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Short communication

Structure elucidation of thioketone analogues of sildenafil detected as adulterants in herbal aphrodisiacs

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ABSTRACT

Two analogues of sildenafil were detected in herbal dietary supplements marketed as aphrodisiacs. Both compounds were identified as thioketone analogues of sildenafil in which the carbonyl group in the pyrimidine ring of sildenafil was substituted with a thiocarbonyl group. The first compound was identified as thiosildenafil, a compound that has recently been reported as an adulterant in health supplements. The structure of the second compound was established using LC–MS, UV spectroscopy, ESI-MS^n, NMR and a hydrolytic process. A detailed study of the hydrolysis products of sildenafil, thiosildenafil, and the second unknown compound proved that the second compound, named thiomethisosildenafil, had a structure analogous to sildenafil in which the N-methylpiperazine moiety had been replaced with 2,6-dimethylpiperazine and the oxygen atom of the carbonyl group in the heterocyclic ring had been replaced with a sulfur atom. Under the hydrolytic reaction conditions employed in this study, thioketones hydrolyze to ketones (e.g., thiosildenafil → sildenafil), making this a valuable technique for the structure elucidation of thiosildenafil analogues. Ten herbal dietary supplements, each as a capsule dosage form, were found to contain 8–151 mg of thiomethisosildenafil per capsule, and one herbal dietary supplement was found to contain 35 mg of thiosildenafil per capsule.

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1. Introduction

Synthetic phosphodiesterase type 5 enzyme (PDE-5) inhibitors are widely used for the treatment of erectile dysfunction (ED). Today, there are three such drugs approved by the U.S. Food and Drug Administration: sildenafil citrate (Viagra®, manufactured by Pfizer), vardenafil hydrochloride (Levitra®, manufactured by Bayer), and tadalafil (Cialis®, manufactured by Lilly). In the last few years, these ED drugs and structurally modified synthetic analogues have been found in "natural" herbal health supplements marketed as aphrodisiacs [1–17]. Most sildenafil analogues found as adulterants have been modified in the piperazine portion of the molecule, but recently, herbal dietary supplements were found to contain thiosildenafil [17] and thiohomosildenafil [16,17], two novel sildenafil analogues modified by replacing a carbonyl group in sildenafil with a thiocarbonyl group. Our laboratory recently detected two thiosildenafil analogues in herbal dietary supplements. Compound **1** was identified as thiosildenafil. Since this compound has already been reported [17], discussions of this compound in our paper will be limited to the presentation of new information, a comparison of its properties to those of compound **2**, and discrepancies observed between our data and that already presented. Compound **2** was characterized using LC–ESI-MS, UV spectroscopy, collision-induced dissociation (CID)-MS^n, GC–MS, NMR, and hydrolytic techniques. It was identified as 5-(5-((3R,5S)-3,5-dimethylpiperazin-1-ylsulfonyl)-2-ethoxyphenyl)-1-methyl-3-propyl-1H-pyrazolo[4,3-d]pyrimidine-7(6H)-thione. The structures of sildenafil, thiosildenafil, methisosildenafil, and this new analogue, named thiomethisosildenafil, are shown in Fig. 1.

2. Experimental

2.1. Materials

Sildenafil citrate was obtained from Pfizer, tadalafil from Lilly, and vardenafil hydrochloride trihydrate from Bayer. Methisosildenafil was previously isolated, purified, and characterized in our laboratory [15]. Reagent grade formic acid and hydrochloric acid and HPLC-grade OmniSolv acetonitrile and OnmiSolv high purity solvent methanol and dichloromethane, and glacial acetic acid, ACS grade were purchased from EM Science (Gibbstown, NJ, USA). 2,6-

