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Cytotoxic quaternary alkaloids from the flowers of *Narcissus tazetta*

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Dedicated to Prof. Dr. Paul J. Scheuer, University of Hawaii at Manoa, on the occasion of his 86th birthday

Intensive chromatographic separation of the polar fraction of an ethanolic extract of the fresh flowers of *Narcissus tazetta* L. (Amaryllidaceae) yielded two new quaternary alkaloids with a phenanthrene skeleton, *N*-methyl-8,9-methylenedioxy-phenantridinium methylsulfate (**1**) and *N*-methyl-8,9-methylenedioxy-phenantridinium malate (**2**). The structure determination of the alkaloids was based on one- and two-dimensional NMR studies including HMQC, and HMBC studies, and mass spectroscopic analysis. The existence, in **1**, of the methylsulfate group was confirmed by X-ray diffraction analysis. Cytotoxic activities for **1** and **2** against a panel of cancer cell lines are also reported.

1. Introduction

In continuation of our ongoing studies of the constituents of the Egyptian Amaryllidaceae plants [1–4] we have investigated the fresh flowers of *Narcissus tazetta* L. (Amaryllidaceae). To date 48 alkaloids with different skeletons have been reported from different *Narcissus* species [5–25]. Many of the Amaryllidaceae alkaloids have shown a variety of biological activities [26].

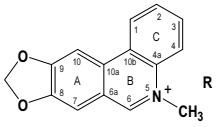
2. Investigations, results and discussion

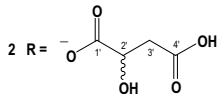
Compound **1** was purified as yellow needles. It is a quaternary alkaloidal salt and behaves as two different compounds in the MS (the substituted phenanthrene skeleton will be referred to as M_1, while M_2 is the methylsulfate part). The EIMS showed ions at m/z 238 and 111, corresponding to M_1 $[C_{15}H_{12}NO_2]^+$, and M_2 $[SO_4CH_3]^+$, respectively. Moreover, two different base peaks were observed at m/z 223 and 64, corresponding to the stable fragments $[C_{14}H_9NO_2]^+$ and $[SO_2]^+$, resulting from loss of CH_3 and CH_3O_2 fragments from M_1 and M_2, respectively. Investigation of the ^{1}H NMR spectrum of **1** (Table 1) showed resonances for 15 protons, seven of which appeared in the aromatic region ranging from 9.70–7.76 ppm, together with two three-proton singlets at δ 3.67 and 4.64, and a two-proton singlet at δ 6.42. Interpretation of the aromatic region showed four protons to be contiguous (H-1 to H-4) suggestive of an *ortho*-substituted ring (e.g. ring C in **1**), together with two singlets at δ 7.76 and 8.46, indicative of *para*-substituted protons arising from a tetra-substituted ring (e.g. ring A in **1**). The remaining downfield singlet at δ 9.70 could be ascribed to an iminic proton in a heteroaromatic-ring (e.g. ring B in **1**). The singlets at δ 4.64 and δ 3.67 can be assigned as an *N*-methyl group and as the methyl group of the methylsulfate radical. In addition the signal at δ 6.42 was assigned as the CH_2 of a methylenedioxy moiety. The ^{13}C NMR spectrum of **1** showed resonances for 16 carbons: two quartets, one triplet, seven doublets, and six singlets. The assignments of the protonated carbons are based on an HMQC experiment, while assignment of the quaternary carbons was secured from long-range ^{1}H-^{13}C coupling in the HMBC experiment (Fig. 1, Table 2).

Table 1: Proton chemical shift assignments of compounds 1 and 2

	1	**2**		**1/2**
^{1}H	δ (ppm)[a]	δ (ppm)[a]	δ (ppm)[b]	M (J_{Hz})
1	8.93	8.87	9.03	dd (8.1, 1.2)
2	8.02	8.10	8.08	dd (8.1, 7.2)
3	8.10	7.96	8.01	ddd (8.0, 7.2, 1.2)
4	8.44	8.38	8.43	brd (8.0)
NCH_3	4.64	4.62	4.59	s
6	9.70	9.64	9.86	s
7	7.76	7.71	7.87	s
OCH_2O	6.42	6.41	6.45	s
10	8.46	8.36	8.65	s
$S-OCH_3$	3.67			s
2′		4.31	3.83	dd (7.3, 5.4)
3′a		2.78	2.47	dd (16.0, 5.4)
3′b		2.54	2.29	dd (16.0, 7.3)
COOH			11.20[c]	

