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Non-enzymatic reduction of a 1,2,4-thiadiazolium derivative

Fa Zhang,[a,*] Carmelita Estavillo,[a] Margie Mohler[b] and Jane Cai[a]

[a]*Analytical Development, Johnson & Johnson Consumer and Personal Products Worldwide, Skillman, NJ 08558, USA*
[b]*Ciencia Group, LLC, Yountville, CA 94599, USA*

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Abstract—It was discovered that 2,3-bis-(2-methoxy-phenyl)-5-phenylamino-[1,2,4]-thiadiazolium bromide (**1**), a 1,2,4-thiadiazolium derivative, could be reduced to the corresponding imidoylthiourea, 1-[(2-methoxy-phenyl)-(2-methoxy-phenylimino)-methyl]-3-phenyl-thiourea (**3**), by some biologically interesting reducing reagents including glutathione, cysteine, and ascorbic acid. The reduction also occurred in Sprague–Dawley rat and Yorkshire swine plasma, suggesting that thiol containing biological molecules existing in the plasma are mainly responsible for this reaction. A facile method for preparation of **3** from **1** was established by using 2-thioethanol as reaction reagent as well as solvent. The structure of **3** was fully characterized using nuclear magnetic resonance (NMR) and mass spectrometry with electrospray ionization source (ESI-MS). Those new findings could shed light on the development of 1,2,4-thiadiazolium derivatives for their potential pharmaceutical applications.

1,2,4-Thiadiazolium derivatives could serve as melanocortin receptor modulators.[1,2] Due to the fact that melanocortin receptors modulate physiological functions such as feeding behavior, nerve regeneration, drug addiction, and even dermatological conditions, 1,2,4-thiadiazolium derivatives could be potentially used for treatment of metabolic, central nerve system (CNS), and dermatological disorders. Even though there are numerous reports regarding the synthesis and pharmacological investigations on 1,2,4-thiadiazolium derivatives,[1–6] there are very limited reports on the chemical properties of this type of compound. As a matter of fact, pharmaceutical properties including chemical properties of new chemical entities (NCE) are one of the major factors to impact their developability and attrition rate in the pharmaceutical development processes. Understanding the chemistry of NCEs has become critical to facilitate those processes. Kurzer and co-authors[7] reported the isomerization of 2-aryl-5-arylamino-3-arylimino-Δ^4-1,2,4-thiodiazolines to 2-guanidinobenzothiazoles. Kevin and co-authors[2] found that 2-(4-methylphenyl)-3-phenyl-5-(4-methoxyphenylamino)-[1,2,4] thiadiazolium bromide could be reduced to 1-(4-methoxyphenyl)-3-[*N*-(4-methylphenyl)benzimidoyl]-2-thiourea by dithioerythritol in dichloromethane. Kevin and co-authors[8] also reported that 2,3-bis-(2-methoxy-phenyl)-5-phenylamino-[1,2,4]-thiadiazolium bromide (**1**), which is a 1,2,4-thiadiazolium derivative, could go through a thermally promoted rearrangement reaction in solution to form the corresponding 2-amidinobenzothiazole (**2**). In this report, it was found that **1** could be reduced to the corresponding imidoylthiourea (**3**) by 2-thioethanol and some biologically interesting reducing reagents including glutathione, cysteine, and ascorbic acid. The reduction also occurred through incubation of **1** with Sprague–Dawley rat and Yorkshire swine plasma. This reduction is a chemical reaction and non-enzymatic process. The experimental procedures are described in endnote.[9]

The reaction of 2,3-bis-(2-methoxy-phenyl)-5-phenylamino-[1,2,4]-thiadiazolium bromide (**1**) with 2-thioethanol was discovered to be a convenient method to synthesize 1-[(2-methoxy-phenyl)-(2-methoxy-phenylimino)-methyl]-3-phenyl-thiourea (**3**). 2-Thioethanol serves as a reducing reagent and reaction solvent. Then 0.47 g of **1** was dissolved in 30 mL of 2-thioethanol to give a clear yellow solution. The reaction solution was kept at room temperature for about 10 min followed by addition of 210 mL of water to generate a light yellow precipitate. The precipitate was isolated by centrifugation. The precipitate was washed with water for several times and then dried over P_2O_5 under vacuum

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* Corresponding author. Tel.: +1 908 874 1333; fax: +1 908 904 3891; e-mail: fzhang1@cpcus.jnj.com

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to give a light yellow solid (yield >55%) which was subjected to HPLC-UV, ESI-MS, and NMR analysis, and identified to have a structure of **3**. HPLC peak area% assay indicated that the synthesized **3** has a purity of higher than 97.9%. The structures are presented in Figure 1. It should be pointed out that compound **3** may also exist in its tautomeric form, i.e. the double bond between C(3) and the phenylimino nitrogen may also shift to result in a double bond between C(3) and the adjacent thiourea nitrogen accompanied by H shift from the thiourea nitrogen to the phenylimino nitrogen. The absolute tautomeric structure of **3** has not been determined yet.

