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THIOPHILIC REACTIONS OF PSEUDOPTEROLIDE: POTENTIAL IMPLICATIONS FOR ITS BIOLOGICAL ACTIVITY

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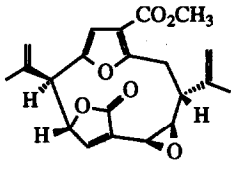
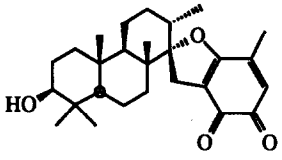
ABSTRACT. **Pseudopterolide, a marine natural product possessing a vinyl epoxide, has been shown to inhibit a variety of biological processes including cell division. We examined the ability of pseudopterolide to add to the sulfhydryl groups of a number of thiol-containing substances and found that it initially binds covalently to the sulfhydryl groups via an S_N2' addition of sulfur to the α,β-unsaturated lactone with concomitant opening of the epoxide. Subsequent addition of the sulfhydryl group to the exocyclic enone results in the formation of a 1,2-adduct via an initial Michael addition reaction followed by an apparent intramolecular S_N' elimination. We suggest that the biological actions of pseudopterolide may be caused by the addition of this compound to the thiol groups of biologically active molecules.**

Pseudopterolide (**1**), an irregular diterpenoid derived from the common red sea whip *Pseudopterogorgia acerosa*, inhibits several biological processes.[2] Initially identified as an anti-inflammatory agent, **1** was subsequently shown to inhibit the division of cultured mammalian cells, and fertilized sea urchin eggs.[3] More detailed analysis revealed that low concentrations of pseudopterolide inhibited cell division without affecting spindle organization or chromosome movement, while higher concentrations of **1** did inhibit spindle formation, amino acid uptake, translation and DNA synthesis.[4] The selective inhibition of cytokinesis by **1** in sea urchin embryos resembles the action of the fungal cytotoxin cytochalasin D[5] and Stypoldione (**2**), an ortho-quinone derived from the tropical marine alga *Stypopodium zonale*.[6] Early results have indicated that various sulfur containing compounds including cysteine and glutathione react with **2** by addition to the quinone ring with subsequent formation of an aromatic ring.[7] Compounds that react with sulfhydryl groups can inhibit the ATPase activity of myosin and the binding of myosin to actin, two functions presumed to be important for cytokinesis. When antibodies directed against myosin are used, cytokinesis is blocked selectively.[8] Although **1** possesses a unique chemical structure that is distinct from the cytochalasins and **2**, in the present study, we determined that pseudopterolide, like stypoldione and cytochalasin, can react covalently with sulfhydryl groups from a number of different types of thiol-containing compounds including thiophenol, cysteine, N-acetylcysteine, N-acetylcysteine methyl ester, and reduced glutathione. Our data suggest that the wide spectrum of biological activity of **1** may be due to a common mechanism: its addition to sulfhydryl groups at or near sensitive reactive sites on cellular proteins, and/or by adding to the sulfhydryl group of glutathione, thereby affecting the function of sensitive sulfhydryl-dependent proteins indirectly.[9]

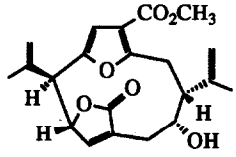
Structurally, this highly functionalized compound is roughly cylindrical in shape with the plane of the α,β-unsaturated lactone being closely parallel to the plane of the furan ring. Both these rings are in turn perpendicular to the pseudopterane skeleton. This overall arrangement gives the molecule an open "outside" and a highly congested "inside".[10] The presence of a cis-disubstituted epoxide activates the α,β-unsaturated lactone making this unique arrangement more reactive towards nucleophilic addition than when only the lactone is present, e.g., as in **3**.[11]