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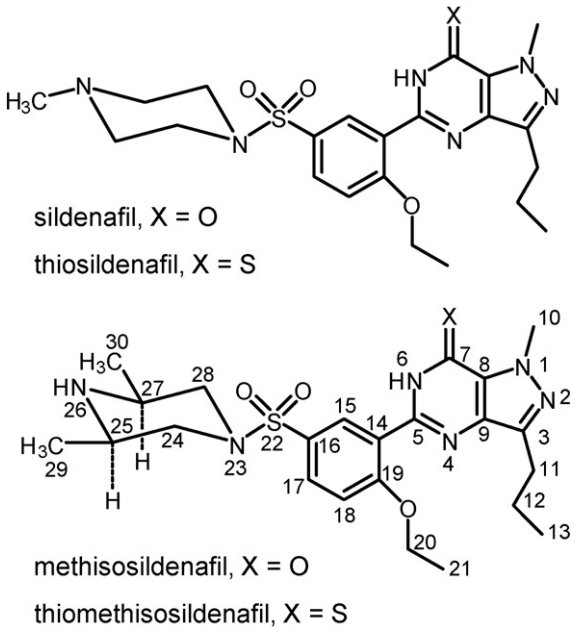

Fig. 1. Structures of sildenafil, thiosildenafil (compound **1**), methisosildenafil and thiomethisosildenafil (compound **2**).

Dimethylpiperazine was purchased from Aldrich (Milwaukee, WI, USA). Water was purified to a resistivity of 18 MΩ cm using a Milli-Q Water System (Millipore, Bedford, MA, USA). Herbal dietary supplements were appropriated from distributors' warehouses.

2.2. Extraction of herbal products in preparation of samples for LC with photodiode array (PDA) and MS detection and CID-MS^n analysis

The contents of 10–12 capsules (fewer if fewer capsules were available) were combined and mixed briefly via a vortex mixer to serve as a composite for analysis. A 30 mg portion of the capsule composite was mixed with 5.0 ml MeOH using a vortex mixer for 30 s, ultrasonic bath for 20 min, and vortex mixer again for 30 s. The mixture was centrifuged (Fisher Scientific, Centrific, Model 228, 3300 rpm maximum) for 15 min, and the supernatant liquid was removed and used for analysis.

2.3. LC with photodiode array (PDA) and ESI-MS detection

LC–PDA-MS was conducted on an Agilent 1100 system with a photodiode array detector and a single quadrapole mass spectrometer, model G1946D, using electrospray ionization in positive ion mode. The UV signal was monitored at 230 nm and set to collect a spectrum for each component detected. The analytical column was a Zorbax SB-C_{18} stationary phase, 150 mm × 4.6 mm, 5 μm particle size (Agilent Technologies, Wilmington, DE, USA). Mobile solvent A was 7 mM formic acid in water and mobile solvent B was 7 mM formic acid in MeCN; the flow rate was 1 ml min^{-1}. A gradient was used for the mobile phase starting with 15% B for the first 5 min, changing linearly to 90% B over 5–15 min, and holding at 90% B for 5 min. The column was re-equilibrated for 7 min prior to the start of the next run. Alternatively, in the analysis of hydrolysis reaction solutions, mobile phase A was 0.1% formic acid in water, mobile phase B was 0.1% formic acid in MeCN, and the mobile phase gradient was changed linearly from 18% to 32% B over 0–4 min, then 32–62% B over 4–16 min.

2.4. Isolation of the two compounds from herbal dietary supplements

The two thioketone analogues, detected in different adulterated herbal products, were isolated from these products using the procedures that follow. Melting points were recorded on a Thomas-Hoover Melting Point Apparatus and were corrected using melting point standards from the National Physical Laboratories, Teddington, England.

*Compound **1** (thiosildenafil)*: The combined contents of four capsules, weighing 1.27 g, were mixed with silica gel in a slurry of mobile solvent consisting of 3% methanol in dichloromethane. The slurry was added to the top of a silica gel column, and the column was eluted. Fractions containing the compound of interest were combined and evaporated yielding 145 mg of residue. The material was recrystallized from methanol giving 92 mg of light yellow crystalline solid, m.p. 182.1–182.9 °C.

*Compound **2** (thiomethisosildenafil)*: The contents of eight capsules, weighing a total of 3.4 g, were mixed with 20 ml MeOH, shaken for 2 min, and centrifuged. The supernatant liquid was decanted and evaporated on a rotary evaporator. The solid material remaining in the centrifuge tube was extracted three more times in the same manner. The combined extracts were recrystallized from 50 ml MeOH to give 294 mg of fluffy faintly yellow crystalline solid, m.p. 199.7–200.7 °C.

