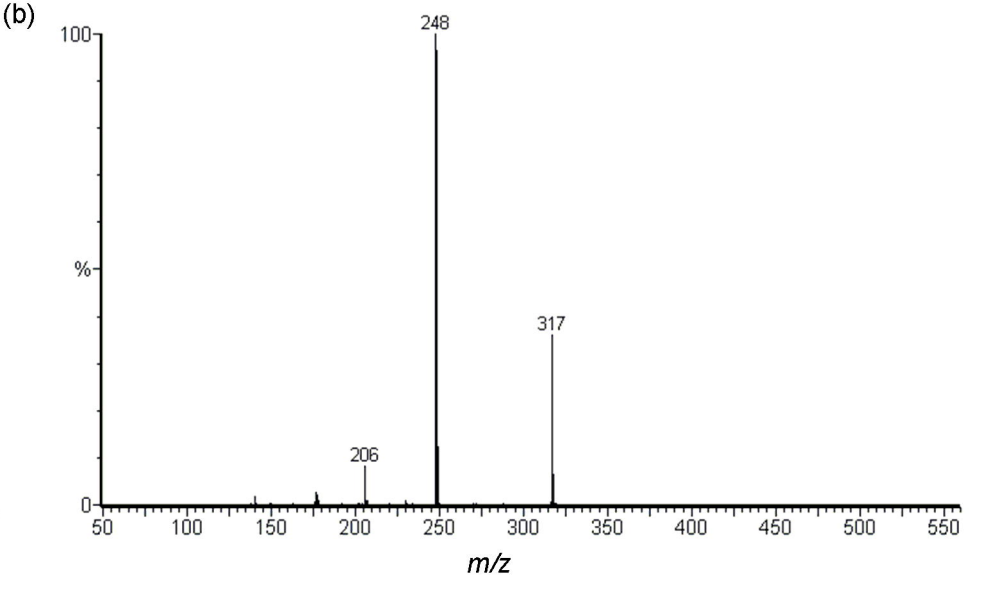


Figure 5

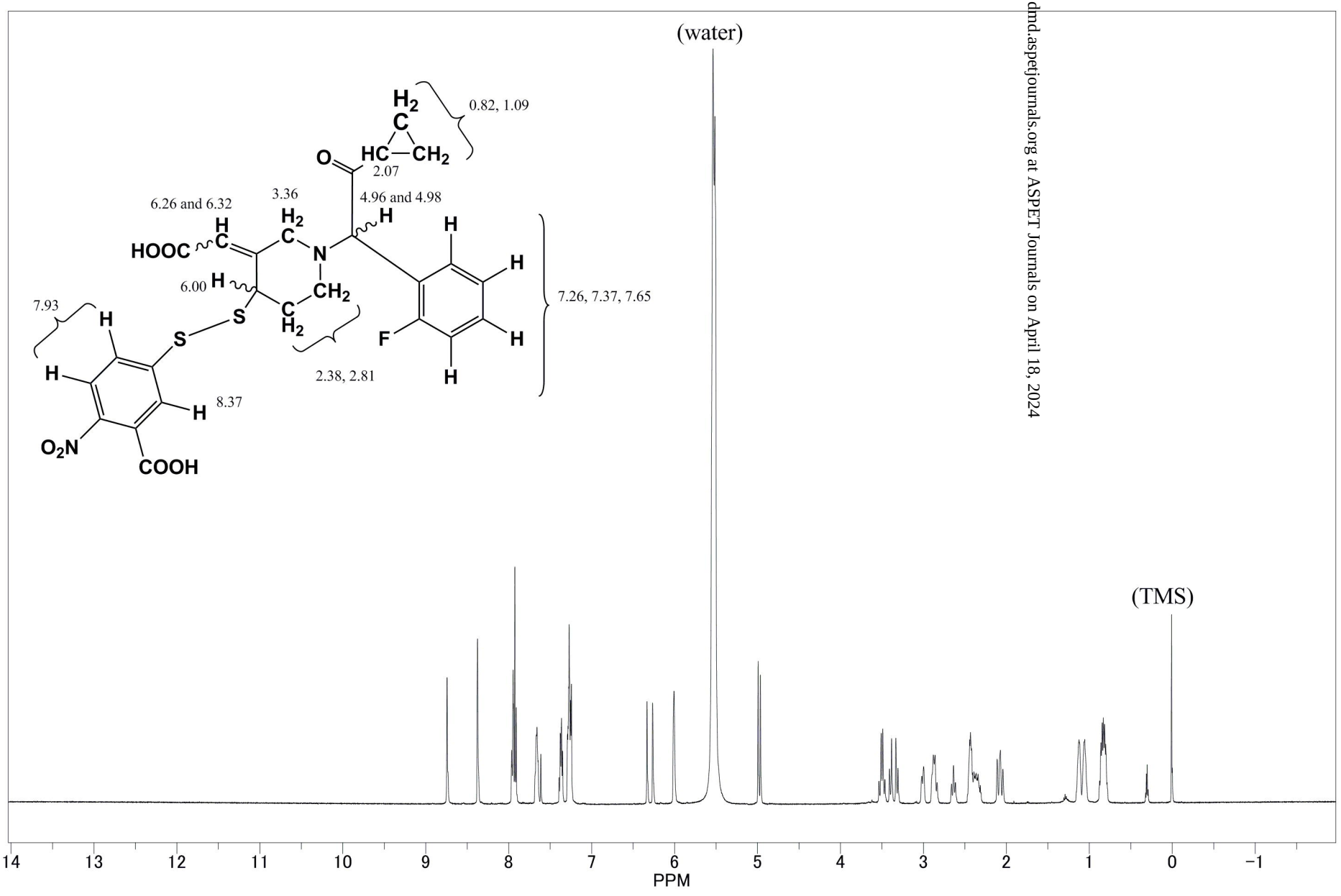
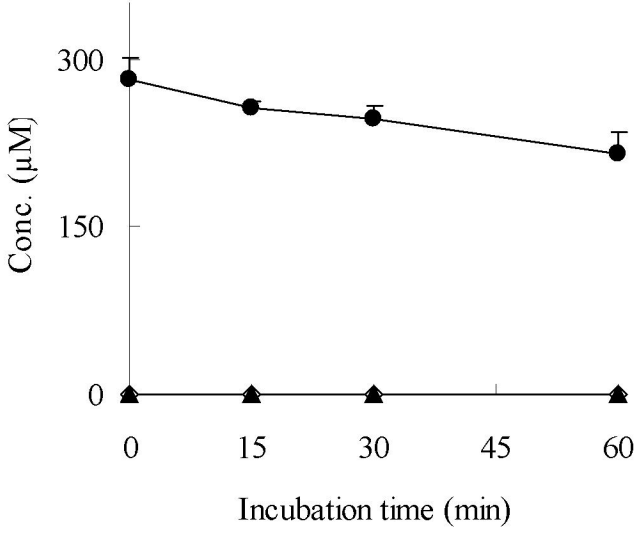


