

# Development of Enantioselective Polyclonal Antibodies to Detect Styrene Oxide Protein Adducts

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Styrene has been reported to be pneumotoxic and hepatotoxic in humans and animals. Styrene oxide, a major reactive metabolite of styrene, has been found to form covalent binding with proteins, such as albumin and hemoglobin. Styrene oxide has two optical isomers and it was reported that the (*R*)-enantiomer was more toxic than the (*S*)-enantiomer. The purpose of this study was to develop polyclonal antibodies that can stereoselectively recognize proteins modified by styrene oxide enantiomers at cysteine residues. Immunogens were prepared by alkylation of thiolated keyhole limpet hemocyanin (KLH) with styrene oxide enantiomers. Polyclonal antibodies were raised by immunization of rabbits with the chiral immunogens. Titration tests showed all six rabbits generated high titers of antisera that recognize (*R*)- or (*S*)-coating antigens accordingly. No cross-reaction was observed toward the carrier protein (BSA). All three rabbits immunized with (*R*)-immunogen produced antibodies that show enantioselectivity to the corresponding antigen, while only one among the three rabbits immunized with (*S*)-immunogen generated antibodies with enantioselectivity of the recognition. The enantioselectivity was also observed in competitive ELISA and immunoblot analysis. Additionally, competitive ELISA tests showed that the immunorecognition required the hydroxyl group of the haptens. Immunoblot analysis demonstrated that the immunorecognition depended on the amount of protein adducts blotted and hapten loading in protein adducts. In summary, we successfully developed polyclonal antibodies to stereoselectively detect protein adducts modified by styrene oxide enantiomers.

Styrene (**1**) is widely used as an industrial material for the production of synthetic rubbers, plastic, insulation, fiberglass, and

automobile parts.<sup>1</sup> Workplace exposures to styrene are the highest in factories fabricating reinforced plastics products. Humans are potentially exposed to this widespread environmental contaminant.<sup>1–3</sup> Styrene causes both pneumotoxicity and hepatotoxicity in animals and humans. Styrene is mainly metabolized by cytochrome P450 to styrene oxide (**2**, SO), which was reported to be cytotoxic, mutagenic, and potentially carcinogenic. Styrene oxide is a known electrophilic species chemically reactive to nucleophiles of macromolecules, such as nucleotides and proteins, to form covalent binding adducts both in vitro and in vivo.<sup>4–6</sup> It has been reported that SO can covalently bind to a variety of nucleophilic functional groups in proteins, including the sulfhydryl group of cysteine, imidazole of histidine, the carboxylic acid group of aspartic and glutamic acid, amino group of lysine, and the *N*-terminal amino group of proteins.<sup>5,7–9</sup> Among those nucleophilic amino acids, cysteine has been reported as the most reactive amino acid residue to be modified by SO.<sup>8,10</sup> Albumin and hemoglobin SO adducts have been identified both in humans and animals. A dose–response relationship between the quantities of SO adducts and styrene exposure was observed in styrene-exposed workers.<sup>11,12</sup> Formation of protein covalent binding with xenobiotics or their active metabolites has long been recognized as a possible mechanism of chemical toxicity.<sup>13</sup> Immunochemical approaches have been successfully developed to detect protein

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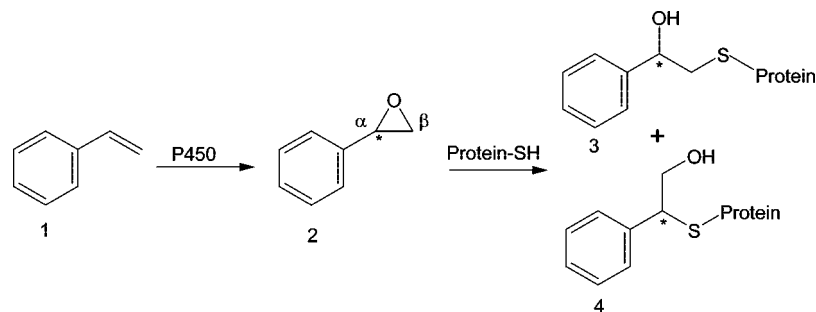
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## Scheme 1



adducts modified by reactive metabolites of xenobiotics, such as atrazine,<sup>14</sup> acetaminophen,<sup>15</sup> bromobenzene,<sup>16</sup> halothane,<sup>17</sup> naphthalene,<sup>18</sup> and trichloroethylene.<sup>19</sup> Recently, we developed polyclonal antibodies to recognize the proteins modified by racemic SO.<sup>20</sup>

Styrene oxide has one chiral center with two optical isomers as shown in Scheme 1. It was reported that the (*R*)-styrene oxide (*RSO*) was preferentially formed in mice, especially in the lung,<sup>21</sup> whereas the (*S*)-styrene oxide (*SSO*) was preferentially generated in rats.<sup>22–24</sup> In human volunteers, the cumulative excretion of the (*S*)-enantiomer of styrene glycol and mendelic acid were higher than the *R* form after exposure to styrene.<sup>25</sup> In human liver microsomes, cytochrome P450-mediated styrene oxidation showed the production of more *SSO* relative to *RSO*.<sup>26</sup> It was also found that *SSO* was preferentially hydrolyzed than *RSO* in human liver microsomes.<sup>26</sup> Animal studies showed that the (*R*)-enantiomer of SO was more toxic than the (*S*)-enantiomer in mice.<sup>27</sup> The mechanism of styrene-induced cytotoxicity is not fully understood. We proposed that the electrophilic epoxide metabolite of styrene attacks the nucleophilic amino acid residues, such as cysteine, of cellular proteins (Scheme 1), and the covalent modification of cellular proteins might initiate the consequent acute toxicity. The availability of enantioselective antibodies against SO modified proteins would provide insight into the role of SO chirality in styrene cytotoxicity both in vitro and in vivo.

Immunoassay offers a powerful tool to investigate the toxicological importance of protein modification in experimental animals and exposed humans. Antibody–antigen recognition is a highly

specific process by which antibodies are capable of recognizing antigens stereoselectively.<sup>28,29</sup> The purpose of this study was to develop enantioselective polyclonal antibodies to detect protein adducts modified by SO enantiomers. These antibody-based immunoassays are expected to facilitate our investigation on the biochemical mechanism of styrene-induced toxicity.