2.5. Collision-induced dissociation (CID) MS

Positive ion electrospray ionization (ESI) CID-MS was conducted on a Thermo-Electron LCQ Deca XP ion trap mass spectrometer using direct infusion at 3 μl min^{-1}. The ion transfer capillary temperature was 250 °C, sheath gas 5 (arbitrary units), auxiliary gas 0 (arbitrary units), capillary voltage 7 V, and spray voltage 5 kV. The collision energy varied from 38% to 45%.

2.6. Hydrolysis

Portions of 1–2 mg of sildenafil, compounds **1** and **2** were placed in separate test tubes, dissolved in 3 ml 6.1 M HCl, sealed with a PTFE-lined screw-cap, and heated at 105 °C for 21 h. A 0.5 ml portion of the reaction solution was evaporated, dissolved in 1.0 ml MeOH, and transferred to an HPLC vial for LC–PDA-MS analysis of the sulfonic acid portion of the molecule. To analyze the amine portion of the molecule, 20 drops of the reaction solution were evaporated in a small beaker, and the residue was extracted into 1 ml of ether pre-saturated with ammonia. Since some solid material remained undissolved, the ether was centrifuged and decanted to a vial for GC–MS analysis.

2.6.1. Identification of the amine hydrolysis product using GC–MS

The amine generated by acid hydrolysis of the unknown component was analyzed by GC–MS on an Agilent gas chromatograph, model 6890N, with a Agilent 5975B Inert XL EI/CI MSD using a Zebron capillary column, ZB-5MS, 30 m × 0.32 mm × 1 μm film thickness (Phenomenex, Torrance, CA) under the following conditions: injector: 220 °C, 1 μl injection volume, splitless mode; carrier gas: helium at 1.3 ml min^{-1} constant flow; oven temperature: 60 °C (hold = 0), increased to 130 °C at 12 °C min^{-1}, increased to 320 °C at 30 °C min^{-1}; MS transfer line heater: 280 °C; MS Quad: 280 °C; MS source: 230 °C; EM voltage: 1423; MS scan range 50–800 Da.

2.6.2. Identification of the sulfonic acid hydrolysis products using LC–PDA-MS

The LC–PDA-MS method described in Section 2.3 was used for the analysis of the sulfonic acid hydrolysis products. Products

generated from the hydrolysis of sildenafil, compounds **1** and **2** were compared by retention time, UV spectra and mass spectra.

2.7. NMR of compound **2**

Approximately 10 mg of the crystalline solid (compound **2**), which was isolated from the herbal product, was dissolved in $CDCl_3$ (Cambridge Isotopes Laboratories) for NMR analysis. ^{1}H-, 2-D ^{1}H–^{1}H-Correlation Spectroscopy (gCOSY), ^{1}H–^{13}C correlation spectroscopy (gHMQC), and Nuclear Overhauser Enhancement Spectroscopy (NOESY) NMR data were recorded on a Inova 500 MHz instrument (Varian Inc.). Collection conditions for ^{1}H include a spectral window of 10,000 Hz, 8 transients, 38,000 complex data points and a 4 μs (45°) pulse duration. For gCOSY gHMQC and NOESY a ^{1}H spectral window of 4386 Hz was used and 2048 complex data points were collected for 300 increments (gCOSY and NOESY) and 600 increments (gHMQC). GCOSY and gHMQC were processed in absolute value mode while NOESY (0.6 s mix time) was processed in phase-sensitive mode.

3. Results and discussion

Compound **1**, isolated from herbal dietary supplements in our laboratory and characterized by LC–ESI-MS, UV, MS, CID-MS^n and hydrolysis, was identified as thiosildenafil, a compound that has recently been detected as an adulterant in an herbal dietary supplement and thoroughly characterized by Zou et al. [17]. The thiosildenafil compound identified by their group was purified chromatographically and obtained as a yellow amorphous powder with a melting point of 172–174 °C. Thiosildenafil was isolated in our laboratory by extraction, silica gel chromatography and crystallization to give a light yellow crystalline solid with a melting point of 182.1–182.9 °C. The difference in melting points between the two laboratories may be attributed to differences in purity or crystalline state.

