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Identification of the bright-greenish-yellow-fluorescence (BGY-F) compound on cotton lint associated with aflatoxin contamination in cottonseed

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Abstract

In order to characterize the structure of the bright-greenish-yellow-fluorescence (BGY-F) compound on cotton lint associated with aflatoxin contamination in cotton seed, various in vitro and in vivo natural BGY-F reaction products were prepared. Under similar high pressure liquid chromatography separation with variable wavelength and programmable fluorescence detection (HPLC–UV/FL), combined with atmospheric pressure ionization and mass spectral determinations it was found that the BGY-F reaction products prepared from three preparations: (a) kojic acid (KA) + peroxidase (soybean peroxide or horseradish type VI and type II) + H_2O_2, or (b) detached fresh cotton locules + KA + H_2O_2, or (c) attached field cotton locules that were treated with a spore suspension of aflatoxigenic *Aspergillus flavus*, all resulted in identical chromatographic characteristics, and all exhibited a molecular weight of 282. Further characterization of the BGY-F reaction product with ^{1}H- and ^{13}C-NMR spectroscopic analysis revealed that it was a dehydrogenator dimer of 2 KA, linked through the C-6 positions. Published by Elsevier Science Ltd.

Keywords: *Gossypium hirsutum* L; Malvaceae; Bright-greenish-yellow fluoresence (BGY-F); *Aspergillus flavus*; Aflatoxin; HPLC-UV/F-MS; NMR; Kojic acid; Kojic acid dimer

1. Introduction

Aflatoxigenic *Aspergillus* sp. invasion of developing cottonseed (*Gossypium hirsutum* L./Malvaceae) results in the formation of a characteristic bright-greenish-yellow-fluorescent (BGY-F) reaction material which occurs on cotton lint in the developing cottonboll when the lint is observed under long wave UV light (Marsh, Bollenbacher, San Antonio & Merola, 1955). It is well established (Marsh, Simpson, Craig, Donoso & Ramey, 1973) that BGY-F results from the reaction of host plant peroxidase with the fungal metabolite kojic acid (KA). KA (5-hydroxy-2 (hydroxymethy)-4*H*-pyran-4-one), the precursor of the BGY-F material is produced by both aflatoxigenic *Aspergillus* sp., *A. flavus* and *A. parasiticus* (Parrish, Wiley, Simmons & Long, 1966). It is also reported that the BGY-F material is formed only on the lint of the developing cottonboll whereas aflatoxin contamination forms in the seeds (Lee & Russell, 1981). Marsh et al. (1969) reported that the BGY-F material can be produced in solutions of peroxidase, hydrogen peroxide, and KA. Recently, we reported a HPLC-UV/FL system to isolate the BGY-F material from various in vitro chemical and in vivo natural BGY-F reaction products (Zeringue & Shih, 1998). These BGY-F materials were obtained from reactions we had prepared from (a) KA + NaClO + H_2O_2, (b) KA + peroxidase + H_2O_2, (c) fresh cotton locules treated with KA + H_2O_2, (d)

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Table 1
Summary of major ions and base peak abundances obtained by API/MS (infusion method)[a] from kojic acid (KA) and various BGY-F reaction products

Reaction product number	Reaction conditions	Major ions, *m/z* (relative abundance, %)	Base peak abundance
0	KA, 100 ppm	**141** (100.0), 283, 255, 111, 83	4800
1	KA + NaOCl + H_2O_2, (C_{18})[b]	**141** (2.8), 268, 93, 83 (100), 59	5250
2a	KA + HRP VI[c] + H_2O_2, (C_{18}), $HOAc$[d], (NH_2)[e], NH_4 OAc	**281** (100.0), 181 (55.8), **141** (23.3)	1720
2b	KA + HRP II[c] + H_2O_2	**281** (26.9), 223, 181 (14.9), **141** (100.0), 83	13,400
2c	KA + SBP[c] + H_2O_2	**281** (100.0), 223, 181 (23.1), 141 (57.7)	5200
3	KA + locules, laboratory (C_{18}), HOAc, (NH_2) NH_4OAc[f]	**281** (89), 181 (25.0), 59 (100.0)	10,000
4	Locules, field (C_{18}), HOAc, (NH_2), NH_4OAc	**281** (40.8), 181 (14.4), 59 (100.00)	8700