1



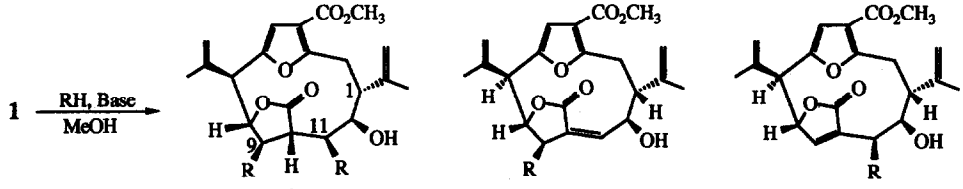
2



3

The reaction of pseudopterolide with one equivalent of PhSH in Et_3N and MeOH gave only the diaddition product (**4a**) possessing an all-*syn* arrangement at the C-9, C-11 and C-12 centres (Table I, entry 1). The stereochemistry of addition was confirmed by observation of ^1H-^1H couplings of 3.2 Hz from H-7 and H-8; 2.4 Hz from H-8 and H-9; 9.4 Hz from H-10 and H-11; respectively. An observation of no coupling between H-11 and H-12 necessitates a dihedral angle of ~90°, as expected from an all-*syn* arrangement. The recovery of half an equivalent of pseudopterolide upon workup alludes to a greater reactivity of the presumed mono-adduct (**5a**) compared to **1** itself.[12]

Table I. Reaction of pseudopterolide with sulfhydryl groups.

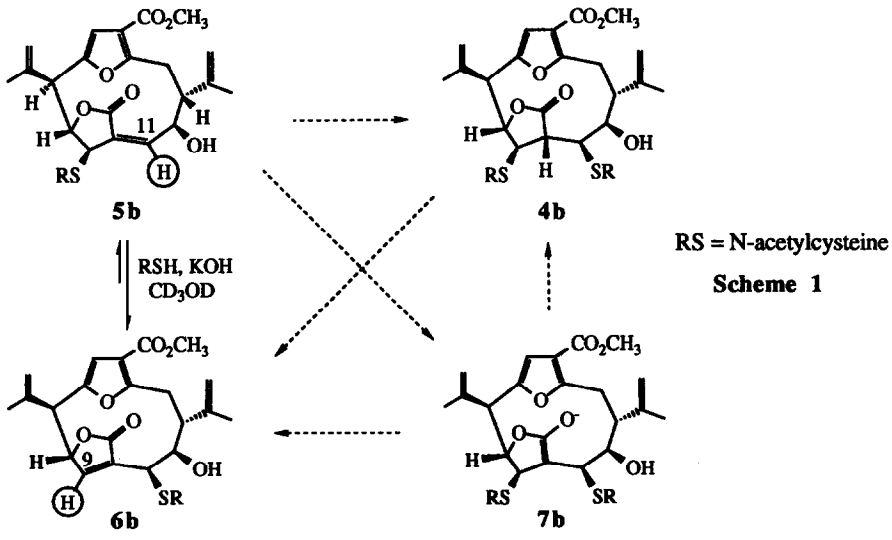


4 **5** **6**

Entry	Product	RH	Base	4	5	6
1	a	PhSH	Et_3N	100	-	-
2		$HSCH_2CH(NH_2)COOH$				
3	b	$HSCH_2CH(NHAc)COOH$	Et_3N	-	100	-
4		$HSCH_2CH(NHAc)COOMe$				
5		$HSCH_2CH(NH_2)COOH$				
6	b	$HSCH_2CH(NHAc)COOH$	KOH	-	25	75
7		$HSCH_2CH(NHAc)COOMe$				
8	a	PhSH	-	-	50	50

The reaction of pseudopterolide with one equivalent of cysteine (Table I, entry 2), or N-acetylcysteine (Table I, entry 3) or N-acetylcysteine methyl ester (Table I, entry 4) under the same conditions gave only the 1,4-mono-adduct (**5b**), with no diaddition product (**4b**) observed within the limits of detection. Similar results were obtained when reduced glutathione was used as the nucleophile. The relative stereochemistry of **5** was deduced from vicinal ^{1}H-^{1}H and ^{13}C-^{1}H coupling constants and from several n.O.e. measurements. Irradiation of the δ 2.04 proton (H-9 in **5b**) was particularly informative since it led to significant enhancements for the H-7, H-11, and H-5 (furanoid), but not for H-8 proton; the other NMR characteristics of **5** were all in accord with the structure and stereochemistry depicted.[10] The formation of **5** was presumed to arise via an S_N2' opening of the epoxide. Since related furanocembranolides such as **3** are quite unreactive,[11] the concurrent release of oxirane ring strain is presumed to be the driving force for this Michael reaction. Infact, an analogous addition of dimethylamine has also been shown to yield the 1,4-adduct.[13] As expected, no products derived from the addition of the amino group of thioamino acids were obtained in these reactions.[14]

