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	GIF (Australia) = 0.564	ESJI (KZ) = 8.771	IBI (India) = 4.260
	JIF = 1.500	SJIF (Morocco) = 7.184	OAJI (USA) = 0.350

SOI: 1.1/TAS DOI: 10.15863/TAS

International Scientific Journal

Theoretical & Applied Science

p-ISSN: 2308-4944 (print) **e-ISSN:** 2409-0085 (online)

Year: 2022 **Issue:** 04 **Volume:** 108

Published: 10.04.2022 **http://T-Science.org**

Issue Article

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IDENTIFICATION OF THE BRIGHT-GREENISH-YELLOW-FLUORRESCENCE (BGY-F) COMPOUND ON COTTON LINT ASSOCIATED WITH AFLATOXIN CONTAMINATION N COTTONSEED

Abstract: *In order to characterize the structure of the bright-yellow-fluorescence (BGY-F) compound on cotton lint associated with aflatoxin contamination in cotton seed, various in vitro and in vivonatural 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 preparation: (a)kojic acid (KA)+peroxidase (soybean peroxide or horseradish type IV 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 products with 1H-and 13C-NMR spectroscopic analysis revealed that it was a dehydrogenator dimmer of 2 KA, linked through the C-6-positions*

Key words: *Acid Dimmer, Aflatoxin; Aspergillus flavus; Bright-greenish-yellow fluorescence (BGY-F);gossypium hirsutum l; HPLC-UV/F-MS; kojic acid; Malvaceae; NMR.*

Language: *English*

Citation: Hamzah, F., & Hamzah, N. (2022). Identification Of The Bright-Greenish-Yellow-Fluorrescence (Bgy-F) Compound On Cotton Lint Associated With Aflatoxin Contamination N Cottonseed. *ISJ Theoretical & Applied Science, 04 (108)*, 133-138.

Soi: http://s-o-i.org/1.1/TAS-04-108-26 ***Doi***: https://dx.doi.org/10.15863/TAS.2022.04.108.26

Scopus ASCC: *1500.*

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 cotton boll when the lint is observed under long wave UV light (Marsh et al., 2012). It is well established, (Marsh et al., 2012). That BGY-F result from the reaction of host plant peroxide, se with the fungal metabolite kojic acid (KA). KA (5-hydroxy-2(hydroxymethy)-4H-pyran-4-one), the precursor of the BGY-F material is produced by both aflatoxigenic Aspergillus sp., A. Flavus and A. Parasiticus (Parrish, et al., 2012). It is also reported that the BGY-F material is formed only on the lint of the developing cotton boll whereas aflatoxin contamination forms in the seed (Lee and Russel, 2012). Marsh et al. (2012) reported that the BGY-F material can be produced in solution 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 and Shih, 2012).

These BGY-F materials were obtaoned from reaction we had prepared from (a) KA+ NaClO+H_1O_2; (b) KA+ peroxidase+ H_2O_2; (c) fresh cotton locules with KA+ H_2O_2 (d).

Detached cotton locules inoculated with 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 chromatograpic 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.

Results and Discussion

Various lyophilized BGY-F preparations were initially dissolved separatelly in H_2O and were subjected to pre-purification by C18 SPE or NH_2 SPE column separations. The dried lyophillized 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 or ions of interest resulting from that analysis are shown in Table 1. As expected, the kojic acid gives a pseudomoleculer ion (M-H)-, at 14 m/z. Other ions in the spectrum of kojic acid include those resulting from a dimmer (283) and from fregmentation 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-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-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.

Table 1. Summary of major ions and base peak abundaces 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 IV[c]+H_2O_2 (C18), HOAc[d], (NH_2)[e] NH_4OAc	**281** (100.0),181 (55.8),141 (23.3)	1720
2b	KA+HRP II[c]+ H_2O_2	**281** (26.9),223,118 (14.9), 141(100.0), 83	13,40
2c	KA+SBPc+H_2O_2	**281** (IWO), 223,181 (23.1), t41 (57.7)	5200
3	KA+ locules,laboratory (C_{18}), HOAc, (NH_2) NH_4OAc[f]	**281** (89), 181 (25,0), 59 (100.0)	10,00
4	KA 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 150ºC, MS set in negative ion mode
[b] Baker bond SPE Polar Plus C_{18} (Octadecyl), 6 ml solid phase extraction column
[c] Peroxsidases from horseradish (HRP VI, HRP II) Peroxsidases from soybean (SBP)
[d] Lyophillized BGY-F product in acidified water (pH 3) with HOAH
[e] Baker bond SPE Amino (NH_2) 6 ml solid phase extraction column
[f] BGY-F compound eluted with 3 column volumes of 0,11 M NH4OAC solution.