[a] Infused at a rate of 10 μl/min at 150°C, MS set in negative ion mode.
[b] Bakerbond SPE Polar Plus C_{18} (Octadecyl), 6 ml solid phase extraction column.
[c] Peroxidases from horseradish (HRP VI, HRP II). Peroxidases from soybean (SBP).
[d] Lyophilized BGY-F product in acidified water (pH 3) with HOAC.
[e] Bakerbond SPE Amino (NH_2) 6 ml solid phase extraction column.
[f] BGY-F compound eluted with 3 column volumes of 0.1 M NH_4OAc solution.

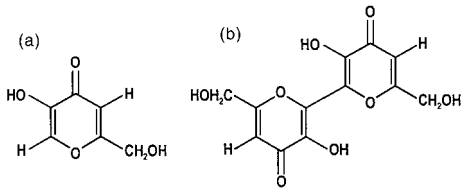

Fig. 1. Structure of kojic acid (A) and proposed structure of the bright-greenish-yellow-fluorescence compound (B).

detached cotton locules inoculated with an aflatoxigenic *Aspergillus flavus* spore suspension, and (e) live developing cotton bolls inoculated with aflatoxigenic *A. flavus*. It was found that in all methods used to form the BGY-F compound with the exception of the reaction of KA + NaClO + H_2O_2, only one product with similar chromotagraphic characteristics was produced, and that compound was probably an oxidized form of KA.

The purpose of this current investigation was to further purify and characterize the structure of the BGY-F compound.

2. Results and discussion

Various lyophilized BGY-F preparations were initially dissolved separately in H_2O, and were subjected to pre-purification by C_{18} SPE or NH_2 SPE column separations. The dried lyophilized products resulting from these pre-purification treatments were dissolved in H_2O:MeOH (50:50, v/v) and were injected by infusion (Harvard syringe pump) into an API/MS (HP9987A/HP5989A) system set in the negative ion mode. The major ions of interest resulting from that analysis are shown in Table 1. As expected, the kojic acid gives a pseudo-molecular ion $(M\text{-}H)^-$, at 141 *m/z*. Other ions in the spectrum of kojic acid include those resulting from a dimer (283) and from fragmentation with loss of ($-CH_2OH$) at 111 amu and additional loss of a carbonyl (C═0) at 83 amu. Reaction product #1 gave no significant ions related to either the kojic acid or the BGY-F compound. The various products from reaction #2 gave a pseudo-molecular ion, $(M\text{-}H)^-$, at 281 *m/z* for the BGY-F and reasonable fragments as well as showing traces of KA (141 *m/z*). The products from reactions #3 and #4 gave the pseudo-molecular ion $(M\text{-}H)^-$ at 281 *m/z* for the BGY-F. The KA does not appear in these spectra. The large peak at 59 *m/z* arises from the acetic acid.

Successful HPLC separations of KA and BGY-F reaction products confirmed that the KA eluted between 11 and 17 min and that the BGY-F eluted between 18 and 22 min (Table 2). The eluant from the HPLC column was split to the API/MS. As a result, it was shown that from 11–17 min reaction products 1,

Table 2
Summary of total ion chromatographic retention time ranges (TICRT) and major ions obtained by API/MS determined in negative ion mode