## EXPERIMENTAL SECTION

**Chemicals and Instruments.** (*R*)-styrene oxide (*RSO*, 98%), (*S*)-styrene oxide (*SSO*, 98%), bovine serum albumin (BSA, 99%), hemocyanin from keyhole limpet *Megathura crenulata* (KLH, 5.8 mg/mL PBS solution), horseradish peroxidase (HRP) conjugated antirabbit IgG secondary antibody, MagicMark XP Western protein standard, tris(2-carboxyethyl)phosphine (TCEP), and methyl 3-mercaptopropionate were purchased from Sigma-Aldrich (St. Louis, MO). *N*-Succinimidyl(3-(2-pyridyldithio))-propionate (SPDP), Supersignal West Pico chemiluminescence substrate kit, and AminoLink Coupling Resin were purchased from Pierce (Rockford, IL). Other reagents were of analytical grade. BD Falcon (Bedford, MA) 96-well microtiter plates were used in ELISA. The absorbance was measured with a microplate reader (VERSA<sub>Max</sub>, Molecular Devices, Sunnyvale, CA). The ELISA absorbance data were analyzed by SigmaPlot for Windows 9.0. SDS-PAGE was performed on a Bio-Rad mini electrophoresis system. Membrane transfer was conducted on a Bio-Rad Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell. Structural identification of synthetic compounds was performed on a 300 MHz NMR spectrometer (Varian Associates, Palo Alto, CA) and an LC/MS system including an Agilent 1100 HPLC system interfaced with a Sciex API 2000 tandem quadrupole mass spectrometer (Applied Biosystems, Foster City, CA).

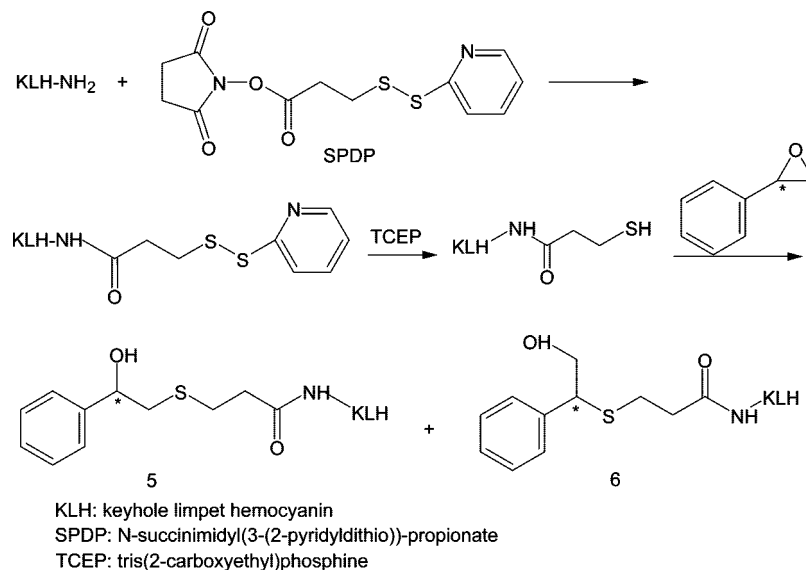
**Synthesis.** *Immunogens (Thiolated KLH–RSO/SSO Adducts, 5 and 6).* Immunogens were synthesized as illustrated in Scheme 2, using the method reported by our laboratory<sup>18,20</sup> with minor modification. SPDP (4.5 mg dissolved in 150  $\mu$ L of dimethyl sulfoxide (Me<sub>2</sub>SO)) was added to 6.0 mL of KLH solution (5.8 mg/mL) dropwise while stirring. After stirring for 1.5 h at room temperature, TCEP (52.6 mg dissolved in 300  $\mu$ L of PBS) was added to this mixture dropwise with stirring. After stirring for 3 h at room temperature under nitrogen, the pH of the mixture was adjusted to 9–10 with diluted NaOH solution, and then the mixture was divided into two aliquots. To each aliquot, 30  $\mu$ L of *RSO* or *SSO* diluted in 150  $\mu$ L of Me<sub>2</sub>SO was added dropwise with stirring. The mixture was stirred overnight at

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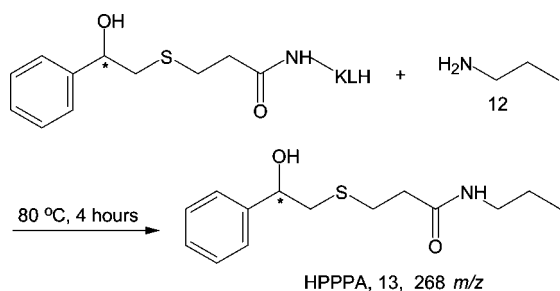
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## Scheme 2



## Scheme 3



room temperature under nitrogen. The next day, the products were dialyzed five times against 1 L of deionized water and lyophilized separately. Treatment of the thiolated KLH with *RSO* or *SSO* gave the (*R*)-immunogen and (*S*)-immunogen, respectively.

**Verification of Synthetic Immunogens.** In order to ensure success in the synthesis of the immunogens, i.e. (*R*)-immunogen and (*S*)-immunogen, we characterized the protein adducts as follows. The lyophilized immunogens (5.0 mg) were separately suspended in 4.0 mL of propylamine (**12**), followed by addition of 18 M NaOH solution (0.5 mL). The mixture was heated in a tightly closed tube with stirring at 80 °C in an oil bath for 4 h. After the mixture was cooled down to room temperature, the excess of propylamine was evaporated under a stream of nitrogen. The remaining was resuspended in 200  $\mu$ L of acetonitrile, vortexed for 3 min, and centrifuged at 12 000 rpm for 10 min. The supernatant was collected and subjected to LC/MS analysis. MS analysis was performed in selective ion monitoring (SIM) mode to monitor  $m/z$  268 [ $M + H$ ]<sup>+</sup> (Scheme 3). The control sample was treated by the same procedure without exposure to SO.

**BSA-*RSO* and BSA-*SSO* Adducts.** Protein adducts, bovine serum albumin-(*R*)-styrene oxide (BSA-*RSO*) and bovine serum albumin-(*S*)-styrene oxide (BSA-*SSO*), were synthesized as coating antigens (*R*)- and (*S*)-coating antigens for ELISA and positive control in Western blot analysis. To 2 mL of BSA solution (10 mg/mL in PBS buffer, pH 7.4), 20  $\mu$ L of *RSO* or *SSO* dissolved in 100  $\mu$ L of Me<sub>2</sub>SO was added and stirred overnight at room temperature. The resulting mixtures were extensively dialyzed

against deionized water and dried by lyophilization separately. In order to prepare BSA-*RSO* and BSA-*SSO* with a variety of hapten loading, BSA (10 mg/mL) was incubated with *RSO* or *SSO* at various concentrations (1.0, 5.0, and 10 mM) in PBS buffer (pH 7.4, 50% of Me<sub>2</sub>SO) with stirring overnight at 4 °C. A similar procedure of dialysis and lyophilization was performed as above.