[a] Recorded in CD_3OD
[b] Recorded in DMSO-d_6
[c] Very broad signal (hump)





The resonance at δ 3.67 in the ^{1}H NMR spectrum of **1** was misleading; this signal was initially assigned as a solvent impurity. After examination of the MS spectrum, it was clear that methylsulfate is present as a counter ion. The appearance of the fragmentation ions at m/z 111 $[CH_3SO_4]^+$, 64 $[SO_2]^+$, and 48 $[SO]^+$ further supported the existence of the methylsulfate group. The crystalline nature of **1** allowed confirmation of the methylsulfate group by X-ray diffraction analysis (Fig. 2).

Compound **2** was obtained as a yellow solid. Similarly to **1**, compound **2** displayed EIMS peaks at m/z 238 and 223. The ion for the malate radical at m/z 133 was not observed. The FABMS (glycerol) of **2** displayed two ions at m/z 330 for $[C_{15}H_{12}NO_2 + \text{glycerol}]^+$, and 225 for $[C_4H_5O_5 + \text{glycerol}]^+$. Other significant fragmentation ions of the malate part of **2** are presented in the Scheme.

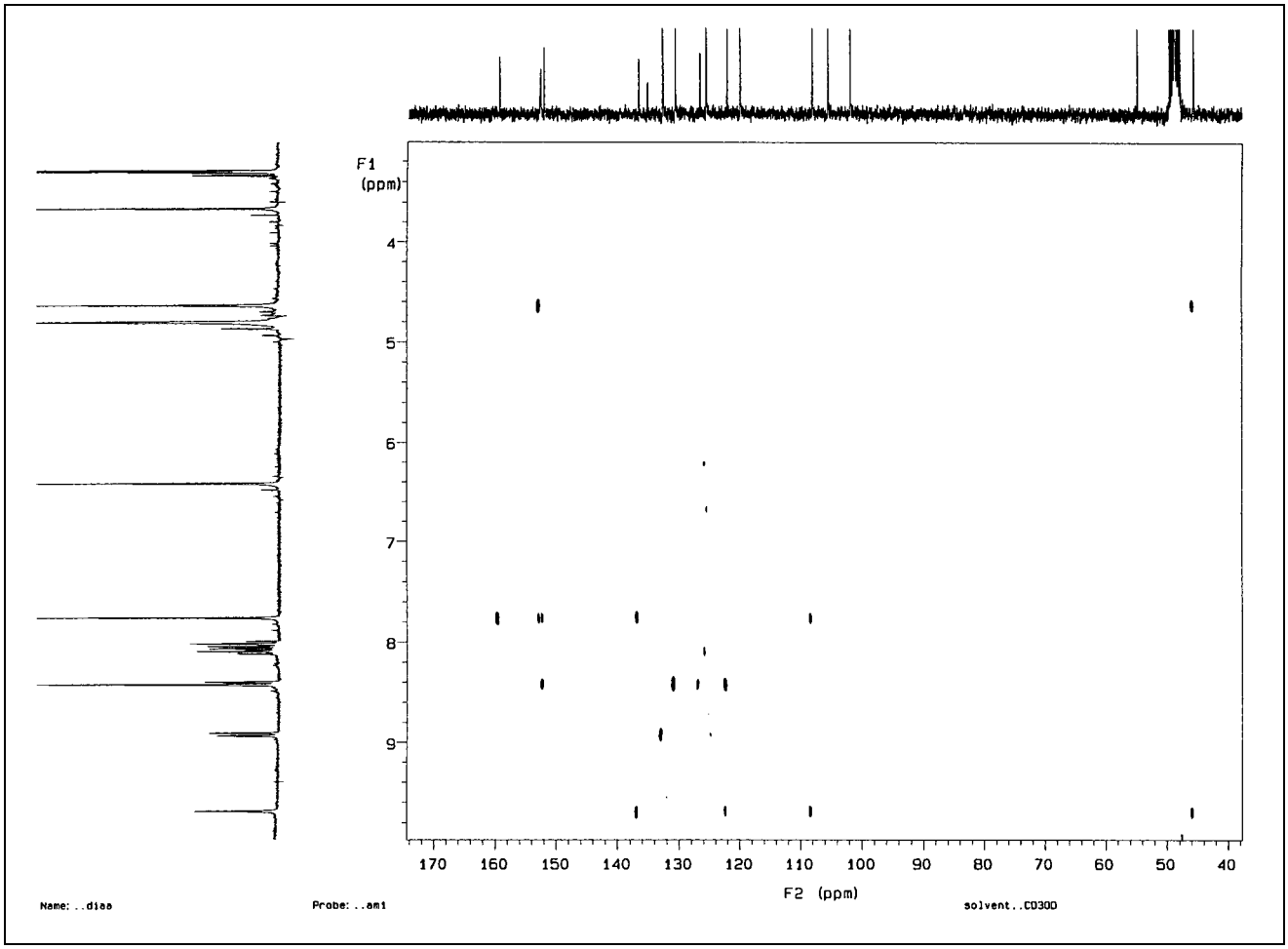


Fig. 1: HMBC spectrum of compound **1**

Comparison of the NMR data (Tables 1 and 2) of **2** with that of **1** showed that **2** lacked the signals at δ 3.67/55.06 but contained new resonances due to the malate group. These resonances include a ^{1}H doublet of doublet at δ 4.31 (H-2′, 69.08: C-2′), a doublet of doublet at δ 2.78 (H-3′a) and a doublet of doublet at δ 2.54 (H-3′b, 41.33: C-3′), together with resonances at δ 178.55 (C-1′) and 175.53 (C-4′), suggestive of two carboxylic acid moieties.