Reaction product number	Reaction conditions	TICRT	Major ions, m/z (relative abundance, %)	Base peak abundance
0	KA, 5000 ppm	11.92–15.05[a]	**141** (100.0), 142	2000
1	KA + NaOCl + H_2O_2	11.19–14.17	249 (100.0), 155, **141** (40.0), 205	7000
2	KA + HRP VI[b] + H_2O_2	18.45–21.22	**141** (100.0), ***281*** (92.4), 140, 157, 283, 255	330
2	KA + HRP VI + H_2O_2	12.33–16.52	**141** (100.0), 142	2700
2	KA + HRP VI + H_2O_2, (C_{18})[c]	18.77–20.81	**141** (100.0), ***281*** (10.3), 417, 255, 283	1560
2	KA + HRP VI + H_2O_2, (C_{18})	12.02–16.47	**141** (100.0), 142	5250
2a	KA + HRP VI + H_2O_2 (C_{18}), HOAc[e], (NH_2)[d], NH_4,OAc[f]	18.83–20.03	**141** (100.0), 233, 283, 255, 245, **281** (26.1)	230
2a	KA + HRP VI + H_2O_2 (C_{18}), HOAc, (NH_2), NH_4OAc[e]	11.97–13.96	**141** (100.0), 140	580
2b	KA + HRP II + H_2O_2	18.92–21.85	**141** (100.0), ***281*** (31.8), 140, 255, 155, 181 (6.4), 127	1570
2b	KA + HRP II + H_2O_2	12.34–16.20	**141** (100.0), 248, 245	6400
2c	KA + SBP + H_2O_2	18.23–21.25	**141** (100.0), **281** (17.7), 250, 127, 157, 180	1240
2c	KA + SBP[b] + H_2O_2	12.45–16.26	**141** (100.0)	3250
3	KA + locules, laboratory (C_{18})	18.19–21.43	***281*** (100.0), 140, (15.8), 249, 254, 154, 205	570
3	KA + locules, laboratory (C_{18})	12.70–16.52	**141** (100.0), 249, 255, 155, 204, 170, 401, 284	930
3	KA + locules, laboratory (C_{18}), HOAc, (NH_2), NH_4OAc	18.09–19.71	**141** (100.0), ***281*** (85.2), 233, 256	54
3	KA + locules, laboratory (C_{18}), HOAc, (NH_2), NH_4OAc	11.87–16.47	**141** (100.0), 249, 155, 233, 255, 283	240
4	Locules, field (C_{18})	18.76–22.21	***281*** (100.0), 205, 154, 248, 140 (8.6), 191, 255, 379	405
4	Locules, field (C_{18})	12.60–14.37	248 (100.0), **141** (98.3), 265, 155, 171, 363, 205, 220, 283, 379	290
4	Locules, field (C_{18}), HOAc, (NH_2), NH_4OAc	17.25–22.70	***281*** (100.0), 233, **141** (19.0), 348	315
4	Locules, field (C_{18}), HOAc (NH_2), NH_4OAc	12.18–16.67	**141** (100.0), 283, 227, 155, 233, 265, 249, 205	320

[a] Mobile phase MeOH:0.1% TFAA:TEA (120:75:3), column flow rate, 150 μl/min at 170°C.
[b] Peroxidases from horseradish (HRP VI, HRP II), peroxidases from soybean (SBP).
[c] Bakerbond SPE Polar Plus C_{18} (Octadecyl) 6 ml solid phase extraction column.
[d] Bakerbond SPE Amino (NH_2) 6 ml solid phase extraction column.
[e] Lyophilized BGY-F product in acidified H_2O (pH 3) with HOAc.
[f] BGY-F compound eluted with 3 column volumes of 0.1 M NH_4OAc solution.

Table 3
^{1}H-[a] and ^{13}C-NMR[b] spectral data (ppm) for KA and KA-dimer

Assignment	KA		KA-dimer	
	δ_H	δ_C	δ_H	δ_C
2		168.8	167.5	
3	6.33	110.7	6.6	109.6
4		177.1		178.9
5		144.8		146.6
6	8.03	142.3		142.7
7	4.28	60.4	4.28	60.2
5-OH	9.07		–[c]	
7-OH	5.67		–[c]	

[a] DMSO-d_6.
[b] D_2O.
[c] Not detected due to the broadening resulting from the fast exchange with the water protons present in the sample.