**Methyl 3-(2-hydroxyphenylethylthio)propanoates (MHPPs, 7–10).** Compounds **7–10** were synthesized as competitors (analytes) for competitive ELISA and Western blot. They mimic the epitopes of protein adducts by SO at cysteine residues. Enantiomeric SO (360 mg, 3 mmol), either *RSO* or *SSO*, was dissolved in 5 mL of THF/water (10:1), followed by addition of methyl 3-mercaptopropanoate (372 mg, 3.1 mmol) and triethylamine (909 mg, 9 mmol). The reaction mixture was stirred at room temperature overnight, and then most of the solvent was removed under reduced pressure. The residue was extracted with ethyl acetate (10 mL  $\times$  3), and the combined organic phase was washed with saturated sodium bicarbonate solution and brine, dried over anhydrous sodium sulfate, and concentrated under a vacuum. The residue was chromatographed on silica gel to give the corresponding methyl 3-(2-hydroxy-2-phenylethylthio)propanoate (MH2PP (**7/9**), 281 mg, 39%) and methyl 3-(2-hydroxy-1-phenylethylthio)propanoate (MH1PP (**8/10**), 187 mg, 26%). The structures of the synthetic competitors were confirmed by NMR. MH2PP, <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  2.63 (m, 2 H), 2.77–2.98 (m, 4H), 3.73 (s, 3H), 4.78–4.82 (m, 1H), 7.32–7.40 (m, 5H). MH1PP, <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  2.52–2.57 (t,  $J = 7.5$  Hz, 2 H), 2.71–2.77 (m, 2H), 3.69 (s, 3H), 3.88–3.91 (m, 2H), 3.99–4.04 (m, 1H), 7.30–7.37 (m, 5H).

**Methyl 3-(2-phenylethylthio)propanoate (MPP, 11).** A mixture of 2-phenylethanethiol (138 mg, 1.0 mmol) and methyl acrylate (95 mg, 1.1 mmol) was stirred at room temperature until TLC analysis showed that most of 2-phenylethanethiol was consumed, and then the reaction mixture was subject to silica gel chromatography without further workup to give MPP (**11**, 96 mg, 42.8%) as a colorless oil. MPP, <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  2.61–2.66 (t,  $J =$

7.5 Hz, 2H), 2.81–2.84 (m, 4H), 2.89–2.94 (m, 2H), 7.21–7.35 (m, 5H).

**3-(2-Hydroxy-2-phenylethylthio)-N-propylpropanamide (HPPPA, 13).** MH2PP (**7/9**, 50 mg, 0.2 mmol) was dissolved in 1.0 mL methanol, followed by addition of 3.0 mL of propylamine and stirred at room temperature until TLC analysis showed that most of MH2PP was consumed. Then the reaction mixture was subject to silica gel chromatography without further workup to give HPPPA (35 mg, 67%). HPPPA,  $^1\text{H NMR}$  ( $\text{CDCl}_3$ ):  $\delta$  0.88–0.93 (t,  $J = 7.4$  Hz, 3H), 1.46–1.56 (m, 2H), 2.40–2.53 (m, 2H), 2.72–2.93 (m, 4H), 3.15–3.21 (m, 2H), 4.76–4.80 (m, 1H), 6.14 (s, 1H), 7.28–7.38 (m, 5H). LC/MS:  $m/z$  268  $[\text{M} + \text{H}]^+$ .

**Immunization of Rabbits.** Six female New Zealand white rabbits (Herbert's Rabbitery, Plymouth, CA) weighing 2.5–3.0 kg were divided into two groups and immunized with (*R*)- and (*S*)-immunogens, respectively. Each immunogen (100  $\mu\text{g}$ ) was dissolved in 0.5 mL of PBS buffer (pH 7.4) and emulsified with 0.5 mL of Freund's complete adjuvant. The rabbits were injected subcutaneously with the emulsion (1.0 mL/rabbit) at multiple sites on the back. The rabbits were boosted with a 2 week interval by the same procedure except that the Freund's complete adjuvant was replaced by Freund's incomplete adjuvant. Test bleeds were collected before each boosting injection, and the titer and enantioselectivity was examined by ELISA. The rabbits were boosted until no further increase in antibody titer was observed.

**Analysis of Antiserum Titer.** The titer of the antisera obtained from the rabbits immunized with (*R*)-immunogen or (*S*)-immunogen was determined by measuring the binding of serial dilutions (1:400 to 1:12 800) to microtiter plates coated with coating antigens BSA–RSO, BSA–SSO, or native BSA. Coating antigen solution (100  $\mu\text{L}$ , 20  $\mu\text{g}/\text{mL}$ ) in PBS (10 mM phosphate-buffered saline, pH 7.4, 0.15 M NaCl) was added to each well of 96-well microtiter plates and incubated at 4 °C overnight. The coating antigen solution was discarded, and the plates were washed three times with PBST (10 mM phosphate-buffered saline, pH 7.4, 0.15 M NaCl, 0.05% Tween-20). After washing, 150  $\mu\text{L}$  of 5% nonfat milk–PBST buffer was added to each well and incubated at room temperature for 1.5 h. The plates were washed three times with PBST buffer after incubation, and 100  $\mu\text{L}$  of serially diluted antiserum in PBST was added to each well. After incubation at room temperature for 2 h, the plates were washed three times in the same manner, and 100  $\mu\text{L}$  of HRP conjugated secondary antibody (antirabbit IgG, 1:10 000) in PBST was added to each well. After incubation at room temperature for 1 h, the plates were washed three times as before, and 100  $\mu\text{L}$  of substrate solution (0.3 mM tetramethylbenzidine, 0.4 mM  $\text{H}_2\text{O}_2$  in 0.1 M sodium acetate buffer at pH 5.5) was added to each well. After incubation for 15 min at room temperature, the colorimetric development was quenched by adding 50  $\mu\text{L}$  of 4 N  $\text{H}_2\text{SO}_4$  solution to each well. The absorbance at dual wavelengths (450–650 nm) was measured. To evaluate its affinity, each antiserum was tested on all three coating antigens. The titration curves were fitted by four-parameter logistic equation using SigmaPlot. The enantioselectivity index was defined as the ratio of  $\text{EC}_{50}$  (antiserum dilution which produced 50% highest absorbance) obtained from (*R*)- or (*S*)-coating antigens ( $\text{REC}_{50}$  or  $\text{SEC}_{50}$ ).

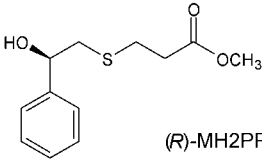
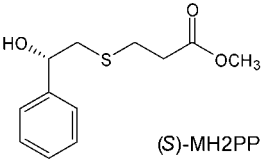
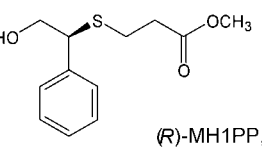
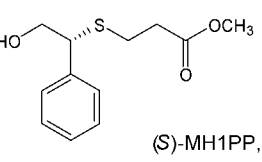
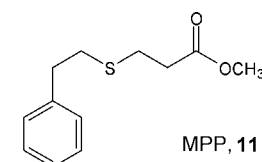
**Competitive ELISA.** Totally five competitors (**7–11**) were used in the competitive ELISA (Table 1). A volume of 100  $\mu\text{L}$  of coating antigen solution (20  $\mu\text{g}/\text{mL}$  of BSA–RSO or BSA–SSO in PBS) was added to each well of 96-well microtiter plates and incubated at 4 °C overnight. Serial dilutions of the competitors (MHPPs or MPP,  $1\text{--}10^{-7}$  mM except for that of (*R*)-MH2PP, i.e.,  $10\text{--}10^{-6}$  mM) were prepared in diluted antisera (1:3200) in 5% nonfat milk–PBST buffer. The resulting mixtures were incubated at 4 °C overnight. The following day, the same procedure in the titer analysis was followed. The absorbance at dual wavelengths (450–650 nm) was read. The obtained absorbance values were converted using the equation: control (%) =  $A/A_0 \times 100\%$ , where  $A$  represents the absorbance values in the presence of a competitor and  $A_0$  is the absorbance without a competitor. The resulting competition curves were fitted by a four-parameter logistic equation using SigmaPlot.  $\text{IC}_{50}$  values (the concentration that produced 50% inhibition) were obtained from the regression curve. The cross activity was defined as the ratio of  $\text{IC}_{50}$  from the same antiserum to the corresponding enantiomer.

