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BIOSYNTHESIS OF BIKAVERIN IN FUSARIUM OXYSPORUM. USE OF ^{13}C NUCLEAR MAGNETIC RESONANCE WITH HOMONUCLEAR ^{13}C DECOUPLING TO LOCATE ADJACENT ^{13}C LABELS*

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Bikaverin obtained by supplementing cultures of Fusarium oxysporum with singly and doubly ^{13}C labeled acetate was enriched by approximately 0.5 atom percent with the ^{13}C isotope. At this low enrichment ^{13}C NMR spectra of samples labeled from (1-^{13}C)- and (2-^{13}C)acetate did not show, unequivocally, the pattern of isotopic incorporation. Small sample size, poor solubility and difficulties in the assignment of resonances also restricted the amount of information that could be obtained from the ^{13}C NMR spectrum of the sample labeled from (1,2-^{13}C)acetate. The difficulty was overcome by using ^{13}C homonuclear single-frequency decoupling in conjunction with ^{1}H heteronuclear decoupling to locate bonded ^{13}C-^{13}C pairs. The carbon skeleton of bikaverin was shown to be biosynthesized entirely by condensation of acetate units and the pattern of assembly was established.

Cultures of Fusarium oxysporum characteristically develop a deep red color which changes to dark purple as the culture ages. The biochrome responsible has been identified as bikaverin,[1,2,3] a wine-red compound with indicator properties first isolated by KREITMAN and coworkers[4] who referred to it as lycopersin. The name bikaverin has been adopted, despite precedence of the older lycopersin, to avoid confusion with the carotenoid pigment lycopersene.[5] Extensive chemical investigations,[5,6,7] an X-ray crystallographic study[8] and recent synthesis,[9] have established the structure I.

Bikaverin is unique among natural products in containing a benzoxanthone ring system. KJAER and colleagues,[7] in noting its probable polyketide

origin, remarked on the unusual length and cyclisation pattern of the presumed precursor chain, and suggested that orsellinic acid might function as a starter unit. However, no biogenetic studies have been conducted and a preliminary account[10] of the present investigation was the first evidence on the mode of formation of this compound. Our study on bikaverin was beset with several difficulties including low isotopic incorporation, insolubility of the metabolite in solvents suitable for nuclear magnetic resonance (NMR) examination, and uncertainties in signal assignment due to the number of similar quaternary carbon atoms. Although not unique to this investigation these difficulties in combination thwarted the elucidation of bikaverin biogenesis by established ^{13}C-labeling techniques, including ^{13}C NMR analysis of a sample enriched from a doubly labeled precursor.[11,12] To overcome these problems single ^{13}C frequency homonuclear decoupling was used to provide unambiguous identification of the ^{13}C-^{13}C biogenetic pairs in bikaverin resulting from incorporation of doubly labeled acetate. The information from the decoupling experiments not only established the polyketide origin of bikaverin but also revealed the pattern in which acetate units are assembled within the molecule.

Materials and Methods

Microbiological

A single spore isolate of Fusarium oxysporum SCHLECHT, HLX 1218-5, was supplied by Dr. D. BREWER from the culture collection of the Atlantic Regional Laboratory. It was maintained on potato-dextrose agar. A vegetative inoculum was grown in the following medium; D-glucose (2%),

ammonium tartrate (0.46%), potassium dihydrogen phosphate (0.1%), magnesium sulfate heptahydrate (0.05%), sodium chloride (0.01%), calcium chloride (0.01%) and 1% (v/v) of a trace mineral solution containing cupric sulfate pentahydrate (40 mg), boric acid (6 mg), ammonium molybdate tetrahydrate (4 mg), manganese sulfate monohydrate (7.5 mg), zinc sulfate heptahydrate (880 mg) and ferrous sulfate heptahydrate (100 mg) in 1 liter of water. The culture obtained by inoculating 50 ml of this medium with mycelium from an agar slant and incubating 4 days was blended and introduced as a 4% (v/v) inoculum into 50 ml of the same medium. After 2 days incubation the evenly-dispersed mycelial suspension in this secondary culture was used as a 4% (v/v) inoculum for biosynthetic experiments.