Table 2: Carbon chemical shift assignments of compounds 1 and 2[a]

	1		**2**		
^{13}C	δ (ppm)[b]	HMBC with H[b]	δ (ppm)[b]	δ (ppm)[c]	HMBC with H[c]
1	125.86	H-3	125.84	124.72	H-3
2	130.95	H-4	130.93	129.46	H-4
3	132.96	H-1	132.95	131.46	H-1
4	120.22		120.17	119.55	
4a	135.47		135.41	133.65	H-1, NCH_3
NCH_3	45.88	H-6	45.85	45.18	
6	152.21	H-7, NCH_3	152.69	151.86	H-7, NCH_3
6a	136.83	H-7 ($^2J_{CH}$), H-6 ($^2J_{CH}$)	136.78	134.39	H-7 ($^2J_{CH}$)
7	108.33	H-6	108.28	107.13	
8	152.83	H-10, H-7 ($^2J_{CH}$)	152.17	150.01	H-10, H-7 ($^2J_{CH}$)
OCH_2O	105.75		105.75	104.17	
9	159.53	H-7	159.50	157.20	H-7, H-10 ($^2J_{CH}$)
10	102.15		102.11	101.25	
10a	122.38	H-6, H-10 ($^2J_{CH}$)	122.32	120.39	H-10
10b	126.88	H-4, H-10	128.14	124.94	H-2, H-4, H-10
$S-OCH_3$	55.06				
1′			178.55	176.43	H-2′ ($^2J_{CH}$), H-3′a, H-3′b
2′			69.08	65.71	H-3′a, H-3′b ($^2J_{CH}$)
3′			41.33	41.69	
4′			175.53	171.86	H-3′a, H-3′b ($^2J_{CH}$)

[a] Long-range connectivities in HMBC spectra are given; Long-range couplings were via $^3J_{CH}$ unless otherwise noted in parentheses. The HMBC spectrum was recorded with long-range delay optimized for 7 Hz [b] Recorded in CD_3OD [c] Recorded in DMSO-d_6.