**Western Blot Analysis.** A total of 2  $\mu\text{g}$  of protein was loaded and resolved by SDS-PAGE. The protein bands resolved were then transferred to a PVDF membrane using a semidry transfer cell (Bio-Rad) at 15 V for 30 min. Following transfer, the membrane was washed in Tris-buffered saline with Tween-20 (TBST, 10 mM Tris, 0.15 M NaCl, 0.05% Tween-20, pH 7.5) once and blocked with 5% nonfat milk–TBST buffer for 1 h at room temperature. After the membrane was blocked, it was incubated with antiserum no. 1254 (antiserum against the (*R*)-immunogen, 1:2 000) in 5% nonfat milk–TBST buffer overnight at 4 °C. The next day, the membrane was washed with TBST four times for 2 min and then incubated with a HRP conjugated antirabbit antibody at a 1:10 000 dilution in 5% nonfat milk–TBST buffer for 1 h at room temperature. The membrane was again washed with TBST buffer four times, and the protein bands were detected by Supersignal West Pico chemiluminescence kit and visualized using a Perkin-Elmer Geliance 600 imaging system (Perkin-Elmer, Fremont, CA). Immunoblot treated with antiserum no. 1257 (antiserum against the (*S*)-immunogen, 1:2 000) was performed in parallel.

**Exposure of WI-38 Cells to Styrene Oxide Enantiomers.** Human lung cell line WI-38 cells were cultured in RPMI 1640 medium (Mediatech Inc., Manassas, VA) supplemented 10% FBS (HyClone, Logan, UT). WI-38 cells were seeded on 6-well plates at a density of  $2 \times 10^5$  cells per well. The culture medium was discarded after being cultured for 24 h, and fresh medium containing 0.5 mM or 2.0 mM RSO or SSO was supplemented. The cells were cultured for another 24 h, and the cells were harvested by digestion and washed with PBS three times. The cells were lysed by sonication on ice for 1 min. The protein concentration of the cell lysate was determined by the BCA protein determination kit.

**Immobilization of BSA–RSO and BSA–SSO to Agarose Beads.** AminoLink Coupling Resin (Pierce, Rockford, IL) was utilized as a beaded agarose support. BSA (20 mg in 2 mL buffer) was immobilized to 2 mL of the resin following the manufacturer's instruction. By comparison of the BSA concentration before and after immobilization, the coupling efficiency was found to be about 80%. After immobilization of BSA, 10  $\mu\text{L}$  of RSO or SSO dissolved

**Table 1. Structures of Analytes, IC<sub>50</sub> Values, and Calculated Cross Activity of Antisera Numbers 1254 and 1257**

Structures of analytes	Antiserum no. 1254		Antiserum no. 1257	
	IC <sub>50</sub> (mM)	Cross reactivity	IC <sub>50</sub> (mM)	Cross reactivity
 (R)-MH2PP, <b>7</b>	5.0	100%	>10	<3.6%
 (S)-MH2PP, <b>9</b>	No inhibition	Negligible	0.36	100%
 (R)-MH1PP, <b>8</b>	0.39	100%	>1	<2%
 (S)-MH1PP, <b>10</b>	0.9	43%	0.02	100%
 MPP, <b>11</b>	No inhibition	Negligible	No inhibition	Negligible

in 100  $\mu$ L of Me<sub>2</sub>SO was added to the resin with 1 mL of 0.1 M PBS at pH 9.2 and mixed overnight at 4 °C. The resulting resin was packed into a column and washed with 10 mL of 0.1 M PBS pH 7.2 for use.

**Purification of Antibodies.** In order to enhance the enantioselectivity of the polyclonal antibodies, the antisera were purified by use of the affinity columns prepared above. Briefly, antiserum no. 1254 or no. 1257 (10  $\mu$ L) was diluted in 0.5 mL of 0.1 M PBS (pH 7.2) and slowly loaded onto the affinity column. After standing at room temperature for 2 h, the column was eluted with 12 mL of 0.1 M PBS and fractions were collected. The enantioselectivity of each fraction was evaluated by titration analysis. The column was regenerated by elution with 8 mL of 0.1 M glycine-HCl (pH 2.5) buffer and then 16 mL of 0.1 M PBS (pH 7.2).

## RESULTS AND DISCUSSION

The biochemical mechanism of styrene-induced cytotoxicity has not been fully understood. It was suggested that styrene oxide

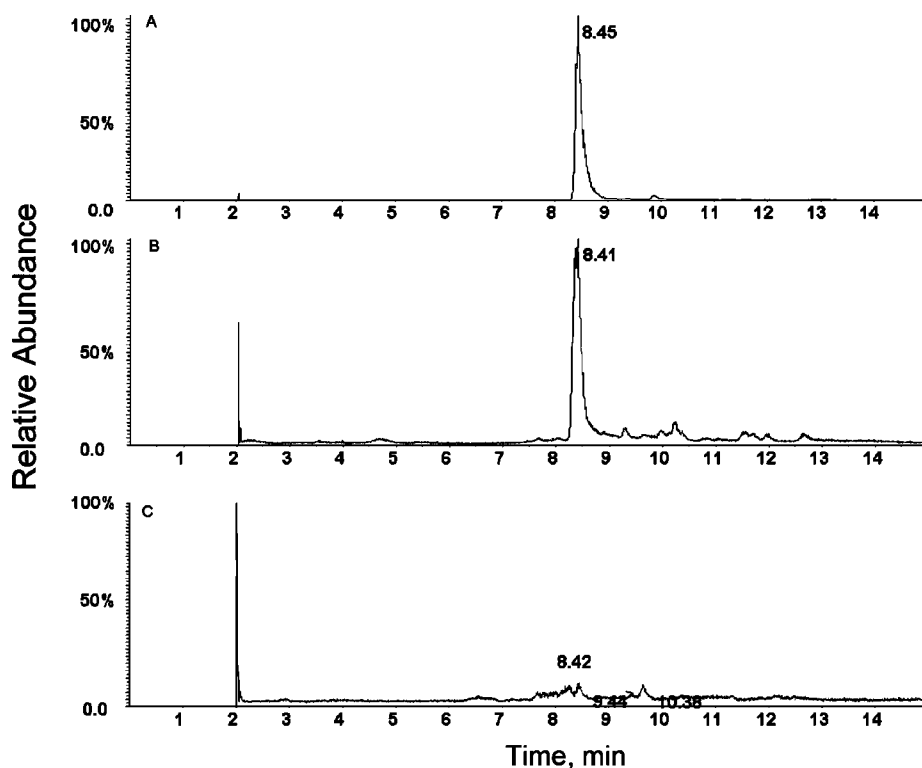
(SO) as a major metabolite is associated with the cytotoxicity of styrene. We proposed that protein modification by SO may play an important role in styrene toxicity. Recently, we prepared a polyclonal antibody as an experimental tool to detect racemic SO modified proteins.<sup>20</sup> The effort in the development of antibodies to selectively detect protein adducts modified by SO enantiomers was inspired by the findings of toxic effects of SO with enantiomeric differentiation.<sup>19–25</sup> The availability of the antibodies would assist us to investigate the enantiomeric role of SO in styrene-mediated toxicity. A number of immunoassays developed for analysis of chiral drugs, such as propranolol,<sup>30</sup> alprenolol,<sup>31</sup> and pentobarbital,<sup>32</sup> ractopamine,<sup>33</sup> finrozole,<sup>34</sup> and S-20499,<sup>35</sup> have been reported. Some efforts have been made to develop enanti-