To our surprise, the addition of one equivalent of cysteine (Table I, entry 5), or N-acetylcysteine (Table I, entry 6), or N-acetylcysteine methyl ester (Table I, entry 7) to **1** in MeOH and KOH as base gave two products in a 1:3 ratio - the 1,4-adducts (**5b**) and the hitherto unobserved 1,2-adducts (**6b**), respectively. The single most striking difference between the major isomer, **6b**, and **5b** was the replacement of the olefinic proton signal at δ 5.83 (with vicinal coupling of 7.4 Hz to H-12) in the latter by an olefinic signal in **6b** at δ 7.04 with weak coupling (1.6 Hz) to H-8, showing the migration of the double bond into the lactone ring. The formation of the *syn* 1,2-adduct (**6b**)[15] can be rationalized by the conjugate addition of sulfhydryl groups to the newly formed exocyclic enone (**5b**) with subsequent intramolecular S_N' displacement of the thiol group from the intermediate enolate **7b** or the diadduct **4b** (Scheme 1). The synclinal relationship of the HO- and the RS- groups in either **4b** or **7b** may be responsible for such an efficient elimination.[2,13] Although, the formation of **6b** can also be explained by addition of nucleophile to an intermediate allyl cation,[10] the basic conditions of the reaction precludes this possibility. In fact, treatment of **1** with PhSH under mild Lewis acid catalysis resulted in the formation of **5a** and **6a** in ca 1:1 ratio (Table I, entry 8).[16]



RS = N-acetylcysteine

Scheme 1

The possibility that the 1,2-adduct could arise from a Michael addition of the sulfhydryl group to the initially formed 1,4-adduct (**5**) followed by a retro-Michael elimination reaction was verified by monotoring the reaction of the preformed **5b** (Fig. 1a) and N-acetylcysteine in KOH and CD_3OD using ^1H NMR spectroscopic techniques (Scheme 1). On the order of mixing, an appearance of the C-9 proton in **6b** at the expense of the C-11 proton in **5b** was observed (Fig. 1b). As expected, the final equilibrium amounts of **5b** and **6b** were again in a 1:3 ratio (Fig. 1c).

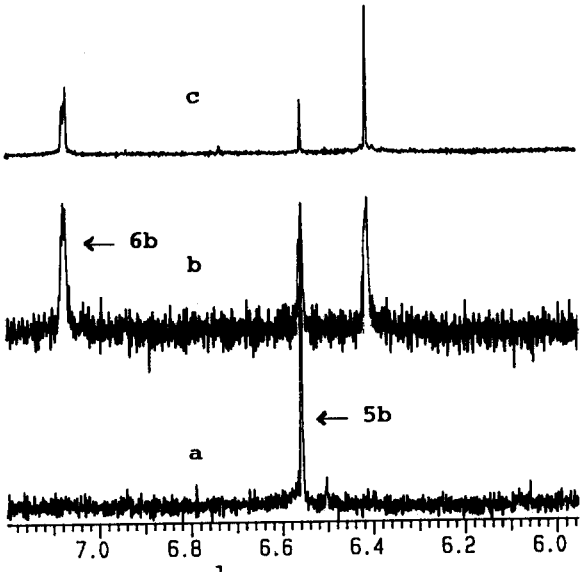


Figure 1. ^1H NMR Spectra of 5b Containing Equimolar Amounts of N-Acetylcysteine in KOH/MeOH

The reactivity of pseudopterolide towards sulfur containing nucleophiles exhibits some interestingly unexpected results. While pseudopterolide is converted directly into the diadduct by the reaction of thiophenol in Et_3N and MeOH, a 1,4-adduct is the only isolated product when **1** is reacted with cysteine, N-acetylcysteine, N-acetylcysteine methyl ester, or glutathione under the same reaction conditions. Two products, the 1,4-adduct and the 1,2-adduct, are formed in a 1:3 ratio when the addition of thioamino acids is carried out in KOH as the base. The conversion of 1,4-adduct into the 1,2-adduct under basic conditions is an equilibrium process and can be accomplished via the conjugate addition of the sulfhydryl group to the exocyclic enone followed by an apparent intramolecular S_N' elimination.

If the formation of the contractile ring in sea urchin embryos during mitosis is dependent upon sulfur containing substances in the cell, then pseudopterolide possesses the ability to covalently bind with some or all of these sulfur groups. The possibility that pseudopterolide is reacting with the contractile ring itself alludes to the result that it does react with cysteine in more than one fashion and cysteine is an amino acid found in the filamentous actin and myosin rich proteins making up the ring.