The MS–MS spectrum on the protonated molecular ion of thiosildenafil recorded on an ion trap mass spectrometer in our laboratory differs from the one recorded on a triple quadrapole mass spectrometer by Zou et al. [17], but is practically identical to the spectrum recorded on an ion trap instrument by Lee [19].

Other than the discrepancies in melting points and MS–MS spectra, both of which have been accounted for, the compound isolated in our laboratory and identified as thiosildenafil is in full agreement with the data presented by Zou et al. [17]. Since thiosildenafil has previously been characterized, the remainder of this paper is focused on the structure elucidation of compound **2**, but will include new data on compound **1** and data on compound **1** for comparison to compound **2**.

3.1. LC–PDA-MS

The total ion chromatograms for a mixture of the three ED drugs (vardenafil, sildenafil and tadalafil) and two adulterated herbal dietary supplements and are shown in Fig. 2, and UV and MS data for these compounds are given in Table 1. The isotopic ratio of the

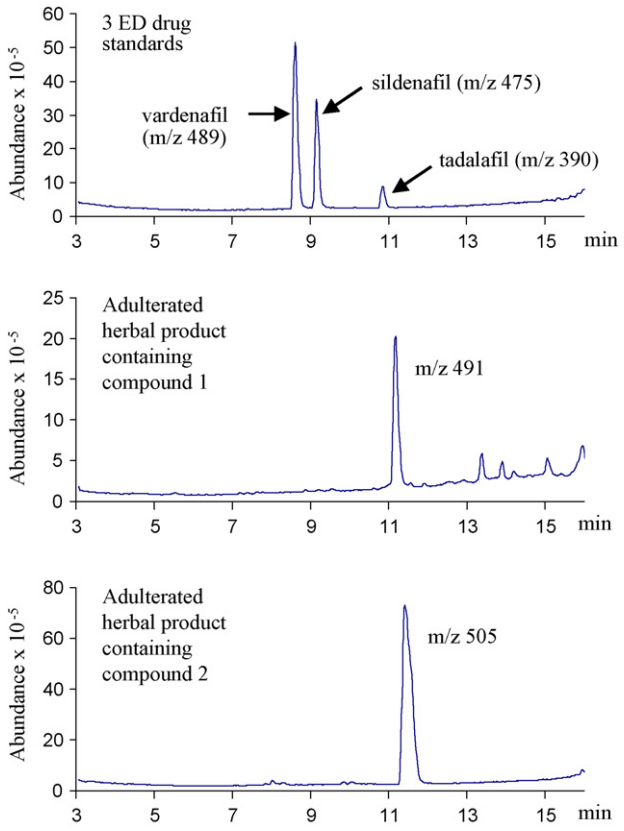

Fig. 2. Total ion chromatograms of a mixture of sildenafil, vardenafil, and tadalafil standards, an adulterated herbal dietary supplement containing compound **1**, and an adulterated herbal dietary supplement containing compound **2**.

relative abundances of the peaks at *m*/*z* 507 (*A* + 2) and *m*/*z* 505 (*A*) in compound **2** is 11/100, evidence for the presence of two sulfur atoms in the molecule. Similarly, the (*A* + 2)/A isotopic ratio for compound **1** is 12.2/100. The protonated molecular ion of compound **1** is 14 units below that of compound **2**, suggesting a difference in structure of a CH_2 group.

The UV spectrum of compound **2**, λ_{max} at 227, 248 (sh), 296 and 355 nm, is significantly different from the UV spectra of the three ED drugs, but is completely superimposable with the spectrum of compound **1**, and these spectra resemble the published spectra of thiosildenafil [17] and thiohomosildenafil [16,17]. These UV spectra are quite unique and indicative of a thiosildenafil analogue.

3.2. CID-MS

Major product ions generated from CID on the protonated molecular ions of the three erectile dysfunction drugs, compounds **1** and **2** are shown in Table 1. The sildenafil product ion at *m*/*z* 377 results from fragmentation of the S–N sulfonamide bond with a hydrogen transfer, and the other sildenafil product ions listed in Table 1 are also devoid of the piperazine ring moiety

Table 1
LC–PDA-MS data for the three ED drug standards (top three) and two herbal products (bottom two).