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**Figure 1.** LC/MS profiles of (*R*)-immunogen (B) and thiolated KLH (C) after reaction with propylamine at 80 °C for 4 h. LC/MS profile of authentic synthetic 3-(2-hydroxy-2-phenylethylthio)-*N*-propylpropanamide (A).

oselective antibodies to detect protein adducts modified by chiral ligands, such as ibuprofen.<sup>36</sup> The present study is a follow-up of our earlier antibody work designed to detect protein SO (racemic) adducts.

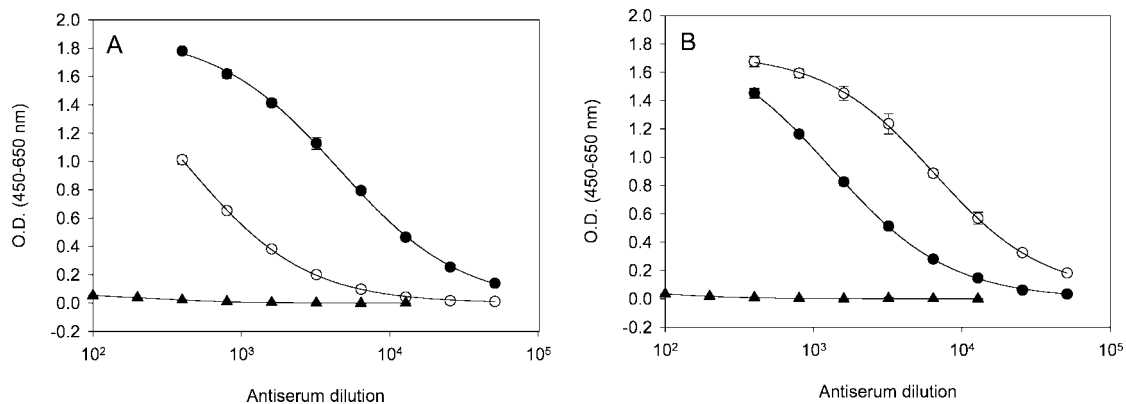
**Synthesis and Verification of the Immunogens.** Immunogens were synthesized following an earlier procedure by which we succeeded in raising polyclonal antibodies against protein adducts modified by SO racemates. Carrier protein KLH was first thiolated by SPDP, and the resulting thiolated protein was reduced by TCEP to generate free sulfhydryl groups, which immediately reacted with *RSO* or *SSO*, respectively (Scheme 2). We learned from our previous work that the thiolation of KLH allowed us to incorporate a spacer between the carrier protein and the hapten to increase the visibility of the hapten.

Verification of the structure of immunogens is an important step to succeed in development of antibodies and is often a challenge in immunochemistry. In the present study, we successfully developed a chemical approach to characterize the incorporation of the hapten to the carrier protein. As shown in Scheme 2, the thiolation of the carrier protein by reacting with SPDP introduces an amide. This provides the possibility to release the carboxyl moiety by transamidation. The transamidation reaction was performed by heating the immunogens in neat propylamine (12), and the reaction was expected to produce amide HPPPA

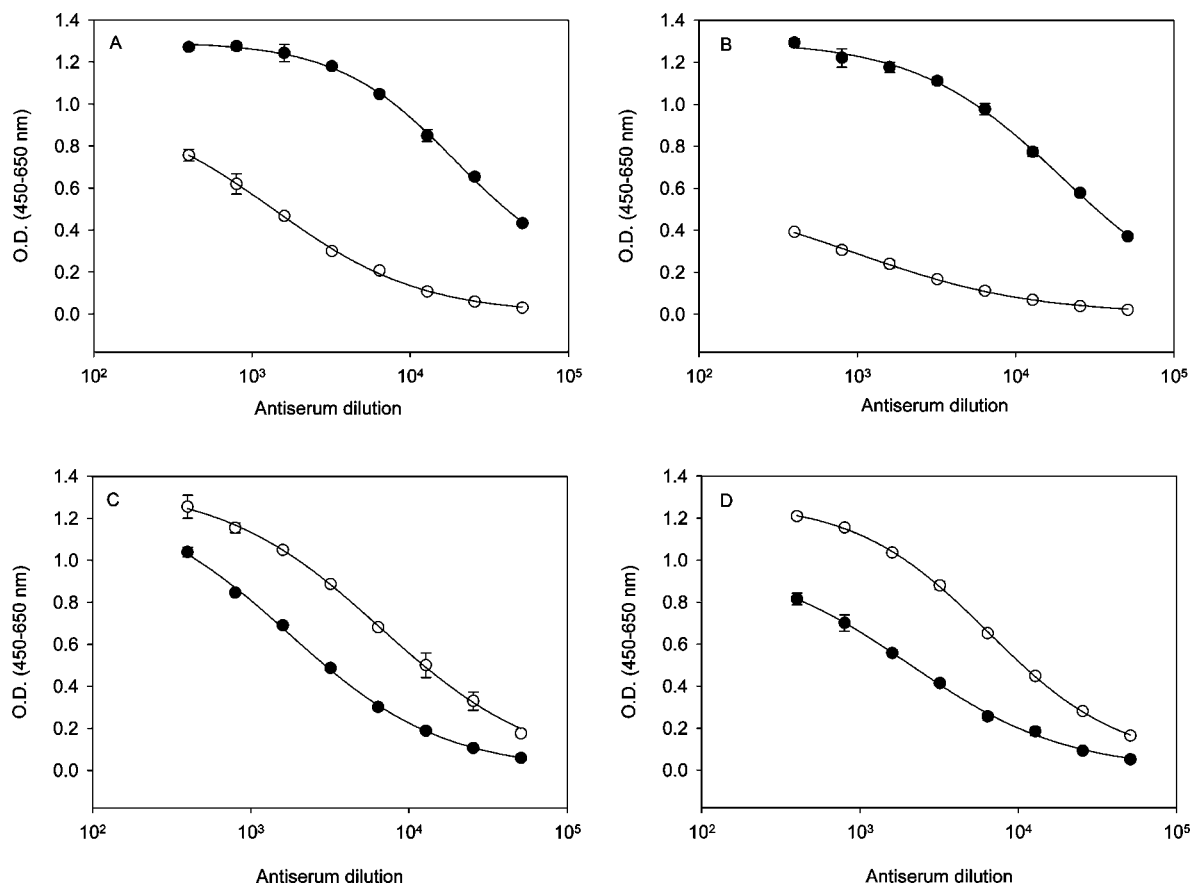
(13, Scheme 3), which can be readily detected by LC/MS. HPPPA was synthesized by conjugating methyl 3-mercaptopropionate with SO, followed by reaction with propylamine. Immunogens, including the KLH adducts modified by either *SSO* or *RSO*, were treated with propylamine and analyzed by LC/MS in the SIM mode. Thiolated KLH as a negative control was treated in parallel. Figure 1 shows the ion chromatography profiles for *m/z* 268, and a peak at 8.4 min was observed in the sample obtained from the immunogens after treatment with propylamine. The retention time of the peak (Figure 1B) was found to be identical to that of authentic standard 13 (Figure 1A). As expected, no such peak was detected in the control sample (Figure 1C). The observed amide 13 indicates the incorporation of the hapten into the carrier protein. Although this approach was unable to provide quantitative information about hapten loading due to the lacking of reaction yields, it allowed us to verify the hapten incorporation into the protein in identity. The assurance of the chemical identities of immunogens was an important prerequisite for the success in the preparation of antibodies.