EXPERIMENTAL. All reactions were carried out in oven dried glassware under a positive pressure of nitrogen. Reagents and solvents were purchased from Aldrich Chemical Company and were used without further purification with the exception of methanol which was dried over magnesium turnings. Preparative TLC was performed using Merck pre-coated silica gel 60 F_{254}, 20x20 cm, 0.25 mm thickness glass backed analytical plates. Analytical TLC analysis was carried out using Merck pre-coated silica gel 60 F_{254}, 0.2 mm thickness aluminum backed sheets cut to the desired size. All ^1H NMR, ^{13}C NMR and special 2-D experiments were recorded on a Varian Gemini 300 spectrometer (300 MHz ^1H and 75 MHz ^{13}C). Mass spectra were recorded on a Finnegan-MAT 8230 mass spectrometer. IR spectra were recorded on a Perkin Elmer System 2000 FT-IR spectrophotometer. Pseudopterolide was extracted and isolated from frozen specimens of *P. acerosa* according to the procedure of Fenical[2] to yield 2.2% by dry weight of the pure compound.

Reaction of PhSH with pseudopterolide: Synthesis of 4a, 5a and 6a. Pseudopterolide (7.0 mg, 0.019 mmol) was mixed with Et_3N (0.028 mL, 0.020 mmol) and thiophenol (0.02 mL, 0.020 mmol) in methanol (0.2 mL). The reaction mixture was stirred at room temperature for 0.5 h after which time TLC analysis revealed the disappearance of all the thiophenol. Prep TLC purification (hexanes:EtOAc, 4:1; R_f 0.32) yielded the desired product in 48.0% yield (96.0% based on recovered pseudopterolide). ^1H NMR ($CDCl_3$, δ) 1.65 (d, J = 9.5 Hz, 1 H), 1.70 (brs, 6 H), 2.77 (dd, J = 17.7, 2.8 Hz, 1 H), 2.95 (dd, J = 9.5, 3.4 Hz, 1 H), 3.03 (brd, J = 3.3 Hz, 1 H), 3.18 (dd, J = 17.7, 12.6 Hz, 1 H), 3.31 (dd, J = 3.4, 2.3 Hz, 1 H), 3.42 (ddd, J = 12.6, 5.5, 2.8 Hz, 1 H), 3.60 (brd, J = 11.7 Hz, exch with CD_3OD, 1 H), 3.79 (dd, J = 11.7, 5.5 Hz, 1 H), 3.83 (s, 3 H), 4.54 (brs, 1 H), 4.88 (brs, 1 H), 4.91 (brs, 1 H), 4.93 (brs, 1 H), 5.01 (dd, J = 3.3, 2.3 Hz, 1 H), 6.48 (s, 1 H), 7.33 (m, 10 H); ^{13}C NMR ($CDCl_3$, δ), 18.19, 21.37, 30.09, 46.65, 49.96, 50.75, 51.60, 52.08, 57.61, 76.52, 86.95, 111.65, 113.87, 114.96, 115.50, 127.74, 129.10 (2 C), 129.16, 129.61 (2 C), 131.99, 132.82 (2 C), 134.03 (2 C), 134.46, 140.92, 145.37, 150.00, 159.96, 163.21, 176.87; HREI MS m/z 590.1797 (M^+, calcd for $C_{33}H_{34}O_6S_2$, 590.1797).