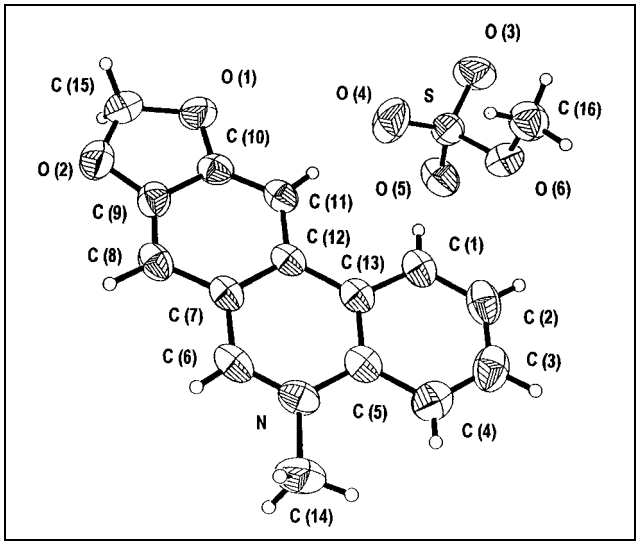
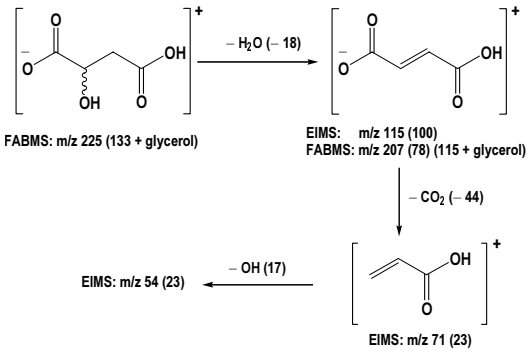


Fig. 2: Computer-generated X-ray crystal structure of compound **1**

The NMR data for **2** are reported here in two different solvents (CD_3OD and DMSO-d_6). The spectrum in DMSO-d_6 showed a very broad ^1H resonance at $\sim\delta$ 11.20 for the proton of a carboxylic acid group at either C-1′ or C-4′. The ^{13}C NMR data for the malate part are comparable with those reported for malic acid [27]. The ^{13}C NMR spectrum of **2** displayed resonances for 19 carbons: one quartet, two triplets, eight doublets, and eight singlets (based on DEPT and HMQC (Fig. 3)). Assignment of the quaternary carbons is based on ^1H-^{13}C long-range correlations in an HMBC experiment (Table 2).

Scheme



Both compounds **1** and **2** showed cytotoxic activity ($IC_{50} = 5$ μg/ml) against cancer cell lines including mouse lymphoma (P-388, ATCC: CCL 46), human colon carcinoma (HT-29, ATTC: HTB 38), and human lung carcinoma (A-549, ATTC: CCL 8).

The alkaloids *N*-methyl-8,9-methylenedioxy-phenantridinium chloride [28] and bicolorine (*N*-methyl-8,9-methylenedioxy-phenantridinium) [11] have been previously reported from *Lapiedra martinezii* and *Narcissus bicolor*, respectively, and possess the same substituted phenanthrene skeleton as **1** and **2**. The assignment of the first alkaloid was based primarily on ^1H NMR and MS data [28]. Both ^1H and ^{13}C NMR data were reported for bico-