Compound	RRT	UV λ_{max} (nm)			MS^1 (*m*/*z*)	MS^2 (*m*/*z*)					
Sildenafil	1.00	215	224	294	475	377	313	311	299	283	
Vardenafil	0.94	215	226 (sh)	246 (sh)	489	461	377	376	329	312	299
Tadalafil	1.19	221	285	291	390	302	273	268	262	250	135
Thiosildenafil	1.22	227	296	355	491	407	343	341	329	313	
Thiomethisosildenafil	1.25	227	296	355	505	448	393	329	327	315	299

[3,9,15,18]. Major product ions from compound **1** (thiosildenafil) are 30 Da higher than those seen in sildenafil (Table 1), 16 mass units attributed to the presence of a thioketone instead of a ketone and 14 mass units resulting from migration of the methyl group on the piperazine nitrogen atom to the thiocarbonyl sulfur atom, a phenomenon that has been previously reported [19]. On the other hand, the major product ions in compound **2** are 16 Da higher than those from sildenafil (Table 1), which supports the replacement of an oxygen atom with sulfur, and further indicates that the additional mass of 14 Da in compound **2** is in the piperazine moiety of the molecule, which is lost during fragmentation, and that there is no alkyl group on the piperazine nitrogen atom with which to migrate.

3.3. Acid hydrolysis of sildenafil, compound **1** (thiosildenafil) and compound **2** (thiomethisosildenafil)

Acid hydrolysis proved to be a useful tool in the structure elucidation of sildenafil and vardenafil analogues [9,14,15]. Acid-catalyzed hydrolysis of the sulfonamide bond was followed by LC–PDA-MS analysis to characterize the resulting sulfonic acid products and GC–MS analysis to characterize the resulting amine product.

3.3.1. Characterization of the sulfonic acids generated by hydrolysis

The sulfonic acid products generated by hydrolysis of compounds **1** and **2**, and sildenafil were compared by LC–PDA-MS. Total ion chromatograms of the hydrolysis products are shown in Fig. 3, and the proposed hydrolytic reaction pathways for these three compounds are shown in Fig. 4. Peak numbers in Fig. 3 correspond to compound numbers in Fig. 4. Based on the LC–PDA-MS data, the following three hydrolytic reaction processes are proposed. First, sulfonamide S–N bond hydrolyzes to generate a sulfonic acid and an amine. The loss in mass from this process depends on the structure of the piperazine ring and aids in structural elucidation. Both compounds **1** (*m*/*z* 491) and **2** (*m*/*z* 505) are hydrolyzed to **5** (*m*/*z* 409). Second, the thioketone hydrolyzes to a ketone with a net loss in mass of 16. Thiosildenafil (**1**) hydrolyzes to sildenafil (**3**), and thiomethisosildenafil (**2**) hydrolyzes to methisosildenafil (**4**), both of which are major products of hydrolysis, and both of which are accompanied by the release of hydrogen sulfide that is detected by smell. Thus, the hydrolytic technique provides additional evidence for these two structures, and also demonstrates that this technique is a valuable technique for the structure elucidation of thiosildenafil derivatives. Third, the pyrazolo-pyrimidine ring hydrolyzes with a net addition of water to the molecule, and thus, a net gain in mass of 18. While the structures of the compounds **7**–**9**, resulting from this latter hydrolytic process, are speculative, the formation of these compounds encompasses a net addition of one equivalent of water accompanied by a complete change in the UV spectrum during the transition.

The structures shown in Fig. 4 are supported by matching mass spectra of corresponding compounds, matching UV spectra of corresponding compounds (data not shown), similar peak height of analogous or common compounds, and a general knowledge of hydrolytic processes.