5a: R_f 0.27 (hexanes:EtOAc, 3:1); ^1H NMR ($CDCl_3$, δ) 1.75 (s, 3H), 1.89 (s, 3H), 2.57 (m, 1H), 3.50 (m, 3H), 3.63 (s, 3H), 3.79 (s, 3H), 3.80 (m, 1H), 4.21 (d, J = 12.5 Hz, exch with CD_3OD, 1 H), 4.92 (brs, 1 H), 4.93 (brs, 1 H), 4.99 (d, J = 4.2 Hz, 1 H), 5.05 (brs, 1 H), 5.33 (d, J = 7.6 Hz, 1 H), 6.36 (s, 1 H), 7.32 (m, 6H); ^{13}C NMR ($CDCl_3$, δ) 18.35, 21.92, 29.34, 49.79, 49.88, 51.65, 51.95, 69.39, 84.79, 112.56, 112.61, 113.89, 116.09, 116.29, 129.31 (2 C), 130.46, 135.20 (2 C), 139.05, 139.10, 145.14, 149.88, 160.54, 163.69, 170.67; HREI MS m/z 480.1609 (M^+, calcd for $C_{27}H_{28}O_6S$, 480.1607). **6a**: R_f 0.15 (hexanes:EtOAc, 3:1); ^1H NMR ($CDCl_3$, δ) 1.94 (s, 3 H), 2.03 (s, 3 H), 2.75 (dd, J = 15.7, 3.5 Hz, 1 H), 3.02 (d, J = 11.8, exch with CD_3OD, 1 H), 3.34 (ddd, J = 12.9, 3.5, 2.1 Hz, 1 H), 3.46 (ddd, J = 11.8, 4.7, 2.1 Hz, 1 H), 3.60 (dd, J = 15.7, 12.9 Hz, 1 H), 3.78 (d, J = 4.5 Hz, 1 H), 3.79 (s, 3 H), 4.06 (d, J = 4.7 Hz, 1 H), 4.80 (brs, 1 H), 5.02 (brs, 1 H), 5.20 (brs, 1 H), 5.35 (dd, J = 4.5, 1.6 Hz, 1 H), 6.34 (s, 1 H), 6.61 (d, J = 1.6 Hz, 1 H), 7.21 (m, 3 H), 7.41 (m, 2 H); ^{13}C NMR ($CDCl_3$, δ) 21.54, 23.09, 29.69, 31.76, 44.43, 48.50, 51.58, 55.8, 75.62, 80.05, 110.53, 115.41, 117.368, 127.20, 129.02 (2 C), 131.62 (2 C), 133.44, 136.77, 140.83, 143.98, 148.09, 150.45, 160.55, 163.80, 172.22; HREI MS m/z 480.1609 (M^+, calcd for $C_{27}H_{28}O_6S$, 480.1607).

Reaction of N-acetylcysteine with pseudopterolide: Synthesis of 5b and 6b. To N-acetylcysteine (19.7 mg, 0.121 mmol) was added 0.65 mL of a 0.35 M methanolic KOH solution. The volume of this solution was made up to 5.0 mL. One equivalent of this solution (0.47 mL, 0.011 mmol) and pseudopterolide (4.2 mg 0.011 mmol) were stirred for 1 h when the reaction mixture was acidified with 2.0 equiv. of $HCl_{(aq)}$ (pH, 7.0) and the solvent removed in *vacuo*. The resulting yellow oil was dissolved in acetone and filtered to remove KCl. Removal of acetone gave 5.8 mg of a crude yellow oil in 96.0% yield. ^1H NMR spectrum of the crude reaction mixture revealed the formation of **5b** and **6b** in a 1:3 ratio. The resulting mixture was purified by prep TLC ($MeOH:CH_2Cl_2:Et_2O$, 8:5:4) to yield the desired products. **5b**: (R_f 0.7); IR (neat, cm^{-1}) 3353, 2925, 1755, 1725; ^1H NMR (CD_3OD, δ) 6.56 (s, 1H), 5.83 (d, J=7.4 Hz, 1H), 4.93 (d, J=4.1 Hz, 1H), 4.34 (dd, J=7.0, 4.2 Hz, 1H), 4.01 (dd, J= 10.5, 7.4 Hz, 1H), 3.94 (d, J=4.1 Hz, 1H), 3.82 (s, 3H), 3.69 (dd, J=15.7, 13.0 Hz, 1H), 3.49 (ddd, J=13.0, 10.5, 3.8 Hz, 1H), 2.92 (dd, J=15.0, 4.2 Hz, 1H), 2.83 (dd, J=15.0, 7.0 Hz, 1H), 2.57 (dd, J=15.7, 3.8 Hz, 1H), 2.04 (s, 1H), 1.95 (m, 3H), 1.81 (m, 3H). **6b**: (R_f 0.4); IR (neat, cm^{-1}) 3369, 2931, 1734, 1721, 1616; ^1H NMR (CD_3OD, δ) 7.04 (d, J=1.6 Hz, 1H), 6.42 (s, 1H), 5.47 (dd, J=4.6, 1.6 Hz, 1H), 5.20 (m, 1H), 5.03 (m, 1H), 4.98 (m, 1H), 4.75 (m, 1H), 4.27 (dd, J=7.9, 4.5 Hz, 1H), 4.07 (d,