**Analysis of Titer.** Immunizations of rabbits were carried out with (*R*)- and (*S*)-immunogens. The titers of the antisera obtained from the rabbits were determined by measuring the binding of serial dilutions (1:400 to 1:12 800) to microtiter plates coated with BSA-*RSO*, BSA-*SSO*, or native BSA. The enantioselectivity of the antibodies was evaluated by the degree of cross-reaction to the counter-enantiomeric coating antigens. The results showed that all six rabbits gave very high titers of antibodies against their own enantiomeric coating antigens accordingly. The three rabbits (numbers 1252, 1253, and 1254) immunized with the (*R*)-immunogen produced enantioselective

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**Figure 2.** Titer analysis of antisera numbers 1254 (A) and 1257 (B) in microtiter plates coated with BSA-RSO (●), BSA-SSO (○), or native BSA (▲). Curves were fitted by four-parameter logistic equation using SigmaPlot. Error bars indicate standard deviations of triplicate determinations.

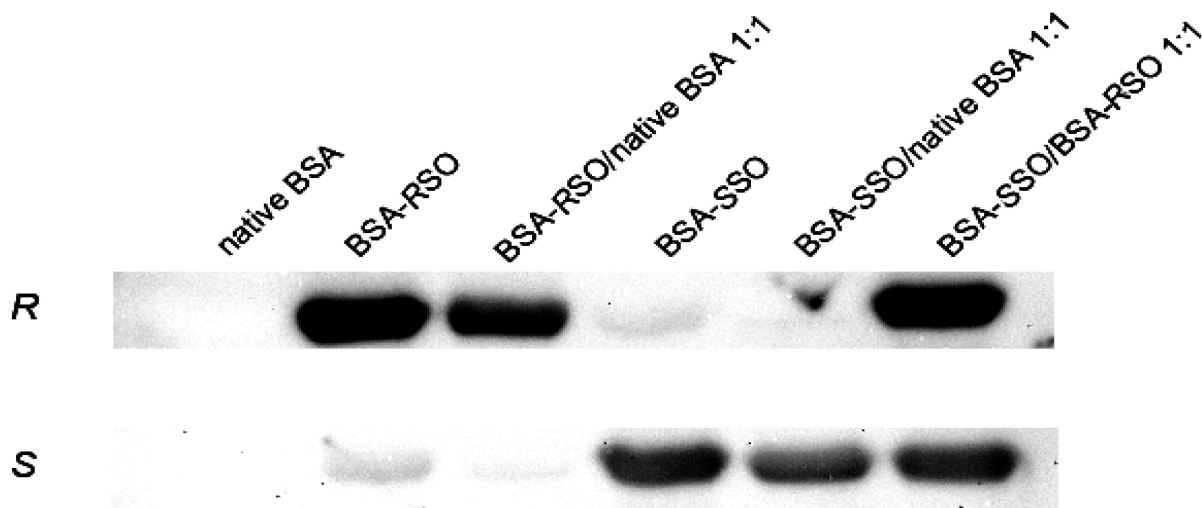


**Figure 3.** Titer analysis of antiserum no. 1254 before (A) and after (B) purification and antiserum no. 1257 before (C) and after (D) purification. Titration was performed in microtiter plates coated with BSA-RSO (●) or BSA-SSO (○).

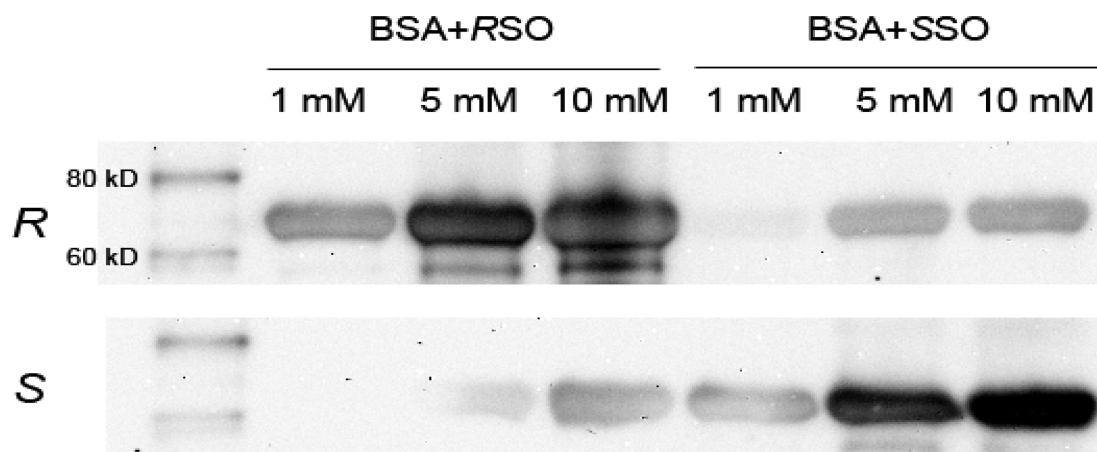
antibodies (Figure S-1 in the Supporting Information), while only one rabbit (no. 1257) among the three of the (S)-group gave enantioselective antibodies (Figure S-1 in the Supporting Information). The antisera obtained from the six rabbits showed little cross-reaction to native BSA. Among the rabbits immunized with the (R)-immunogen, rabbit no. 1254 produced the antiserum with highest titer and enantioselectivity. Figure 2A shows the titration of antiserum no. 1254 against (R)- and (S)-coating antigens as well as native BSA. The enantioselectivity index ( $REC_{50}/SEC_{50}$ ) of antiserum no. 1254 was estimated to be 10. Similarly, Figure 2B shows the titration of antiserum obtained from rabbit no. 1257 immunized with

the (S)-immunogen. The enantioselectivity index ( $SEC_{50}/REC_{50}$ ) for antiserum no. 1257 was approximately 5. Because of the higher titer and enantioselectivity, antisera numbers 1254 and 1257 were selected for use in the remainder of the studies.