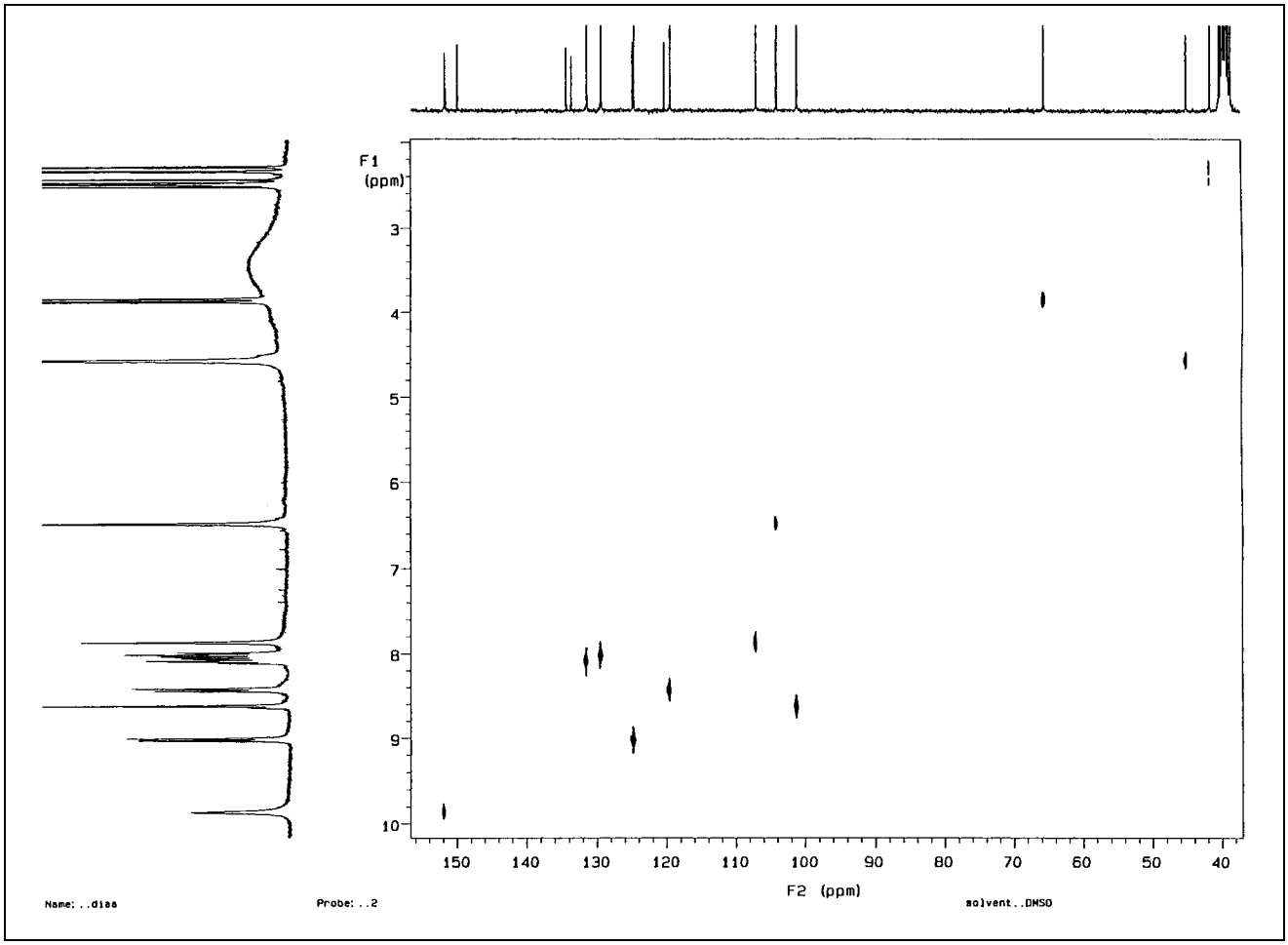


Fig. 3: HMQC spectrum of compound **2**

lorine [11] but the assignment of some ^{13}C resonances are not in agreement with our data. Our assignments for ^{13}C resonances are confirmed and secured from two-dimensional NMR experiments, including HMQC and HMBC.
Compounds **1** and **2** are believed to be genuine natural products and not artifacts because both malic acid and sulphate are common naturally occurring compounds and have been reported frequently from different terrestrial sources [29]. Moreover, more than 500 sulfated compounds have been reported from marine organisms [30]. In addition neither acid nor caustic reagent was used for the extraction, fractionation, separation, or purification of these alkaloids.
To the best of our knowledge compounds **1** and **2** have neither been previously reported from a natural source nor synthesized.

3. Experimental

3.1. Plant material, apparatus and methods

The fresh flowers of *N. tazetta* were collected from plants cultivated at the campus of the Suez Canal University at Ismailia during the flowering period of July and August 1997. Samples were identified and authenticated by Dr. A. Fayed, Professor of Plant Taxonomy at the Department of Botany, Assiut University. A voucher specimen (No. NT1) is deposited at the Herbarium of the Faculty of Pharmacy, Suez Canal University.
Biological assays were performed to determine IC_{50} (μg/ml) for crude extracts and pure compounds on selected cancer cell lines. These cells are mouse lymphoma (P-388, ATCC: CCL 46), human colon carcinoma (HT-29, ATTC: HTB 38), and human lung carcinoma (A-549, ATTC: CCL 8).
M.p.'s were uncorrected. NMR spectra were determined at 300 MHz (for ^1H) and at 75 MHz (for ^{13}C). Mass spectra were performed on a Finnigan MAT-312 at 70 eV. HPLC was conducted on YMC-AQ ODS 5 μm, 120 Å, 250 × 10 mm. TLC was performed on pre-coated silica gel 60 F_{254} and RP-18 F_{254s} (0.25 mm, Merck).

3.2. Extraction and isolation

The fresh flowers of *N. tazetta* (1.8 kg) were crushed and macerated with ethanol 95% for 24 h (2 × 5 l) at room temperature. The combined alcoholic extracts were concentrated under reduced pressure to 200 ml, then diluted with 100 ml H_2O. The resulted solution was extracted successively with n-hexane (3 × 250 ml), dichloromethane (3 × 250 ml), and finally with n-butanol (3 × 250 ml). The n-butanol fraction showed cytotoxicity (IC_{50} = 10 μg/ml). The present work deals with examination of the polar fraction (n-butanol) only. Both n-hexane and dichloromethane fractions are currently under investigation.