2a, 2b, 2c, 3 and 4 showed some presence of KA remaining. Reaction products 2a, 2b, 2c, 3 and 4 all gave the pseudo-molecular ion at 281 m/z for BGY-F between 18 and 22 min. Also present in these spectra at varying intensities was the ion of 141 amu. Based on these MS results, it was concluded that the BGY-F compound has a molecular weight (MWT) of 282 amu, corresponding to two KA molecules minus two protons (Fig. 1).

A larger mixture of reaction product #2 (KA + HRP-Type II + H_2O_2) was prepared to supply a source of the BGY-F product for an NMR structural study (see Experimental). MS results of this product gave the expected pseudo-molecular ion $(M\text{-}1)^-$ at 281 amu as the base peak (the dimer) (100% rel. Ab) and from fragmentation with the loss of (–CH_2OH, C═0, CH_2CO) at 181 amu (46.5% rel. Ab) and loss of $(KA\text{-}H)^-$ at 141 amu (15.9% rel. Ab).

In D_2O, not all exchangeable protons (hydroxyl protons) were seen in the ^{1}H-NMR spectra of KA and the BGY-F derivatives (Table 3). The ^{13}C-NMR spectral data of kojic acid and the BGY-F derivative are summarized in Table 3. Of the several solvents tried — $CHCl_3$, acetone, and DMSO, only DMSO was useful for detection of the hydroxyl group proton at C_5 as well as the alcoholic proton of the hydroxymethyl group in the standard compound (Table 3).

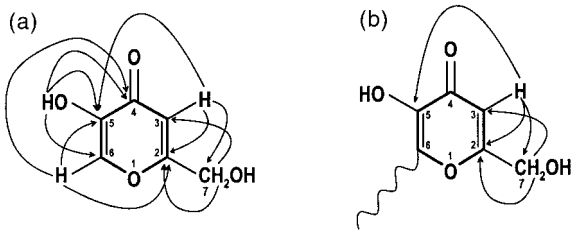

Fig. 2. (A) HMBC connectivities from the same structural segment of kojic acid. Arrowed lines represent connectivities optimized for $^nJ_{CH} = 6.25$ Hz., and (B) Summary of the multiple bond correlation observed in the HMBC spectrum of the bright- greenish-yellow-fluorescence compound.

The ^{1}H- and ^{13}C-NMR spectra of kojic acid and the unknown BGY-F compound were assigned through the use of two-dimensional NMR experiments (HSQC, HMBC) in DMSO-d_6. Results from HSQC spectra established partial carbon connectivities. Assignments of quaternary carbons and carbonyl were obtained from heteronuclear multiple bond correlation (HMBC) experiments. Fig. 2A illustrates the use of HMBC to build structural segments.

The HMBC spectrum of KA was recorded with parameters optimized for $^nJ_{CH}$ 6.25 Hz and $^1J_{CH}$ 166 Hz. If $^nJ_{CH} > 6.25$ Hz, one expects to find a crosspeak in the HMBC spectrum. The lack of connectivities between 3H and C4 indicated that the $^2J_{CH}$ coupling constant is much smaller than 6 Hz. On the other hand, the presence of two crosspeaks for 6H/C6 pair suggests that the $^1J_{CH}$ coupling constant is much greater than 166 Hz. As in furan, the measured one-bond coupling constant of the carbon nearest oxygen is $^1J_{6H-C6} = 198$ Hz, whereas ^1J for C_3 is much smaller, 166 Hz.