Successful HPLC separations of KA and BGY-F reaction products confirmed that the KA eluted betwen 11 and 17 min and that the BGY-F eluted betwen 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 1,2a, 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 betwen 18 and 22 min. Also present in these spectra at varying intensitas was the ion of 141 amu, based on these MS results, it was concluded that the BGY-F compound has a molecullar weight (MWT) of 282 amu, corresponding to two KA molecules minus two protons.

Table 2 Summary of total ion chromatograpic 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 abudance, %)	Base peak abudance
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 Vib+ H_2O_2	18.45-21.22	141 (100.0),281 (92.4), 140,157,283,255	330
2	KA+HRP IV+ H_2O_2	12.33-16.52	141 (100.0), 142	2700
2	KA+ HRP VI+ H2O2, (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[e], (NH_2)[d], NH_4, OAc[e]	11.97-13.96	141 (100.0), 140	580
2b	KA+HRP II+H_2O_2	18.92-2185	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})	18.19-2143	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, 177, 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;1% TFAA:TEA (120:75:3), column flow rate, 150µl/min at 170ºC
[b] peroxidases from horseradish (HRP VI, HRP II), peroxidases from soybean (SBP)
[c] Bakerbond SPE Polar Plus Cis (Octadecyl) 6ml solid phase extraction column
[d] Bakerbond SPE Amino (NH_2) 6ml solid phase extraction column
[e] lyophilized BGY-F product in acidified H_2O (pH3) with HOAc
[f] BGY-F compound eluted with3 column volumes of 0,1 M NH_4Oac solution

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-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) at181 amu (46,5% rel. Ab) and loss of (KA-H)- at 141 amu (15.9% rel. Ab).

Table 3 1H-a and 13 C-NMRb spectral data (ppm) for KA and KA-dimer.

Assigment	KA		KA-dimmer	
	§H	§C	§H	§C
2		168.8	167.5	109.6
3	6.33	110.7	6.6	178.9
4		177.1		146.6
5		144.8		142.7
6	8.03	142.3	4.28	60.2
7	4.28	60.4	-C	
5-OH	9.07		-C	
7-OH	5.67			

a DMSO-d6
b D2O
c not detected due to the broadening resulting from the fast exchange with the water protons present in the sample

In D2O, not all exchangeable protons (hydroxyl protons) were seen in the 1H-NMR spectra of KA and the BGY-F derivatives (Table 3). The 13C-NMR spectral data of kojic acid and the BGY-Fderivative 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 C5 as well as the alcholic proton of the hydroxymethyl group in the standard compound (Table 3)

The 1H- and 13C-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-d6. Results from HSQC spectra established partial carbon connectivities. Assignments of quaternary carbons and carbonyl were obtained from heteronecular multiple bond correlation (HMBC) experiments.

The HMBC spectrum of KA was recoeded with parameters optimized for nJCH 6.25 Hz and 1JCH 166 Hz. If nJCH> 6.25 Hz, one expents to find a cross peak in the HMBC spectrum. The lack of connectivities between 3H and C4 indicated that the 2JCH coupling constant is much smaller than 6 Hz. On the other hand, the presence of two cross peaks for 6H/C6 pair suggests that the 1JCH coupling constant is much greater than 166 Hz. As in furan, the measured one bond couling constant of the carbon nearest oxygen is 1J6H-C6=198 Hz, whereas 1J for C3 is much smaller, 166 Hz. It is significants that the carbonyl resonance (C4) is highly shielded in KA 177 ppm) and not all simillar to other ketones (Levy and Nelson, 2012), (Table 3).