**Competitive ELISA.** Competitive ELISA was conducted to characterize the hapten-selectivity and enantioselectivity of the antibodies. Antisera numbers 1254 and 1257 raised against respective (R)- and (S)-immunogen were diluted to 1:3 200 and used for competitive ELISA in plates coated with the corresponding coating antigens as those for the titration tests. A total of four SO derivatives, including MHPPs 7–10, and MPP were employed



**Figure 4.** Western blot analysis of native BSA, BSA–RSO, BSA–SSO, BSA–RSO/BSA 1:1 mixture, BSA–SSO/BSA 1:1 mixture, BSA–RSO and BSA–SSO 1:1 mixture. Protein bands were incubated with antisera no. 1254 (*R*, 1:2000) and no. 1257 (*S*, 1:2000). Each lane was loaded with 2.0  $\mu$ g of protein.



**Figure 5.** Western blot analysis of BSA exposed to various concentrations (1.0, 5.0, and 10 mM) of *RSO* or *SSO*. Protein adducts bands were treated with antisera no. 1254 (*R*, 1:2000) and no. 1257 (*S*, 1:2000). Each lane was loaded with 2.0  $\mu$ g of protein.

as competitors (analytes). The antisera were preincubated with a serial dilution of the synthetic competitors.

Table 1 lists the  $IC_{50}$  values obtained from the competitive ELISA analysis. As expected, the preincubation of antisera with MHPs **7/8** and **9/10** showed inhibitory effects on the binding of the antibodies to the corresponding coating antigens (Figure S-2 in the Supporting Information). Compounds **7/9** and **8/10** are two pairs of enantiomers derived from *RSO* and *SSO*, respectively. The affinity of the two pairs to the antibodies allowed us to evaluate the enantioselectivity of the antisera raised from (*R*)- and (*S*)-immunogens. Antiserum no. 1254 was preincubated with a serial dilution of the analytes in microtiter plates coated with (*R*)-coating antigen, and antiserum no. 1257 was treated similarly but with (*S*)-coating antigen. As expected, antiserum no. 1254 showed higher affinity to analytes **7** and **8** than **9** and **10** accordingly. However, antiserum no. 1257 showed higher affinity to analytes **9** and **10** than **7** and **8**. This was the additional evidence for the enantiomeric recognition of chiral SO-derived haptens by antisera numbers 1254 and 1257.

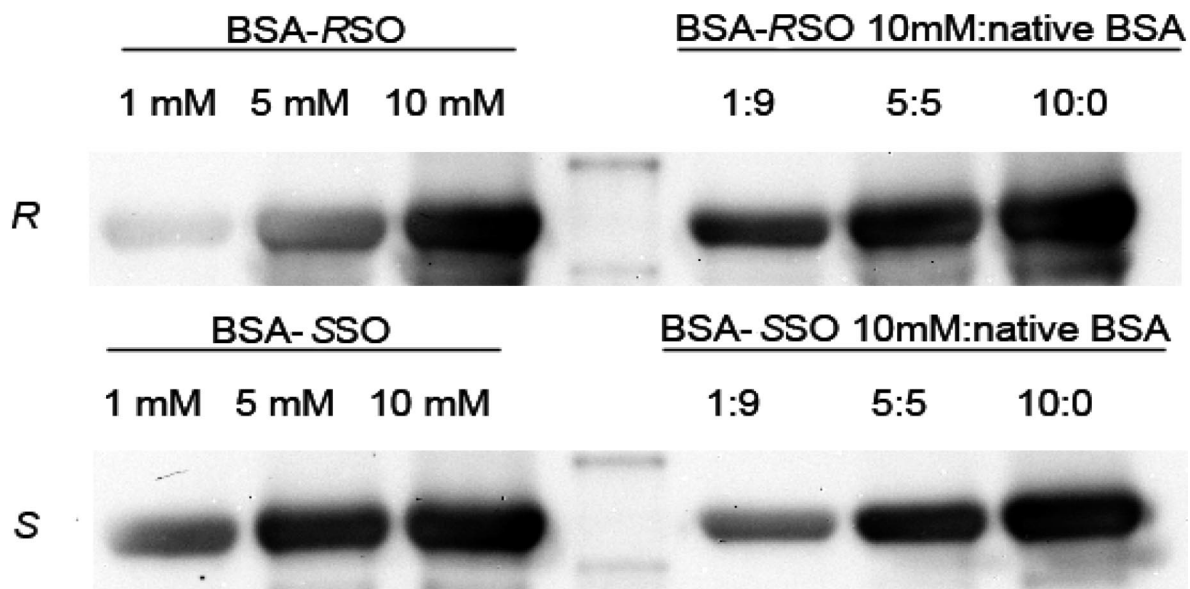
Among the five analytes tested, only MPP failed to inhibit the binding of the antibodies to the coating antigens. This indicates the critical role of the hydroxyl group of the haptens in the

interactions between the antibodies and the haptens. Importantly, this provides strong evidence for the success in the preparation of the antibodies we desired. The immuno-recognition pinpointed the core of the haptens.

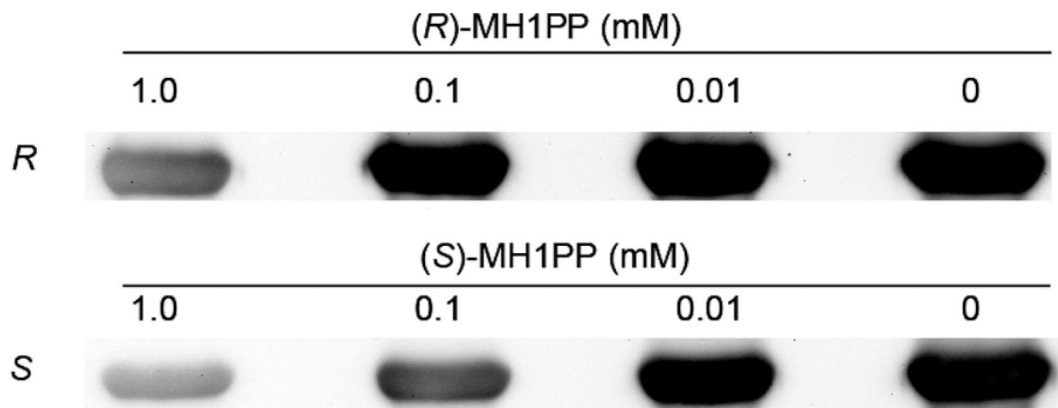
Compounds **7/8** and **9/10** are two pairs of positional isomers. The positional isomers of each pair revealed differentiated inhibition of the antibody-coating antigen binding (Table 1). Interestingly, isomers **8** and **10** were found to be more potent than isomers **7** and **9**, respectively. Isomers **8** and **10** were derived from *RSO* and *SSO* with conjugation at the  $\alpha$ -carbon, and isomers **7** and **9** were derived from the conjugation at the  $\beta$ -carbon. Our earlier studies found that the reaction of SO with *N*-acetyl cysteine produced a 2:1 mixture of the  $\alpha$ -carbon conjugate over the  $\beta$ -carbon conjugate.<sup>20</sup> It is likely that similar preferred nucleophilic conjugation took place when SO reacted with thiolated KLH. This may explain why the polyclonal antibodies revealed higher affinity toward competitors **8/10** rather than **7/9**.