It is significant that the carbonyl resonance (C4) is highly shielded in KA (~177 ppm) and not at all similar to other ketones (Levy & Nelson, 1972) (Table 3), being more like that of an ester. C_3 and C_7 carbon connectivities were established in the HSQS spectrum of the BGY-F compound, there was no H/C crosspeak present at ~143 ppm. In the KA HSQC spectrum, this chemical shift corresponds to the C6 resonance. This observation suggests that in the BGY-F compound no proton is attached to C6. Fig. 2B summarizes the multiple bond correlation observed in the HMBC spectrum of the BGY-F compound. No connectivities to the C4 carbon were present in the HMBC spectrum of the BGY-F compound (Fig. 3), and because this is the only carbonyl carbon in that molecule, the most downfield resonance in the ^{13}C spectrum (~180 ppm) can be assigned to C4.

In kojic acid, positions 3 and 6 have very unequal reactivities (Beelik, 1956). The phenolic hydroxyl group is believed to activate the positions *ortho* and *para* to it. In the case of kojic acid, of the three positions in question, only one *ortho* position at C6 is available for substitution. All the substitution reactions studied, with a single exception, have been restricted to C6 (Beelik, 1956).

The negative ion mass spectrum of the BGY-F compound gives an ion at m/z 281, which suggests the MS 282 for the parent compound. This MS corresponds to a dimer of two kojic acid molecules from which two protons have been subtracted (one per molecule). NMR results indicate the lack of protons at C-6 positions. Moreover, the NMR data strongly suggest a

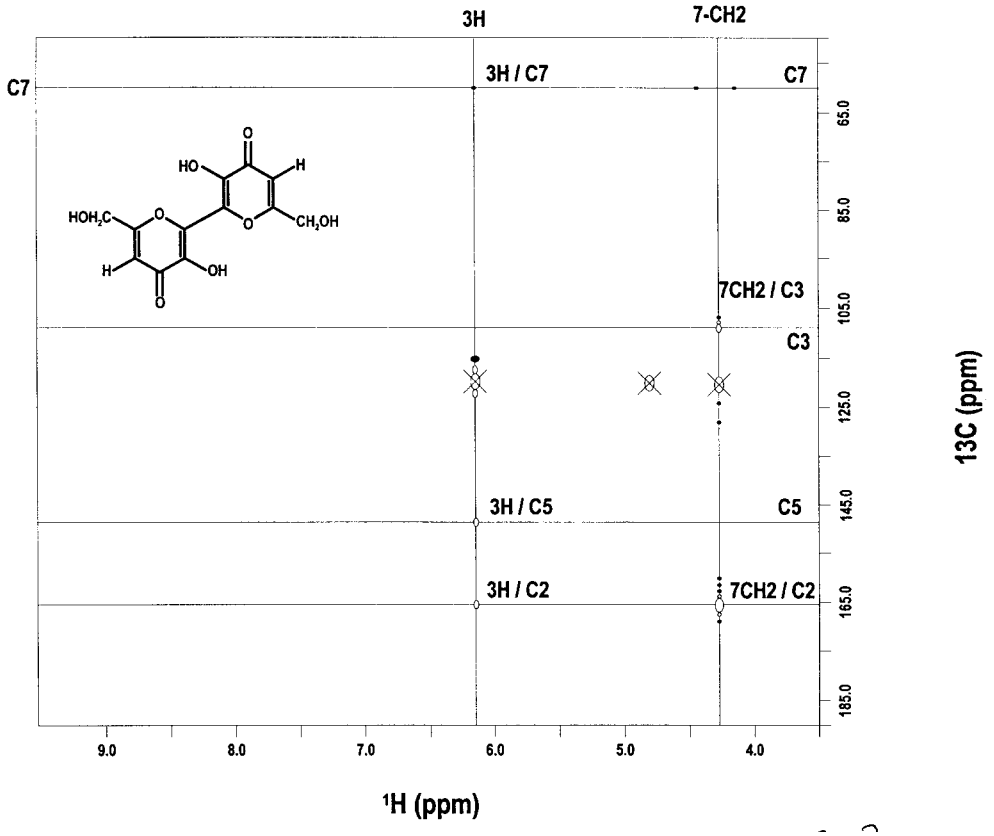

Fig. 3. HMBC spectrum of the bright-greenish-yellow-fluorescence compound.

symmetric species present — only one set of ^{1}H or ^{13}C resonances is seen in the spectra.