J=4.6 Hz, 1H), 3.87 (d, *J*=4.7 Hz, 1H), 3.80 (s, 3H), 3.68 (dd, *J*=15.5, 12.9 Hz, 1H), 3.28 (ddd, *J*=12.9, 3.9, 1.5 Hz, 1H), 3.28-3.32 (dd, *J*=4.7, 1.5 Hz, 1H), 2.98 (dd, *J*=13.7, 4.5 Hz, 1H), 2.76 (dd, *J*=13.7, 7.9 Hz, 1H), 2.58 (dd, *J*=15.5, 3.9 Hz, 1H), 1.96 (s, 3H), 1.95 (s, 3H), 1.94 (s, 3H); MS m/z 533.6 (M^+-H_2O).

Adducts **5b** and **6b** were also characterized as their more stable N-acetylcysteine methyl ester derivatives. **5b** - methyl ester: R_f0.43 (hexanes:EtOAc, 1:4); ^{1}H NMR ($CDCl_3$, δ) 1.81 (s, 3 H), 1.92 (s, 3 H), 2.00 (s, 3 H), 2.63 (dd, *J* = 16.1, 3.1 Hz, 1H), 2.69 (dd, *J* = 14.2, 6.3 Hz, 1 H), 3.03 (d, *J* = 14.2, 4.6 Hz, 1 H), 3.52 (m, 2 H), 3.61 (m, 2 H), 3.74 (s, 3H), 3.83 (s, 3 H), 4.00 (m, 1 H), 4.35 (br, exch with CD_3OD, 1 H), 4.66 (ddd, *J* = 14.2, 6.53, 4.6 Hz, 1 H), 4.81 (d, *J* = 4.1 Hz, 1 H), 4.91 (brs, 1 H), 4.96 (brs, 1 H), 4.99 (brs, 1 H), 5.07 (brs, 1 H), 5.70 (d, *J* = 7.2 Hz, 1 H), 6.20 (d, *J* = 7.0 Hz, 1 H), 6.40 (s, 1 H); ^{13}C NMR ($CDCl_3$, δ) 18.65, 21.99, 23.06, 29.37, 29.69, 32.41, 47.96, 49.50, 50.02, 51.80, 53.11, 69.63, 84.78, 112.66, 114.11, 116.20, 116.42, 130.40, 138.94, 139.01, 145.13, 149.89, 160.56, 163.62, 170.05, 170.46, 170.89; HREI MS m/z 548.1938 (M^+, calcd for $C_{27}H_{32}O_9NSD$, 548.1938). **6b** - methyl ester: R_f0.20 (hexanes:EtOAc, 1:4); ^{1}H NMR ($CDCl_3$, δ) 1.93 (s, 3 H), 1.96 (s, 3 H), 1.98 (s, 3 H), 2.70 (dd, *J* = 15.8, 3.7 Hz, 1 H), 2.91 (d, *J* = 5.5 Hz, 1 H), 2.97 (br, exch with CD_3OD, 1 H), 3.20 (ddd, *J* = 13.0, 3.7, 1.9 Hz, 1 H), 3.37 (s, 3 H), 3.56 (dd, *J* = 15.8, 13.0 Hz, 1 H), 3.71 (d, *J* = 4.4 Hz, 1 H), 3.72 (s, 3 H), 3.78 (dd, *J* = 4.4, 1.9 Hz, 1 H), 3.80 (d, *J* = 4.3 Hz, 1 H), 4.66 (dd, *J* = 7.8, 5.5 Hz, 1 H), 4.78 (brs 1 H), 5.01 (brs 1 H), 5.14 (brs 1 H), 5.16 (brs 1 H), 5.40 (d, *J* = 4.3 Hz, 1 H), 6.35 (s, 1 H), 6.38 (brd, *J* = 7.8 Hz, 1 H), 6.82 (s, 1 H); ^{13}C NMR ($CDCl_3$, δ) 21.51, 22.96, 23.22, 31.73, 36.37, 44.07, 48.35, 51.67, 51.76, 51.93, 52.75, 75.70, 80.19, 110.49, 115.39, 116.11, 117.20, 132.93, 140.81, 144.09, 148.77, 150.44, 160.42, 163.84, 170.02, 171.07, 172.28; HREI MS m/z 547.1878 (M^+, calcd for $C_{27}H_{33}O_9NS$, 547.1878).

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