Compound **3** in the HPLC mobile phase has UV absorption peaks at 275 and 310 nm (sh) with absorption ratio of about 1:0.8. The typical UV spectrum is presented in Figure 2. For mass spectrometric analysis, a solution of **3** in mixture of methanol, water, and formic acid (90:10:0.1, v/v/v) was directly infused into the electrospray ionization (ESI) source (1 μl/min) of a quadruple ion trap mass spectrometer in the positive ion detection mode. The synthesized **3** exhibits a positive ion at *m/e* 392 which is the protonated molecular ion. MS^n ($n = 1, 2, 3$) experiments were also performed and the results are presented in Figure 3 indicating that the ion at *m/e* 392 generates ion at *m/e* 257 which subsequently generates the ion at *m/z* 240. The proposed mass spectrometric fragmentation pathway is presented in Figure 4. ^1H, ^{13}C, ^1H–^1H homonuclear chemical shift correlation (COSY), ^1H–^{13}C heteronuclear multiple quantum coherence (HMQC), and ^1H–^{13}C heteronuclear multiple bond coherence (HMBC) NMR measurements were performed to elucidate the structure of **3**. The results are summarized in Table 1. Both ESI-MS and NMR measurements indicated that **3** has a structure of 1-[(2-methoxy-phenyl)-(2-methoxy-phenylimino)-methyl]-3-phenyl-thiourea.

Glutathione and cysteine occur extensively in many living organisms.[10] Their thiol groups serve as a metal complex center, a redox center, an electron transfer center, and also play vital roles in the structure of proteins. They also have protective functions against oxidative species such as oxygen-containing radicals which are be-

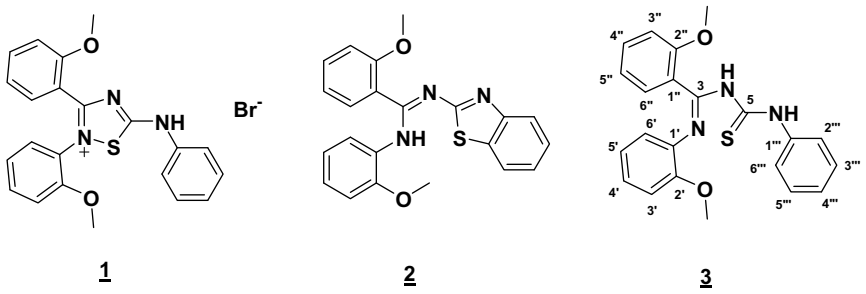

Figure 1. Structures of **1**, **2**, and **3**.

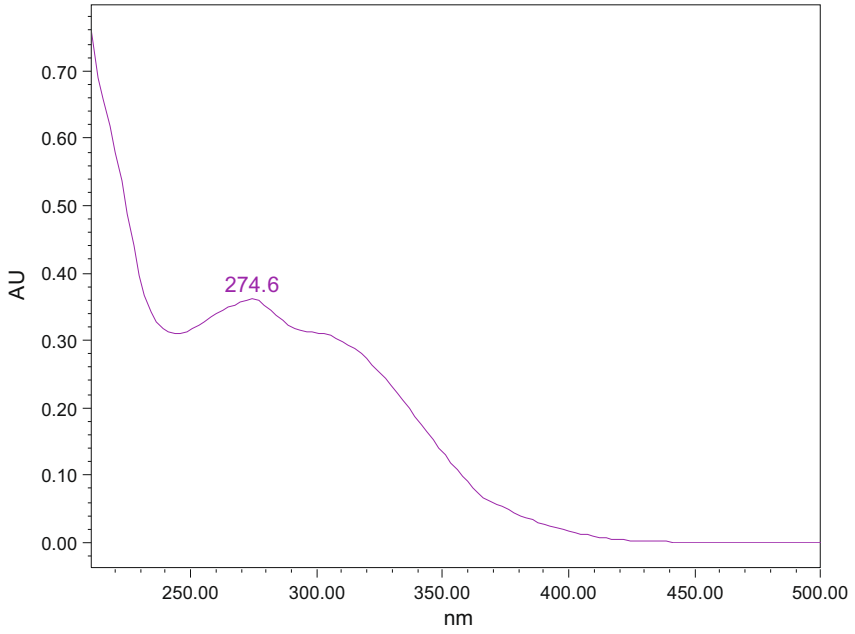

Figure 2. UV spectrum of **3**.