**Purification of Antiserum.** Affinity purification is a conventional and useful method to purify antibodies, and we examined whether affinity chromatography would significantly improve the enantioselectivity of the antibodies. Protein adducts BSA–*RSO* and BSA–*SSO* were coupled to the AminoLink Coupling Resin





**Figure 6.** Western blot analysis of BSA adducts exposed to a selection of concentrations (1.0, 5.0, and 10 mM, left) of *RSO* or *SSO*, and BSA adduct (after exposure to 10 mM of *RSO* or *SSO*) diluted with native BSA (right). BSA-*RSO* bands were incubated with antiserum no. 1254 (*R*, 1:2000) and BSA-*SSO* bands were incubated with antisera no. 1257 (*S*, 1:2000). Each lane was loaded with 2.0  $\mu$ g of protein.



**Figure 7.** Competitive Western blot analysis of BSA-*RSO* and BSA-*SSO* adducts. BSA-*RSO* bands were incubated with antiserum no. 1254 (*R*, 1:2000) mixed with (*R*)-MH1PP at concentrations of 0, 0.01, 0.1, and 1.0 mM. BSA-*SSO* bands were incubated with antiserum no. 1257 (*S*, 1:1000) together with (*S*)-MH1PP at concentrations of 0, 0.01, 0.1, and 1.0 mM. Each lane was loaded with 2.0  $\mu$ g of protein.

individually as described in the experimental procedure. The undesired antibodies were retained on the affinity columns, and the desired antibodies were eluted out. The titers of the purified antisera were evaluated by titration tests. Figure 3 shows the titrations of antisera numbers 1254 and 1257 before and after the affinity purification. The results showed that their enantioselectivity was appreciably improved, but the purification system had limited capacity. It appears that it is not realistic to obtain enantioselective antibody from racemic antibodies by means of affinity chromatography.

**Western Blot Analysis.** The enantioselectivity of antisera numbers 1254 and 1257 was assessed by Western blot analysis. As shown in Figure 4, a chemiluminescent band at the molecular weight of BSA was observed in the lane loaded with BSA-*RSO* adduct incubated with antiserum no. 1254. As expected, no chemiluminescent band was observed in the lane loaded with native BSA. This suggests that the antibodies are able to detect BSA adduct modified with SO and show no cross-reaction toward native BSA. In the same experiment, BSA-*SSO* adduct was loaded

in SDS-PAGE for immunoblot analysis, and no bands with significant chemiluminescence were observed. This indicates little cross-reaction of the antiserum raised from the (*R*)-immunogen toward the (*S*)-antigen, BSA-*SSO* adduct. Similarly, antiserum no. 1257 showed no cross-reaction toward native BSA and minor affinity to the (*R*)-antigen, BSA-*RSO* adduct. These immunoblot results clearly indicate that antisera numbers 1254 and 1257 can differentiate the chirality of the haptens and provide high enantioselectivity of immunorecognition.

In an effort to evaluate the correlation between antibody binding and hapten loading in BSA-*RSO* and BSA-*SSO* adducts, these adducts with various hapten loading were synthesized by exposure of BSA to *RSO* or *SSO* at concentrations of 1, 5, and 10 mM, respectively. After extensive dialysis, the resulting BSA-*RSO* and BSA-*SSO* adducts were subjected to Western blot analysis. As expected, the higher the hapten load, the greater the luminescent intensity in the immunoblot (Figure 5), indicating that the strength of antibody recognition depended on the hapten loading of the protein adducts.

At the same time, the BSA-*RSO* and BSA-*SSO* adducts synthesized by exposure to 10 mM of SO enantiomer were diluted with native BSA at ratios of 1:9 and 1:1 and were analyzed by Western blot. The dilution ratios corresponded to the concentrations of SO enantiomers (1, 5, 10 mM) used the preparation of BSA-*RSO* and BSA-*SSO* adducts. As shown in Figure 6, the pattern of luminescent bands was found similar to that observed in the Western blot of BSA exposed to various concentrations of SO enantiomers. The BSA-*RSO* and BSA-*SSO* adducts without native BSA dilution produced the highest luminescent intensity, followed by those diluted with 2- and 10-fold BSA. This suggested a good quantitative immunoresponse of the antibodies to the protein adducts.

Competitive immunoblot analysis was conducted to determine the hapten selectivity of the antibodies. The membrane blotted with BSA-*RSO* and BSA-*SSO* adducts was cut into four pieces and incubated with antisera numbers 1254 and 1257 containing a series of concentrations (0, 0.01, 0.1, and 1 mM) of (*R*)-MH1PP, **8**, for antiserum no. 1254 or (*S*)-MH1PP, **10**, for antiserum no. 1257, respectively. The resulting membranes were incubated with the secondary antibody and treated with the chemiluminescence kit as above. As shown in Figure 7, the chemiluminescent intensity decreased with the increasing of the concentrations of the competitors coincubated with the corresponding antisera. This indicates that the binding of the antibodies to SO protein adducts is attributable to the antibody recognition of the SO-protein modification at cysteine residues. We did not test the effects of (*R*)-MH2PP and (*S*)-MH2PP in the competitive immunoblot analysis, since competitive ELISA experiments above showed that the affinity of (*R*)-MH2PP and (*S*)-MH2PP to the corresponding antibodies was much weaker than that of (*R*)-MH1PP and (*S*)-MH1PP.

**Western Blot Analysis of Cellular Protein Modified by Styrene Oxide Enantiomer.** To examine the ability of the antisera to detect SO modified cellular protein, WI-38 cellular protein modified by *RSO* or *SSO* were subjected to Western blot analysis. The blotted protein was detected by corresponding numbers 1254 or 1257 antiserum (1:1000), respectively. Several new bands were detected after exposure to *RSO*, and high-density bands were detected in cellular protein exposed to high concentration (Figure S-3 in the Supporting Information). The result showed the antibodies can recognize cellular protein modified by SO, and it was concentration dependent.

In summary, we have successfully developed polyclonal antibodies to recognize chiral SO-derived protein (BSA) adducts modified at cysteine residues with no cross reaction toward native BSA. The antibodies revealed high enantioselectivity of immunorecognition, providing a powerful tool for the investigation of the biochemical mechanism of cytotoxicity induced by styrene.

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#### SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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