Figure 6

(a)



(b)

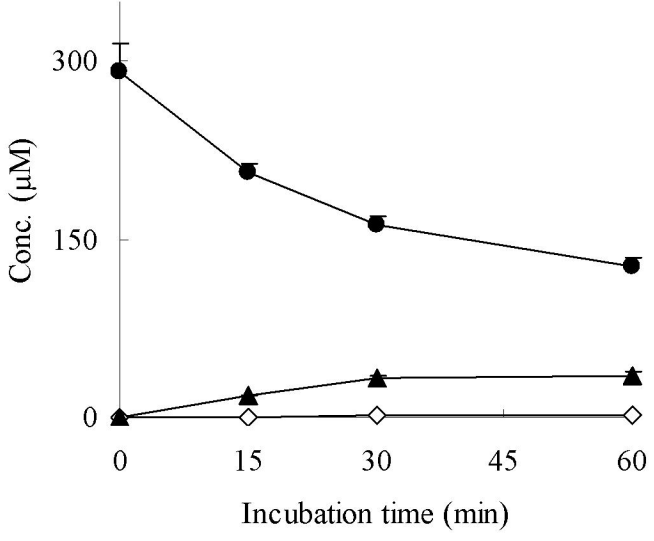


Figure 7

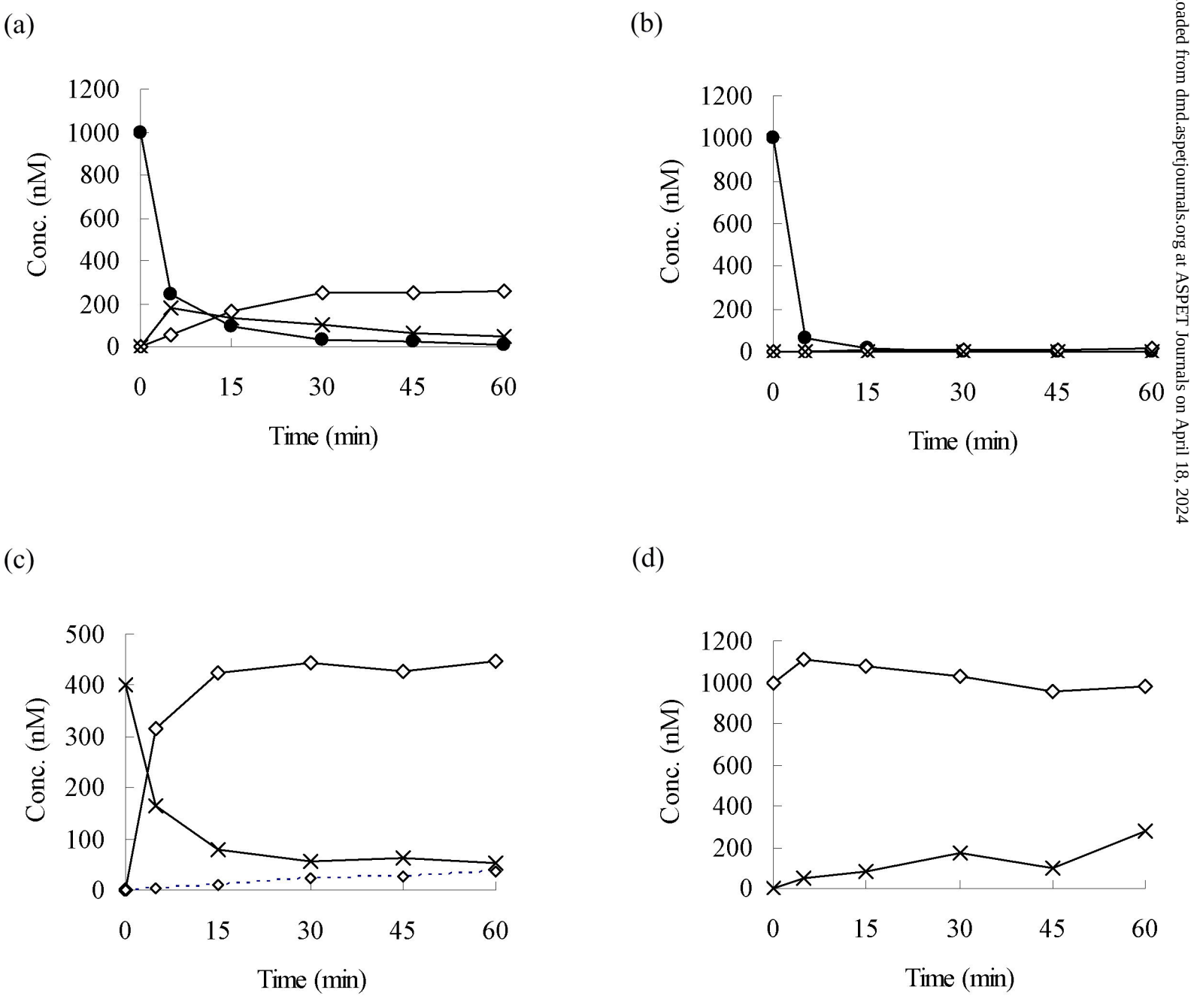
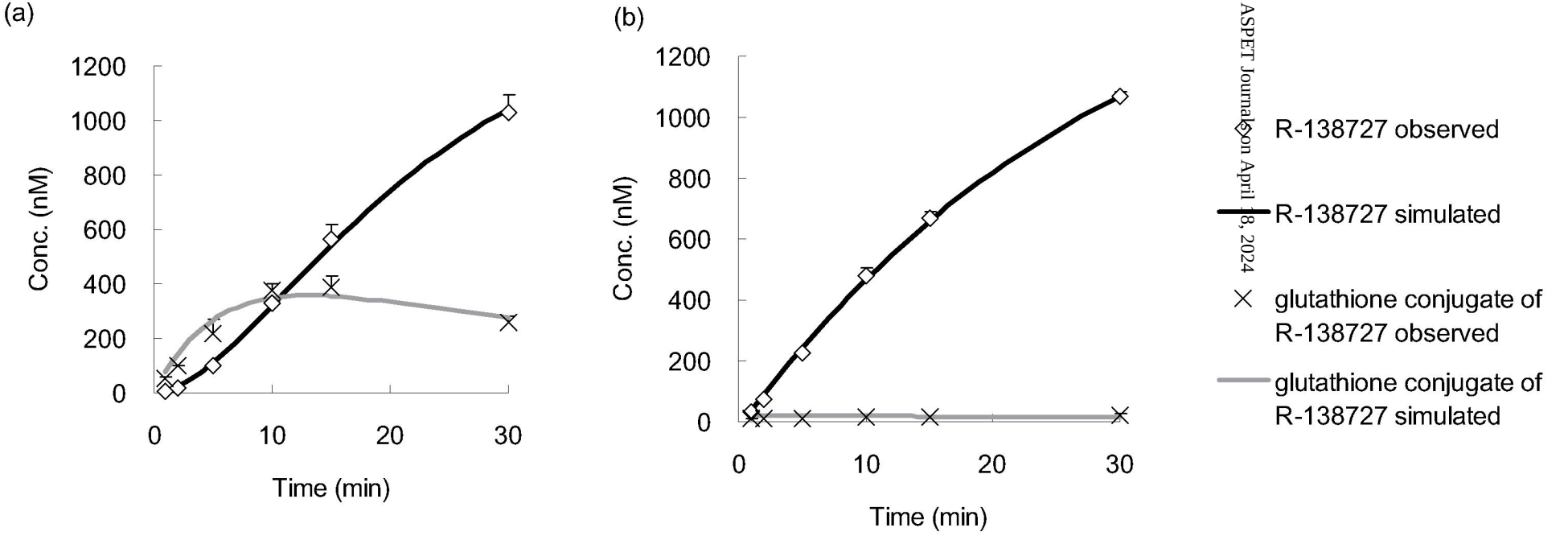


Figure 8



K1= 0.00850 /s	(CV5%)	K1= 0.00569 /s	(CV2%)
K2= 0.143 /s	(CV7%)	K2= 2.82 /s	(CV21%)
K3= 0.0149 /s	(CV35%)	K3= 0.0251 /s	(CV85%)
K4= 0.0416 /s	(CV11%)	K4= 0.0270 /s	(CV6%)