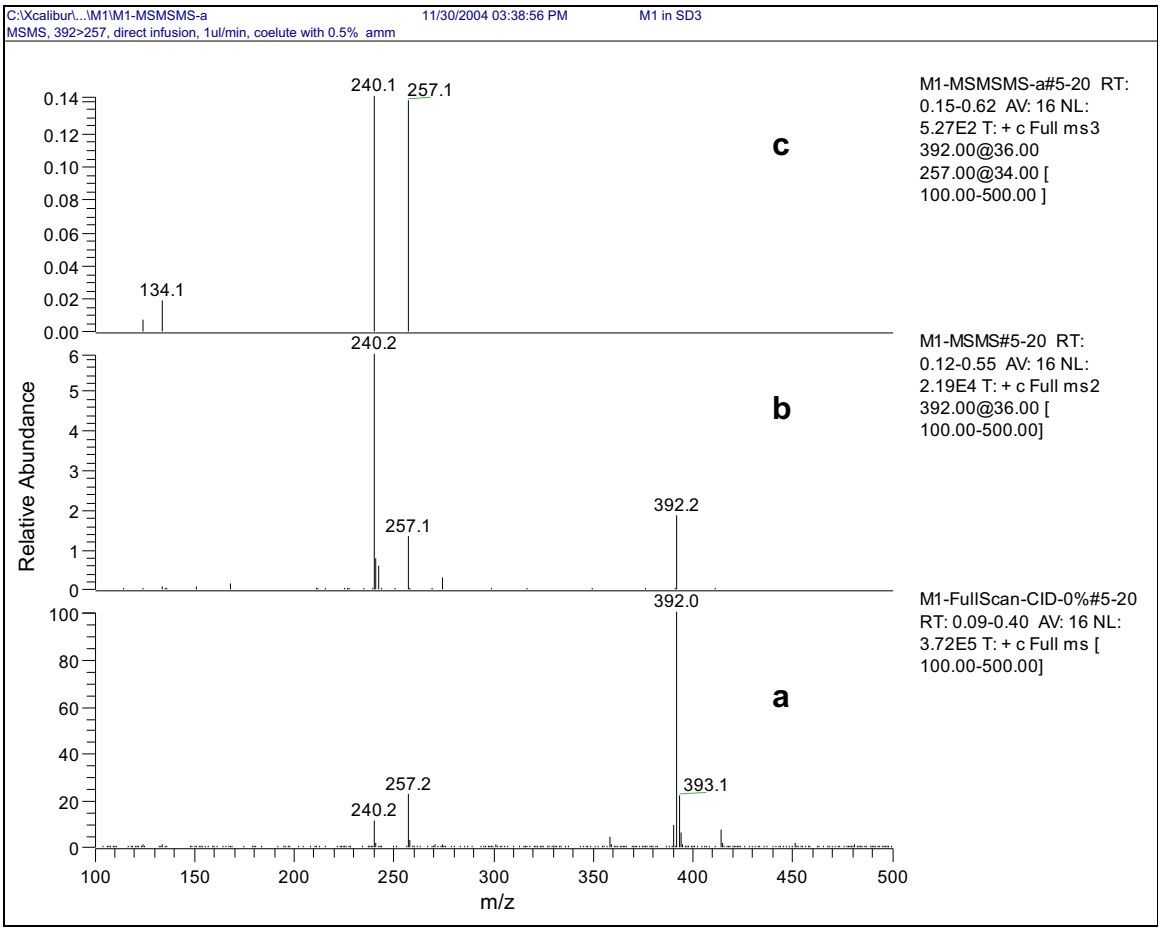

Figure 3. ESI mass spectra of **3**, (a) full scan MS1, (b) full scan MS2 (392 >), and (c) full scan MS3 (392 > 257 >).

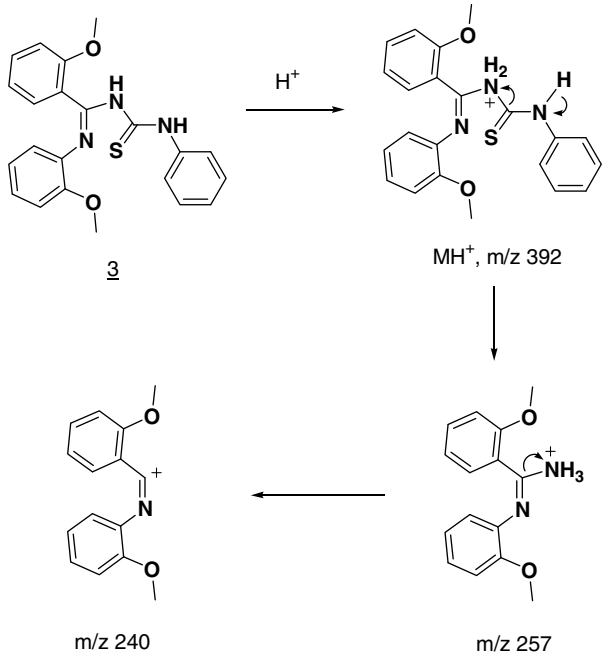

Figure 4. Mass spectrometric fragmentation pathway.