3.3.2. Characterization of the amine generated by hydrolysis

A compound in the hydrolysis solution of the herbal dietary supplement containing compound **2** was detected on the GC–MS system with a retention time of 4.587 min which corresponded in retention time to 2,6-dimethylpiperazine standard (Aldrich, Milwaukee, WI, USA). The mass spectrum of the hydrolysis product matched the mass spectrum of 2,6-dimethylpiperazine standard

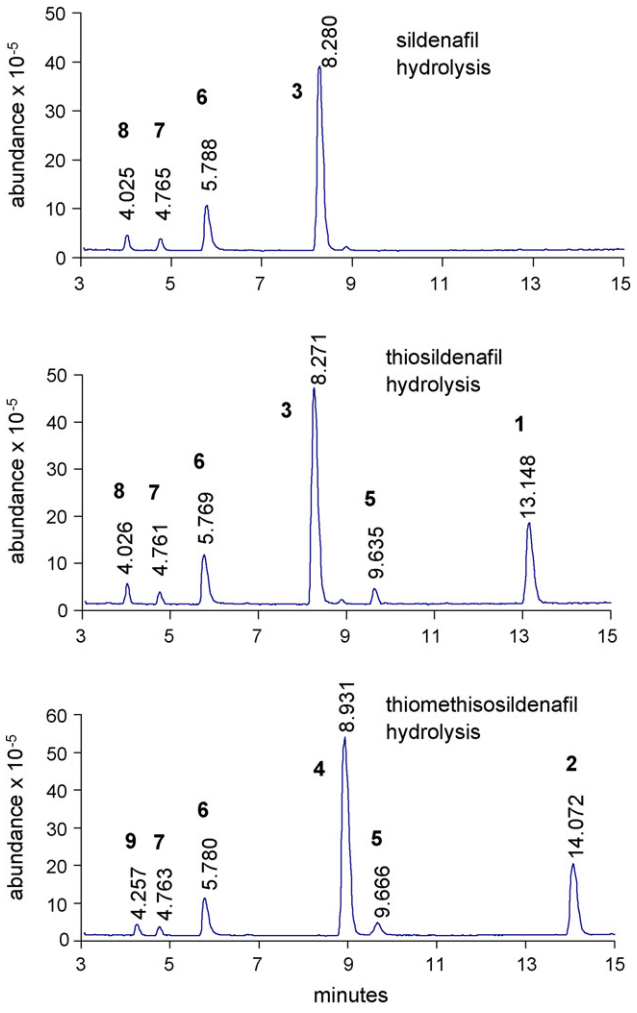

Fig. 3. Total ion chromatograms of the hydrolysis products of sildenafil, thiosildenafil and thiomethisosildenafil. Peak numbers correspond to compound numbers in Fig. 4.

and gave the highest match to this compound in the NIST mass spectral library. The molecular ion was detected at *m*/*z* 114 (17% relative abundance) and other prominent ions were detected at *m*/*z* 99, 71, 70 and 56.

3.4. NMR of compound **2** (thiomethisosildenafil)

The NMR data for thiomethisosildenafil are presented in Table 2. ^{1}H–^{1}H COSY and ^{1}H–^{13}C HMQC experiments clearly demonstrate that the protons at δ1.91 and 3.66 are the two geminal protons, while the proton at δ2.98 is the methine proton. C25 (C27) at δ50.10 correlates with the proton at δ2.98, which shows that this proton is the ring methine proton, and C24 (C28) at δ51.87 correlates with the protons at δ3.66 and 1.91, which shows that these are the ring methylene protons. The proton at δ3.66 is recognized as the equatorial proton lying in the deshielding zone of the S=O group, characteristic of a deshielded equatorial proton of a rigid 6-memebered ring. Indeed, the very observance of equatorial and axial protons indicates that the methyl groups on the piperazine ring are in a cis diequatorial configuration, since a trans configuration (axial–equatorial) would permit the ring to flip. 2,6-Diaxial methyl groups would be energetically unfavorable, so the configuration is established as a cis diequatorial methyl configuration as shown in Fig. 1.