Based on the NMR and MS results, we propose the structure shown in Fig. 1 for the BGY-F compound, a previously unknown kojic acid derivative. The compound has the chemical name 6, 6′-bis[5-hydroxy-2-(hydroxymethyl)-4*H*-pyran-4-one].

3. Experimental

3.1. General analytical procedures

A Hewlett–Packard (HP) Model 1050 pump with a HP 1046 AX programmable fluorescence detector and a HP G1314A variable wavelength detector was used with a UV setting of 280 nm and fluorescence settings of 435 nm (excitation), 494 nm (emission), measured with a 450 nm cut-off filter. Oven temperature was set at 30°C. Analysis was carried out isocratically using MeOH:0.10% TFAA:TEA, (120:75:3) as a mobile phase on a Waters Spherisorb S5 NH_2 (2 × 150 mm) chromatographic column. A 100 μl internal loop injector was used to introduce the analytes onto the column and the sample was eluted with a 150 μl/min flow. A 1:15 post column splitter (high pressure micro splitter valve, 10–32, UpChurch Scientific) was used to introduce 10 μl/min flow into API electrospray interface and into the MS.

3.2. Fungal strain and culture conditions

An aflatoxigenic isolate of a wild-type strain of *A. flavus* (SRRC 1000 A) obtained from Arizona cottonseed was cultured on potato dextrose agar (PDA) Petri plates; spores were extracted from plates for spore suspension preparations utilized in infecting developing cotton bolls.

3.3. Cotton plants and conditions

Cotton plants (Acala SJ-2) were grown in experimental field plots at SRRC in New Orleans, Louisiana, USA, in 1997. At 20–32 days post-anthesis, injections of spore suspensions were deposited on the lint of the developing cotton boll after a 10 mm extracted hole had been produced in the outer carpel surface of the cotton boll.

3.4. Preparation and isolation of the BGY-F reaction product(s)

Reaction product 1 (Production of BGY-F from KA, NaClO, and H_2O_2). 0.1 ml of 31.1% H_2O_2 was

added into a KA solution (25 mg in 20 ml H_2O) and 0.5 ml NaClO was added dropwise over a 20 min period into the KA solution. After 3 h at room temperature, the reaction mixture was lyophilized and stored in the dark at 4°C.

Reaction product 2 (Production of BGY-F from KA in the presence of peroxidase and H_2O_2). 1.0 mg Peroxidase (SBP, HRP Type VI-A and II) and 500 mg of KA were added to 100 ml 0.0003% H_2O_2 solution. The mixture was incubated at room temperature in the dark overnight. The solution was then lyophilized and stored in the dark at 4°C.

Reaction product 3 (Production of BGY-F from fresh locules from cotton bolls that were treated with KA and H_2O_2). Twenty cotton locules were soaked overnight in 40 ml of a 0.1% KA solution (w/v) containing 400 ul of 31% H_2O_2. The fluorescent water solution was collected by filtration with Miracloth. The fluorescent materials on the lint in the locules were extracted three times with H_2O, combined, and then lyophilized. The brownish-yellow product was stored in the dark at 4°C.

Reaction product 4 (Production of BGY-F from live developing cotton bolls that had been inoculated with *A. flavus* (SRRC 1000A)). Developing cotton bolls (20–32 days post anthesis) were each inoculated with 20 ul of an *A. flavus* spore suspension (3.0×10^6 spores/ml). Two weeks after inoculation, the cotton bolls were harvested and examined under long wavelength ultraviolet light. The cotton lint containing BGY-F material was extracted three times with H_2O. The fluorescent water solution was filtered with Miracloth, combined, and then lyophilized. The dark brownish product was stored in the dark at 4°C.