lieved to be part of the cause of certain diseases. Investigation of the reaction between **1** and cysteine or glutathione may provide unique insight into the in vivo fate of **1** and even other 1,2,4-thiadiazolium derivatives if administered pharmaceutically. A mixture of 3.0 mM of **1** and 6.6 mM of glutathione or 10.0 mM of cysteine in mixture (3:7, v/v) of DMSO and phosphate buffer (pH 7.4) was incubated at room temperature for 4 h. The reaction solutions are assayed using HPLC-UV and HPLC-MS. The HPLC-UV chromatogram of the mixture of glutathione with **1** is presented in Figure 5. Figure 5 reveals that only one major peak at 19 min was observed, which was identified to be compound **3** by its identical retention time, UV spectrum, and mass spectrum compared with that of the synthesized **3**. Compound **1** eluted at 14 min and its peak height is not significant indicating almost all of **1** was reduced by glutathione. Similarly, reaction of cysteine with **1** also produced **3** as the major product.

To confirm that it is the thiol groups of glutathione and cysteine that reduce **1** to **3**, the reaction solution described in Figure 5 was incubated with addition of maleimide which is a well-known thiol trapping reagent by forming 1:1 adduct[11] as described in Figure 9. A mixture of 3.0 mM of maleimide, 2.9 mM of **1**, and 6.6 mM of glutathione or 10.0 mM of cysteine in mixture (3:7, v/v) of DMSO and phosphate buffer (pH 7.4) was incubated at room temperature for 4 h. The reaction solutions were analyzed using HPLC-UV. As an example, the chromatogram of the reaction solution from incubation

Table 1. NMR studies on **3**, (a) ^{1}H and ^{13}C chemical shift assignments, (b) 2D correlations

Carbon	^{13}C (ppm, in $CDCl_3$)	^{1}H (ppm, in $CDCl_3$)
(a)		
		14.4 (1H, s, NH)
		8.15 (1H, s, NH)
3	154.2	
5	178.5	
1′	135.5	
2′	151.0	
3′	111.0	6.77 (1H, d)
4′	125.1	6.94 (1H, td)
5′	120.2	6.68 (1H, td)
6′	122.0	6.60 (1H, dd)
1″	121.5	
2″	155.9	
3″	111.1	6.83 (1H, d)
4″	131.9	7.31 (1H, td)
5″	120.5	6.85 (1H, t)
6″	129.0	7.15 (1H, dd)
1‴	138.7	
2‴/6‴	123.9	7.80 (2H, dd)
3‴/5‴	128.6	7.39 (2H, t)
4‴	126.0	7.22 (1H, t)
C(2″)OCH_3	55.4	3.75 (3H, s)
C(2′)OCH_3	55.3	3.73 (3H, s)
(b)		
COSY	7.80 (C(2‴)H/C(6‴)H) and 7.39 (C(3‴)H/C(5‴)H), 7.22 (C(4‴)H) and 7.39 (C(3‴)H/C(5‴)H), 6.77 (C(3′)H) and 6.94 (C(4′)H), 6.94 (C(4′)H) and 6.68 (C(5′)H), 6.68 (C(5′)H) and 6.60 (C(6′)H), 6.83 (C(3″)H) and 7.31 (C(4″)H), 7.31 (C(4″)H) and 6.85 (C(5″)H), 6.85 (C(5″)H) and 7.15 (C(6″)H)	
HMQC	7.80 (C(2‴)H/C(6‴)H) and 123.9 (C(2‴)/C(6‴)), 7.39 (C(3‴)H/C(5‴)H) and 128.6 (C(3‴)/C(5‴)), 7.22 (C(4‴)H) and 126.0 (C(4‴)), 6.77 (C(3′)H) and 111.0 (C(3′)), 6.94 (C(4′)H) and 125.1 (C(4′)), 6.68 (C(5′)H) and 120.2 (C(5′)), 6.60 (C(6′)H) and 122.0 (C(6′)), 6.83 (C(3″)H) and 111.1 (C(3″)), 7.31 (C(4″)H) and 131.9 (C(4″)), 6.85 (C(5″)H) and 120.5 (C(5″)), 7.15 (C(6″)H) and 129.0 (C(6″)), 3.75 (C(2″)OCH_3) and 55.4 (C(2″)OCH_3), 3.73 (C(2′)OCH_3) and 55.3 (C(2′)OCH_3)	
HMBC	7.80 (C(2‴)H/C(6‴)H) and 126 (C(4‴)), 7.39 (C(3‴)H/C(5‴)H) and 138.7 (C(1‴)), 6.77 (C(3′)H) and 120.2 (C(5′)), 6.77 (C(3′)H) and 135.5 (C(1′)), 6.94 (C(4′)H) and 122.0 (C(6′)), 6.94 (C(4′)H) and 151.0 (C(2′)), 6.68 (C(5′)H) and 135.5 (C(1′)), 6.68 (C(5′)H) and 111.0 (C(3′)), 6.60 (C(6′)H) and 151.0 (C(2′)), 6.60 (C(6′)H) and 125.1 (C(4′)), 6.83 (C(3″)H) and 120.5 (C(5″)), 6.83 (C(3″)H) and 121.5 (C(1″)), 7.31 (C(4″)H) and 155.9 (C(2″)), 7.31 (C(4″)H) and 129.0 (C(6″)), 6.85 (C(5″)H) and 111.1 (C(3″)), 6.85 (C(5″)H) and 121.5 (C(1″)), 7.15 (C(6″)H) and 131.9 (C(4″)), 7.15 (C(6″)H) and 155.9 (C(2″)), 3.75 (C(2″)OCH_3) and 155.9 (C(2″)), 3.73 (C(2′)OCH_3) and 151.0 (C(2′))	