3.3. Purification of compounds 1 and 2

The n-butanol fractions were concentrated under vacuum to a brown viscous extract. The resulting extract (2.3 g) was subjected to flash chromatography over alumina, and eluted with ethyl acetate with increasing proportions of MeOH. Ten major fractions were collected. The fractions eluted with EtOAc–MeOH (7 : 3) (F-1) and (6 : 4) (F-2) showed cytotoxicity (IC_{50} = 5 μg/ml). Fraction F-1 (39 mg) contained impure **1,** which was subjected to final purification on a semipreparative HPLC column (YMC-AQ, ODS 5 μm, 120 Å, 250 × 10 mm, 2 ml/min at 254 nm) eluted with MeCN-H_2O (1 : 1) to give **1** (11 mg, crystallized from MeOH). Fraction F-2 (29 mg), contained impure **2**, which was further purified on HPLC (YMC-AQ, ODS 5 μm, 120 Å, 250 × 10 mm, 2 ml/min at 254 nm) eluted with MeCN-H_2O (4 : 6) to give **2** (9 mg).

3.4. Identification of N-methyl-8,9-methylenedioxy-phenantridinium methylsulfate (**1**)

Yellow needles; m.p. 298–300 °C (dec.); $[(C_{15}H_{12}NO_2) \cdot (SO_4CH_3)]^+$; EIMS m/z (Rel. int.%) 238 (36) $[C_{15}H_{12}NO_2]^+$, 223 (100) $[C_{15}H_{12}NO_2{-}CH_3]^+$, 111.9757 (21) calc. 110.9752 for $[SO_4CH_3]^+$, 64 (100) $[SO_2]^+$, 48 (57) $[SO]^+$, 44 (93); HREIMS: calc. for $C_{15}H_{12}NO_2$: 238.0868, found: 238.0870, calc. for SO_4CH_3: 110.9752, found: 110.9756; NMR data: see Tables 1 and 2.

3.5. Identification of N-methyl-8,9-methylenedioxy-phenantridinium malate (**2**)

Yellow solid; m.p. 278–283 ºC (dec.); $[(C_{15}H_{12}NO_2) \cdot (C_4H_5O_5)]^+$; EIMS m/z (Rel. int.%) 238 (20) $[C_{15}H_{12}NO_2]^+$; 223 (100) $[C_{15}H_{12}NO_2{-}CH_3]^+$; 179 (72), 98 (14), 71 (23), 54 (23); FABMS (Glycerol) m/z (Rel. int.%) 330 (43) $[C_{15}H_{12}NO_2$ + glycerol$]^+$, 238 (100) $[C_{15}H_{12}NO_2]^+$, 225 (26) $[C_4H_5O_5$ + glycerol$]^+$, 207 (78) $[C_4H_5O_5 - H_2O$ + glycerol$]^+$, 115 (100) $[C_4H_5O_5{-}H_2O]^+$; NMR data: see Tables 1and 2.

3.6. X-ray crystal structure analyses of 1 [31]

Crystal data: $C_{16}H_{15}NO_6S$, prismatic yellow needle (0.1 × 0.25 × 0.15 mm) grown by evaporation of a solution of **1** in MeOH, monoclinic, $P2_1$, a = 8.4376 (5) Å, b = 10.5329 (6) Å, c = 14.0842 (6) Å; β = 101.695 (3)°, V = 867.016 Å^3, Z = 2, intensities were collected with graphic monochromatized Mo Kα radiation, cell parameters from 25 reflections, T = 293 K. Data collection: Syntex R3 four-circle diffractometer, θ scans, 7467 measured reflections, 4275 independent reflections, 4026 observed reflections [F > 1.5σ (F)], R_{int} = 0.0631, θ_{max} = 27.5°.
The structure was solved by direct methods. All H atoms were refined isotropically, with non-H atoms being refined anisotropically. Data collection: Syntex R3 software. Cell refinement: Syntex R3 software. Data reduction: CRYSTALS [32]. Program(s) used to solve structure: SIR92 [33]. Programs used to refine structure: CRYSTALS. Molecular graphics: CRYSTAN88 [34].

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