BGY-F reaction products were dissolved in H_2O and were filtered through a 0.45 μm PTFE filter. After a C_{18} SPE column was conditioned with one column volume of MeOH and two column volumes of H_2O, the BGY-F product was dissolved in H_2O and passed through the conditioned C_{18} SPE column. The BGY-F material was eluted with six column volumes of H_2O. The C_{18} SPE column was examined for non-eluting BGY-F's by checking the column under a long wavelength UV light, and the BGY-F H_2O eluent was combined and lyophilized. An NH_2 SPE column was conditioned with one column volume of MeOH and two column volumes of H_2O. Lyophilized BGY-F product obtained from the C_{18} SPE column separation was dissolved in acidified H_2O (pH 3, dilute HOAC, 1×10 v/v) dropwise. This BGY-F acidified water solution was loaded onto the NH_2 SPE conditioned column and washed with H_2O. The BGY-F compound was eluted with three column volumes of 0.1 M NH_4OAc solution. The eluent was lyophilized and stored in the darkness at 4°C.

3.5. Further purification of reaction product 2 for NMR structure determination of the BGY-F compound

10 mg HRP (Type II) and 2 g KA was added to 1 l of a 0.00003% H_2O_2 solution and the mixture was incubated at room temperature, in the dark overnight, then lyophilized. The dried reaction product was dissolved in a minimum amount of H_2O, filtered through a Centricon plus membrane (10,000 MWCO); the filtered solution was acidified to pH 3 by adding diluted HOAc (1/10, v/v) dropwise. This acidified water solution was loaded onto a conditioned NH_2SPE column, then the column was washed with 6 column volumes of H_2O. BGY-F compound was eluted with 3 column volumes of 0.1 M $NH_4H_2PO_4$ solution. The resulting eluent was lyophilized and the residue was extracted with three 10.0 ml portions of MeOH. The combined MeOH solutions were filtered through a 0.45 nm PTFE filter and evaporated to dryness. Resultant dried brownish-yellow product was mixed with 300 ml Me_2CO and was centrifuged at 4000 rpm g value for 15 min. The Me_2CO supernant was decanted, and the residue was extracted with three volumes of 10.0 ml 10% Me_2CO in MeOH. After centrifugation, the decanted solution was combined and evaporated to dryness. The remaining brown residue was again extracted with 3 volumes of 10.0 ml 30% Me_2CO in EtOH. Me_2CO was evaporated and the product was lyophilized to dryness. The yellow product was again extracted with 3 volumes of EtOH and then lyophilized to dryness. Final yellow powder (resulted in 0.65% yield) was collected and stored at −10°C.

A Harvard Apparatus 22 syringe pump was used to deliver 10 μl/min of sample into the MS. All the determinations were accomplished on a HP 59987A electrospray unit interfaced to a HP5989A MS-quadrupole MS set in the negative ion mode.

NMR experiments were recorded on a GE Omega PSG 500 MHz spectrometer. The samples (1 mg of unknown compound and 10 mg of kojic acid) were dissolved in 0.7 ml D_2O or DMSO-d_6 in 5 mm Wilmad 528-PP NMR tubes, with ^1H and ^{13}C chemical shifts expressed in ppm downfield from tetramethylsilane.

2D ^1H-detected heteronuclear single quantum coherence (HSQC) experiments in DMSO-d_6 (Norwood, Boyd, Heritage, Soffe & Campbell, 1990) were performed with MLEV-64 ^{13}C decoupling during ^1H acquisition and heteronuclear multiple band correlation (HMBC) spectroscopy was performed according to Bax and co-workers (Summers, Marzilli & Bax, 1986; Bax & Summers, 1986) with delays Δ_1 and Δ_2 set to 3 and 80 ms, respectively.

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