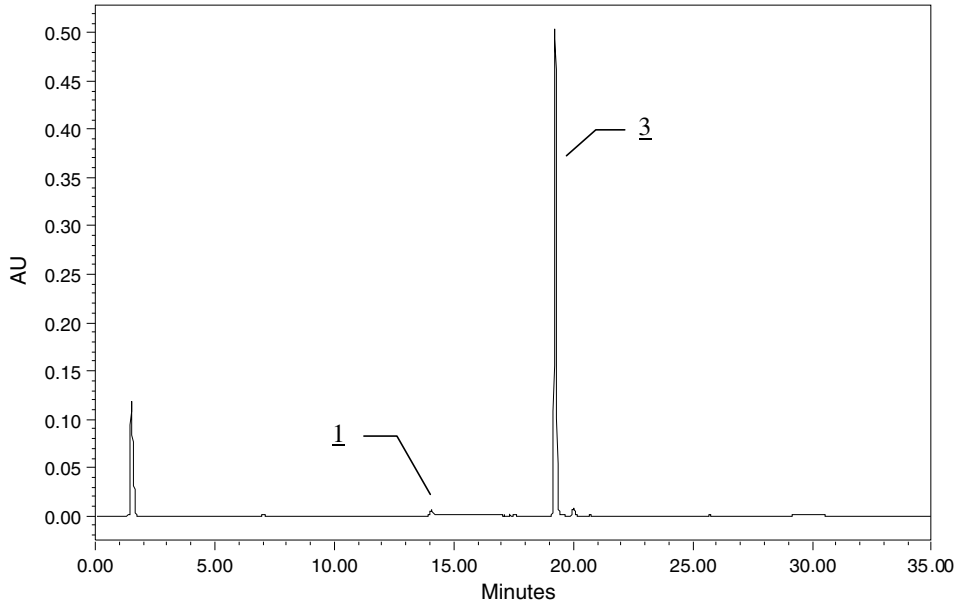

Figure 5. Chromatograms of reaction solutions of **1** with glutathione in mixture (3:7, v/v) of DMSO and phosphate buffer (pH 7.4) at room temperature for 4 h.