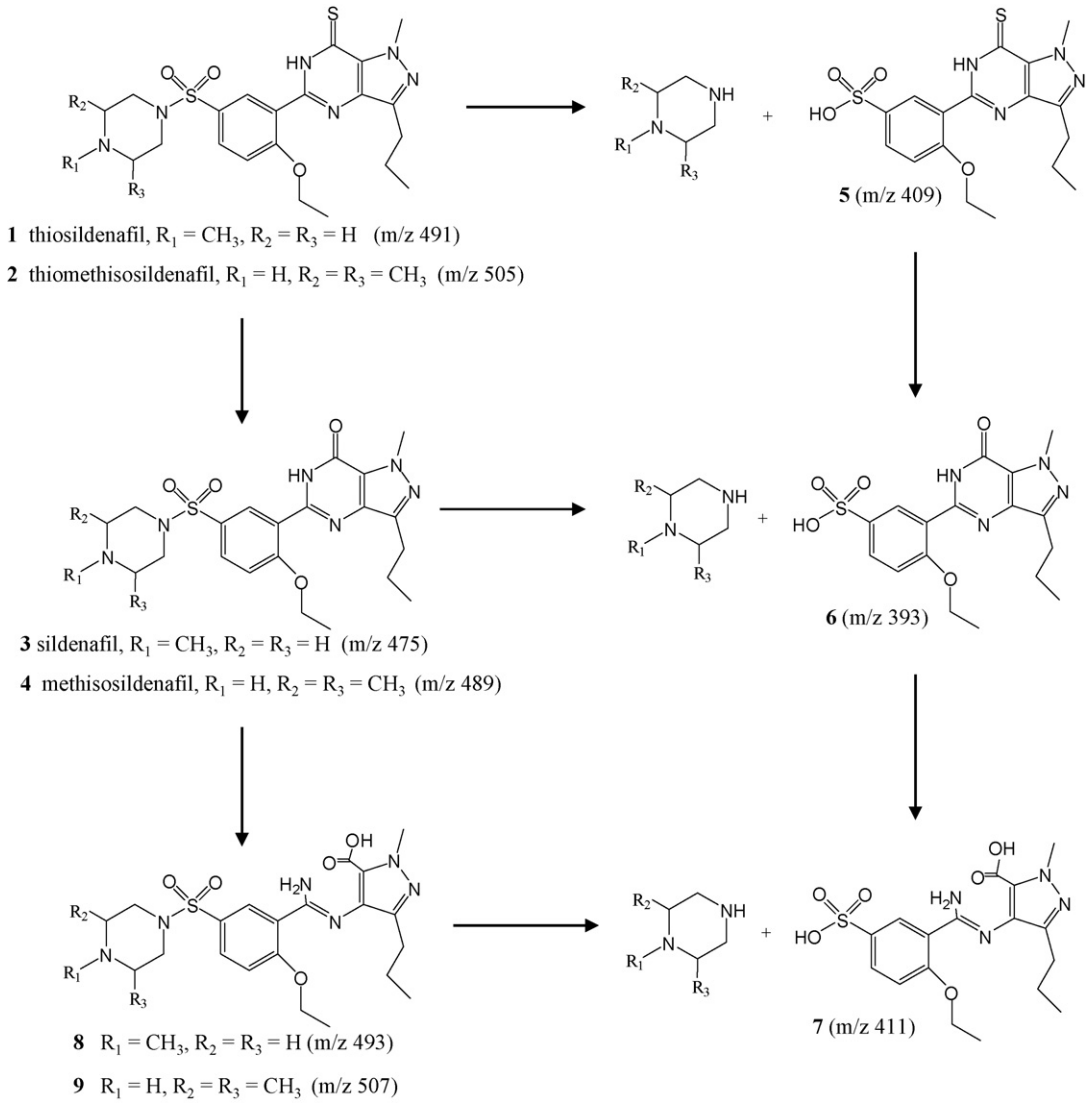

Fig. 4. Proposed hydrolytic pathways for sildenafil, thiosildenafil, and thiomethisosildenafil. Compound numbers correspond to peak numbers in Fig. 3.

In addition to the striking anisotropic effect of the sulfonyl π electrons on the piperazine equatorial proton, the association (through space) of the geminal protons with aromatic protons provides additional evidence for attachment of the sulfur atom to piperazine nitrogen farthest from the methyl groups. 2D-NMR NOESY experiments demonstrate proximity of H-15 and H-17 to H-24 axial and equatorial protons. A somewhat stronger NOE occurs between the geminal piperazine protons and aromatic

Table 2
NMR data for thiomethisosildenafil.