Being more like that of an ester, C3 and C7 carbon connectivities were established in the HSQS spectrum of the BGY-F compound, there was no H/C cross peak present at-143 ppm. In the KA HSQC spectrum this chemical shift corresponds to the C6 resonance. This observation suggest that in the BGY-F compound no proton is attached to C6. No connectivities to the C4 carbon were present in the HMBC spectrum of the BGY-F compound and because this is the only carbonyl carbon in that molecule, the most down-field resonance in the 13C spectrum (~180 ppm) can be assigned to C4.

In kojic acid position 3 and 6 have very unequal reactivities (Beelik, 2012). The phenolic hydroxyl groupies believed to activate to position 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 substitusion. All the substitusion reactions studied, with a single exception, have been restricted to C6 (Beelik,2012).

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 dimmer of two kojic acid molecules from which two protons have been substracted (one per molecule). NMR results indicate the lack of protons at C-6 positions. Moreover, the NMR data strongly suggest a symmetric species present only one set of 1H or 13C resonances is seen the spectra.

Based on the NMR and MS results, we propose the structure the structure, a previosly unknown kojic acid derivative. The compound has the chemical name 6, 6'-bis [5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one].

Experimental

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 (excitatiom), 494 nm (emission), measurred with a 450 nm cut-off filter. Oven temperature was set at 30ºC. Analysis was carried out isocratically using MeOH:0.01% TFAA;TEA, (120:75:3) as a mobile phase on a waters spherisorb S5 NH2 (2x150 mm) chromatographic column. A 100 pl internal loop injector was used to introduce the analyzes onto the column and the sample was eluted with a 150 µl/min flow. A 1:15 post column splitter (high pressure micro spliter valve, 10-32, upchurch

scientific) was used to introduce 10 µl/min flow into Api electrospray interface and into the MS.

Fungal strain and culture conditions

An aflatoxigenic isolate of a wild-type strain of A. Flavus (SRRC 1000A) 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.

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-antheisis, 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.

Preparation and isolation of the BGY-F reaction products (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 0.5ml 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 4ºC.

Reaction product 2 (production of BGY-F from KA in the presence of peroxsidase and H_2O) 1.0 mg peroxidase (SBP,HRP Type VI-A and II) and 500mg 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 4ºC.

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 miraclouth. The fluorscent 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 4ºC.

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 antheis) were each inoculated with 20 ul of A.flavus spore suspension (3.0 x 106 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 theree times with H_2O. The fluorscence water solution was filtered with Miracloth, combined and then lyophilized. The dark brownish product was stored in in the dark at 4ºC.

NGY-F reaction products were dissolved in H2) and were filtered through a 0.45 µm PTFE filter. After a C18 SPE column was conditioned with one column volume of MeOHand two column volume of H_2O, the BGY-F product was dissolved in H_2O and passed through the conditioned C18 SPE column. The BGY-F material was eluted with six column volumes of H_2O. The C18 SPE column was examined for non-eluting BGY-F's by ckecking 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 C18 SPE column separation was disolved in acidified H_2O (pH 3, dilute HOAC 1x10 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 4ºC.

Further nurification 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 11 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, than 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 nmPTFE filtered and evaporated to dryness. Resultant dried brownish-yellow product was mixed with 300 ml Me2CO and was centrifuged 4000 rpmg value for 15min. The Me2CO supernant was decanted and the residue wasextracted with three volumes of 10.0 ml 10% Me2CO 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%Me2CO in EtOH. Me2CO was evaporated and the product was lyophilized to dryness. The yellow product was again extracted with 3 volumes of EtOH an than lyophilized to dryness. Final yellow powder (resulted in 0.65% yield) was collected and stored at— 10ºC.

A Harvard Apparatus 22syringe pump was used to deliver 10 pl/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 (1mg of unknown compound and 10 mg of kojic acid) were dissolved in 0.7 ml D2O or DMSO-d6 in 5 mm Wilmad 528-PP NMR tubes, with 1H and 13C chemical shifts expressed in ppm downfield from tetramethylsilane.

2D 1H-detected heteronuclear single quantum coherence (HSQC) experiments in DMSO-d6 (Norwood, et al., 1999) were performed with MLEV-64 13C decompling during 1H acquisition and heteronuclear multiple band correlation (HMBC) spectroscopy was performed according to bax and co-workers (Summer et al., 1996). With delays A1 and A2 set to 3 and 80 ms, respectively.

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