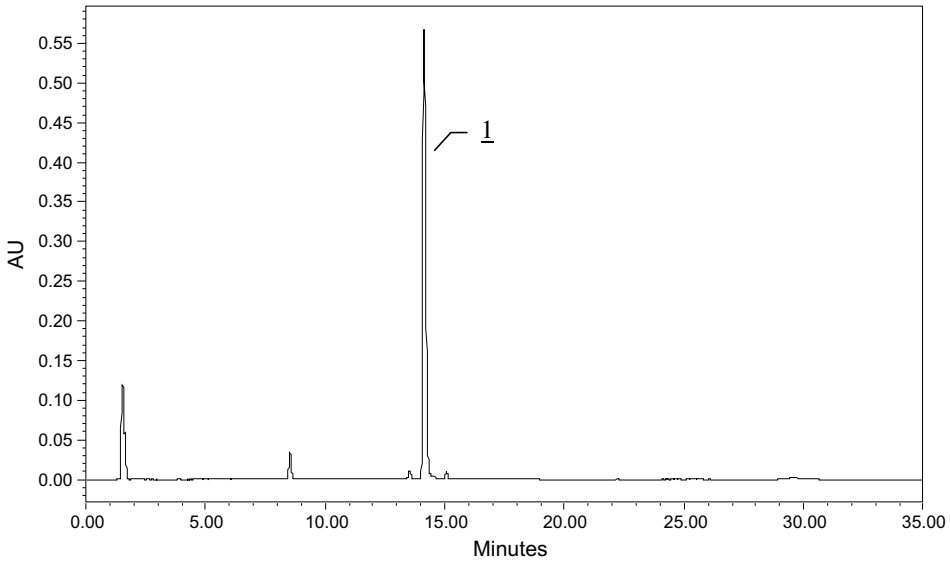

Figure 6. Chromatogram of reaction solutions of **1** with glutathione in the presence of maleimide in mixture (3:7, v/v) of DMSO and phosphate buffer (pH 7.4) at room temperature for 4 h.

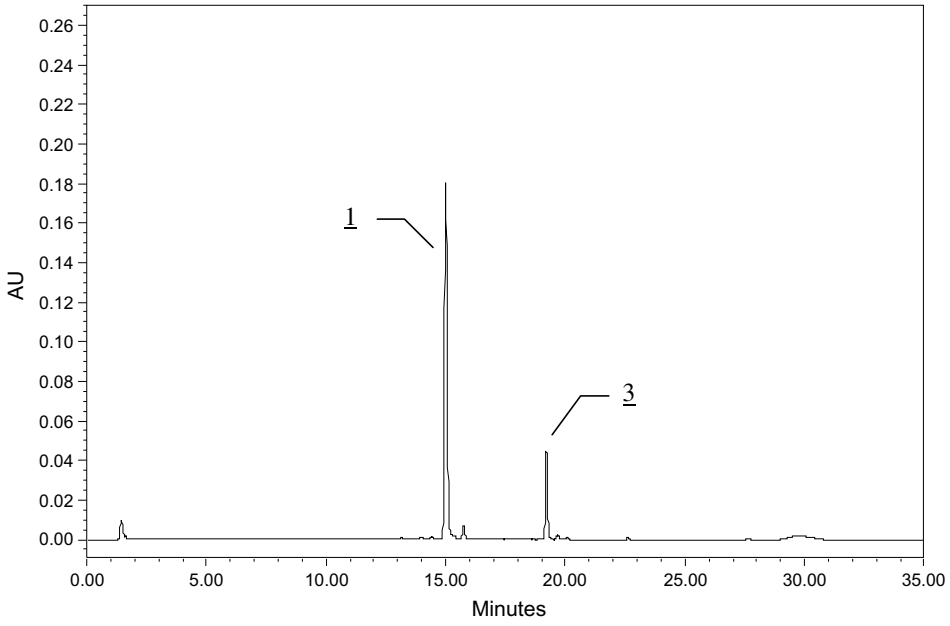

Figure 7. Chromatograms of Yorkshire swine plasma incubated with 3.0 mM of **1** at room temperature for 30 min.

of **1** with glutathione in the presence of maleimide is presented in Figure 6. As expected, in contrast to Figure 5, Figure 6 reveals that the major peak observed is at 14 min, which is responsible for the starting compound **1**. The peak for product **3** at 19 min is very minor. Incubation of **1** with cysteine in the presence of maleimide resulted in similar phenomena. The aforementioned results indicated that the reactions between **1** and glutathione or cysteine were inhibited by trapping the thiol groups of glutathione or cysteine using maleimide.

Similar to glutathione and cysteine, ascorbic acid (vitamin C) is also one of the most important reducing agents in biochemistry and plays an important role in many biochemical processes.[12] Therefore, it is also important to understand the interaction of ascorbic acid with 1,2,4-thiadiazolium derivatives when they are used for therapeutical applications. In this report, the reaction of **1** with ascorbic acid was investigated. A mixture of 2.9 mM of **1** and 10.8 mM of ascorbic acid in mixture of DMSO and phosphate buffer (pH 7.4) was incubated at room temperature for 10 h. The reaction solution was analyzed using HPLC-UV and HPLC-MS. The HPLC-UV and HPLC-MS analysis revealed that one of the major products was compound **3** confirmed by its identical retention time, UV spectrum and mass spectrum comparing with that of the synthesized **3**. Similar to glutathione or cysteine, ascorbic acid could also reduce **1** to **3**,