Group	Atom #	δ (^{1}H, ppm)	Multiplicity	^{1}H–^{1}H COSY	^{1}H–^{13}C HMQC δ (^{13}C, ppm)	NOESY
N-CH_3	10	4.50	3H, S			
$CH_2CH_2CH_3$	11	2.92	2H, t, J = 7.6	H-12	27.48	H-12, H-13
	12	1.83	2H, m	H-11, H-13	21.99	H-11, H-13
	13	0.98	3H, t, J = 7.5	H-12	13.80	H11, H-12
Benzene ring	15	8.81	1H, d, J = 2.1	H-17	130.40	H-24 ax, H-24 eq
	17	7.83	1H, dd, J = 9.1, J = 2.1	H-15, H-18	131.61	H-18, H24 eq, H24 ax
	18	7.16	1H, d, J = 9.1	H-17	112.80	H-17, H-20
OCH_2CH_3	20	4.38	2H, q, J = 7.0	H-21	66.21	H-18, H-21
	21	1.70	3H, t, J = 7.0	H-20	14.48	H-20
Dimethyl-piperazine[a]	24 (28) ax	1.91	2H, br m	H-24 eq, H25	51.87	H-24 eq, H-15, H-17, H-29
	24 (28) eq	3.66	2H, d, J = 10.8	H-24 ax, H-25	51.87	H-29, H-15, H-17, H-24 ax, H-25
	25 (27)	2.98	2H, br m	H-29	50.10	H-24 eq, H-29
	29 (30)	1.03	6H, br m	H-25	19.04	H-24 ax, H-24 eq, H-25

[a] Designations for protons on the piperazine ring: ax = axial proton, eq = equatorial proton. NMR phenomena that occur to a proton on one side of the piperazine ring also occur to the analogous proton on the other side of the ring; only protons on one side of the ring are cited in the table.

proton H-15 than with aromatic proton H-17 indicating that the piperazine ring prefers to be oriented toward the other heterocyclic ring in the molecule.

3.5. Evaluation of herbal dietary supplements

Pure solid materials of thiosildenafil and thiomethisosildenafil that were isolated from herbal dietary supplements and obtained as crystalline solids were used as standards in the analysis of herbal products shown to contain these adulterants. The analytical system used is described in Section 2.3 using 230 nm for detection. Ten herbal dietary supplements were found to contain 69, 67, 151, 149, 135, 68, 8, 72, 91 and 97 mg of thiomethisosildenafil (free base) per capsule, and one herbal dietary supplement was found to contain 35 mg of thiosildenafil (free base) per capsule. The therapeutic dose of sildenafil citrate (Viagra®) is typically 50 mg.

4. Conclusions

Two synthetic thiocarbonyl analogues of sildenafil were detected in "all-natural" herbal dietary supplements. These two compounds were isolated from the herbal products, obtained as crystalline solids, and identified as thiosildenafil and thiomethisosildenafil using LC–MS, CID-MS^n, UV spectroscopy, and NMR. The structures of the compounds were further established by acid-catalyzed hydrolysis followed by LC–PDA-MS to evaluate the resulting sulfonic acid products and GC–MS to evaluate the resulting amine product. Hydrolysis is a powerful technique for structure elucidation of thiosildenafil analogues because the thioketone is hydrolyzed to the corresponding ketone (e.g., thiosildenafil → sildenafil), and the amine generated by hydrolysis can be analyzed by GC–MS and identified through a library search.

Thiosildenafil was found in one herbal product at 35 mg per capsule and thiomethisosildenafil was found in 10 herbal products at 8–151 mg per capsule. The product label on the dietary supplements indicates that the product enhances sexual performance, lists a blend of natural herbal substances as active ingredients, and gives no indication of a synthetic erectile dysfunction drug or related synthetic compound. Consumers who purchase this product, often under the notion that natural products are safer than synthetic ones, are unaware that they are consuming a synthetic compound that has not been evaluated for safety and efficacy.

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