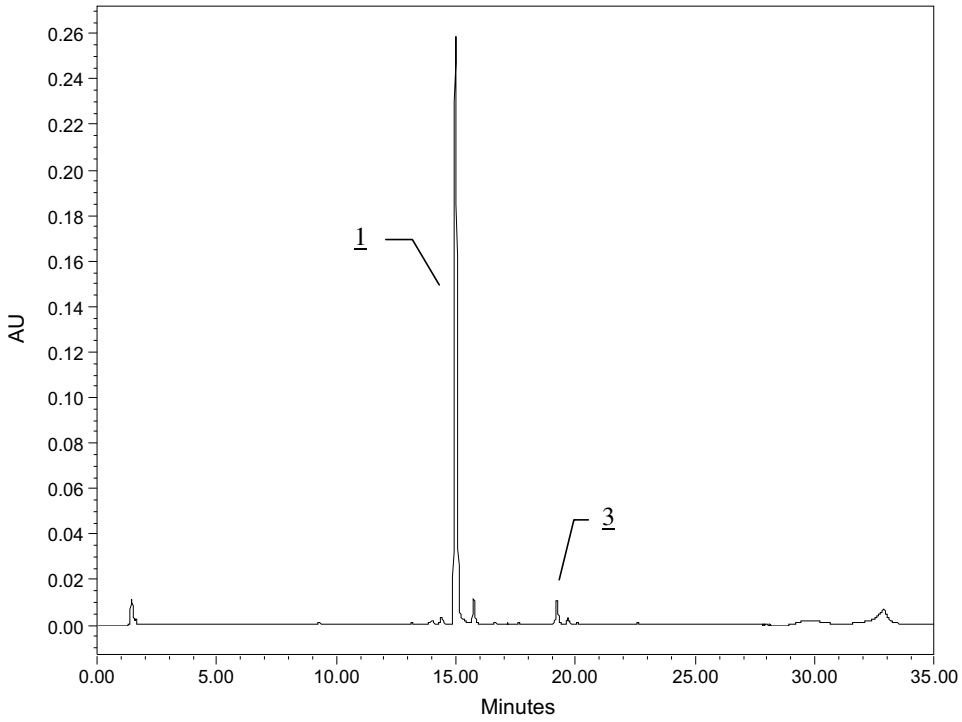

Figure 8. Chromatograms of Yorkshire swine plasma incubated with 3.0 mM of **1** and 4.1 mM of maleimide at room temperature for 30 min.

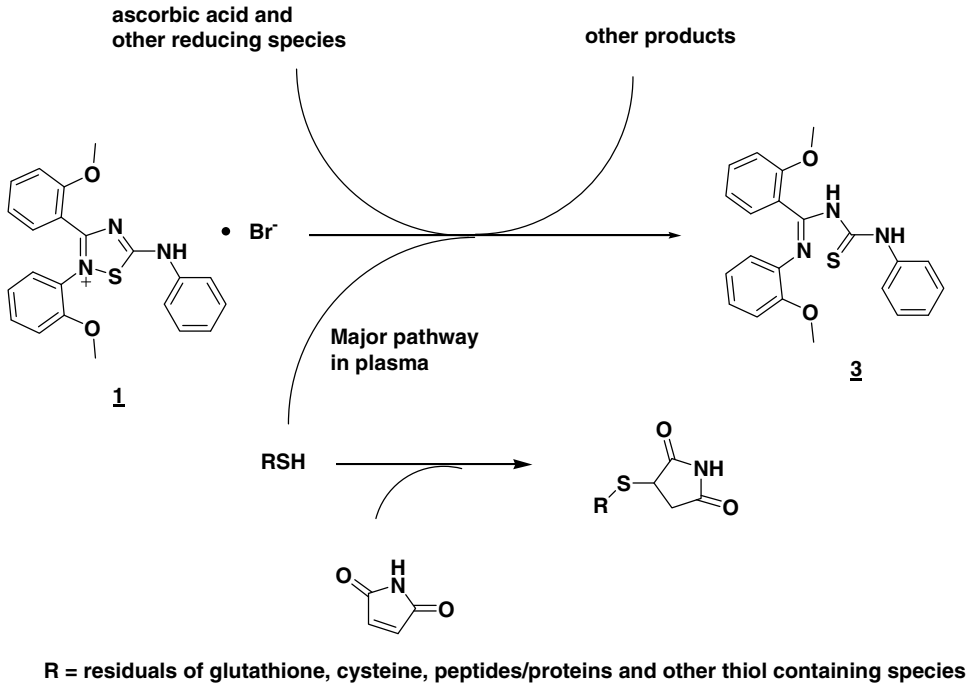

Figure 9. Reduction of **1**.

but the reaction was slower and additional products were formed, which remain to be identified.

The metabolism of a drug molecule is a crucial part in its safety assessment. Investigating the stability of a drug molecule in plasma is one of the common approaches in drug metabolism evaluation. As a preliminary investigation, 3.0 mM of **1** was incubated with Yorkshire swine plasma or Sprague–Dawley rat plasma at room temperature for 30 min. The resulting sample was assayed by HPLC-UV and HPLC-MS. HPLC-UV chromatogram of the Yorkshire swine plasma incubated with **1** is presented in Figure 7, which revealed that besides the peak of **1**, a new product peak at 19 min was observed. This new product peak was identified to be **3** by its identical retention

time, UV spectrum, and mass spectrum, and confirmed by comparing with synthesized **3**. Interestingly, the addition of maleimide into the plasma sample of Figure 7 inhibited the formation of **3**. Figure 8 exhibits the HPLC-UV chromatogram of the sample from incubation of 3.0 mM of **1** with 4.1 mM of maleimide in Yorkshire swine plasma at room temperature for 30 min. The peak of **3** is minimal. The results of Figures 7 and 8 indicate that the major species responsible for the reduction of **1** to **3** in Yorkshire swine plasma might be mainly thiol containing since maleimide can scavenge the thiol groups and prevent their reactions with **1**. For example, the thiol containing species in plasma could be cysteine, glutathione, and other peptides or proteins. Similarly, incubation of **1** in Sprague–Dawley rat plasma also generated **3** as the major product and the reaction could also be inhibited by maleimide. The reaction pathways are illustrated in Figure 9.

Based on the aforementioned results, 2,3-bis-(2-methoxy-phenyl)-5-phenylamino-[1,2,4]-thiadiazolium bromide (**1**), a 1,2,4-thiadiazolium derivative and melanocortin receptor modulator, is sensitive to biologically interesting reducing reagents and could be reduced to the corresponding imidoylthiourea, 1-[(2-methoxy-phenyl)-(2-methoxy-phenylimino)-methyl]-3-phenyl-thiourea (**3**). Because that the reduction occurs via a non-enzymatic pathway, it is important to understand this reaction prior to traditional CYP450 studies in metabolism assessment. It will also be important to evaluate pharmacological and/or toxicological effects of the reduction products. It is obvious that antioxidants should be avoided as preservatives in products formulations containing 1,2,4-thiadiazolium derivatives. The new redox reaction between a 1,2,4-thiadiazolium derivative and reducing reagents observed in this report could also enrich chemical knowledge of the related compounds.

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9. *Experimental procedures.*
   (a) Chemicals and plasma: 2,3-bis-(2-methoxy-phenyl)-5-phenylamino-[1,2,4]-thiadiazolium bromide was obtained from the Laboratory of Process Research at the 'Organisch-Chemisches Institute' of the University of Zurich (Switzerland). HPLC grade methanol, water, and trifluoroacetic acid (TFA) were obtained from J.T. Baker (Phillipsburg, NJ, USA) or Burdick & Jackson (Muskegon, MI, USA). 2-Thiolethanol, L-cysteine, glutathione, and ascorbic acid were obtained from Aldrich (St. Louis, MO, USA). Yorkshire swine plasma and Sprague–Dawley rat plasma were obtained from Biochemed Pharmaceuticals (Winchester, VA, USA).
   (b) HPLC-UV analysis: high performance liquid chromatography was performed using an Agilent 1100 HPLC system (Palo Alto, CA, USA) with diode array UV detection at 326 nm. The UV spectra of the HPLC peaks were obtained using the on-line diode array detector. Mobile phase A is water containing 0.2% (v/v) trifluoroacetic acid (TFA). Mobile phase B is methanol containing 0.2% (v/v) TFA. A binary linear gradient elution using mobile phase A and B was conducted: 0–10 min, linear gradient from 45% B to 60% B, 10–20 min, linear gradient to 90% B at a flow rate of 0.45 mL/min. A Zorbax (Agilent Technologies, Palo Alto, USA) SB-C18 column was used (3.5 μm, 3.0 mm × 150 mm). The column temperature was 35 °C. The injection volume was 10 μl.
   (c) HPLC-MS and direct infusion MS analyses: HPLC-MS analysis was performed on a ThermoFinnigan (San Jose, CA, USA) LCQ mass spectrometer with an electrospray ionization source (ESI) and a quadruple ion trap mass analyzer in the positive ion mode. The heated capillary was kept at 150 °C and the ionization potential of the nebulizer was set at 4 kV. The mass spectrometer was interfaced to a TSP HPLC system which consisted of a vacuum degasser, a P4000 pump, an AS3000 autosampler, and a UV6000LP PDA detector. The HPLC conditions were the same as the HPLC-UV analysis. Direct infusion ESI mass spectrometric analysis was performed by directly infusion into the ESI source at a flow rate of 1 μl/min.
   (d) NMR analysis: NMR measurements were carried out on a Varian (Palo Alto, CA) INOVA 400 NMR spectrometer using $CDCl_3$ as the solvent.
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