Cultures for producing bikaverin were grown in the inoculum medium modified to contain increased amounts of D-glucose (12%) ammonium tartrate (0.55%) and ferrous sulfate heptahydrate (23 mg/ℓ). All cultures in liquid media were grown in 250 ml Erlenmeyer flasks and incubated at 26° on a rotary shaker (220 r.p.m., 3.8 cm eccentricity).

Isotopically labeled substrates were added in sterile aqueous solution as follows: L-(methyl-^{14}C)methionine and L-(methyl-^{13}C)methionine as single additions on the second day to give broth concentrations of 0.75 mM; sodium (1-^{14}C)acetate and (1-^{14}C)acetic acid at various times and concentrations; (1-^{13}C)-, (2-^{13}C)- and (1,2-^{13}C)acetic acid each in 3.3 mmol amounts to 1 liter of culture at 2,4 and 6 days after inoculation.

Chemicals

Lichexanthone (II) was obtained from Dr. C. F. CULBERSON, Duke University, Durham, N. C., and 2,7-dimethoxy-5,8-dihydroxy-1,4-naphthoquinone (III) from

Dr. R. BENTLEY, Department of Biochemistry and Nutrition, University of Pittsburgh.

Sodium (1-^{13}C)acetate, (2-^{13}C)acetate, (1,2-^{13}C)acetate and L-(methyl-^{13}C) methionine (each 90% enriched) were obtained from Merck, Sharp and Dohme Canada Ltd., Pointe Claire, Quebec. Radioactive compounds were supplied by New England Nuclear Corporation, Boston, Massachusetts. Samples of sodium acetate were converted to acetic acid by passing an aqueous solution through a column of cation exchange resin (Dowex 50 × 8) in the hydrogen form.

Isolation of Bikaverin

Cultures were harvested on the eighth day after inoculation. The mycelium from 1 liter of broth was blended with 0.1 M hydrochloric acid (500 ml), washed well with water by vacuum filtration, and Soxhlet extracted with acetone for 36 hours. The filtrate, acidified to pH 2 with hydrochloric acid, and cooled at 4° for 18 hours, deposited a precipitate that was recovered by centrifugation and extracted with chloroform. Evaporation of the combined acetone and chloroform extracts gave a deep-red residue which was leached successively with petroleum ether (bp 30-60°) and water, then dried and extracted with chloroform (350 ml) under reflux. The extract was filtered through a shallow layer of silicic acid and washed first with 0.03% (w/v) aqueous borax and then water to remove norbikaverin. Concentration of the chloroform solution normally yielded at least 60 mg of bikaverin.

NMR Spectroscopy

^{1}H NMR spectra were recorded on a Varian Associates model HA-100 spectrometer. ^{13}C NMR spectra were obtained with a Varian Associates

model XL-100-15 pulse Fourier transform instrument at 25.16 MHz.

Typically, bikaverin (35 mg) was dissolved in 0.3 ml of a 1:1 (v/v) mixture of chloroform-d and trifluoroacetic-d acid contained in a tube of 5 mm external diameter. Tetramethylsilane was used as an internal reference; the spectral width was 5 kHz; 32 K data points were recorded giving a maximum spectral accuracy of ± 0.16 Hz. To retain nuclear Overhauser enhancements during acquisition of the high resolution (HR) ^{13}C NMR spectra, from which ^{13}C-H coupling constants were measured, the proton noise decoupling field was applied for 3.5 sec. between data acquisition periods of 3.2 sec.

Results

Isotopic Enrichment from Labeled Substrates

In preliminary experiments with ^{14}C-labeled substrates it was observed that radioactivity from L-(methyl-^{14}C)methionine was incorporated into bikaverin with about 15-fold dilution. The supplement did not affect the yield, whereas sodium acetate in concentrations as low as 2 mM severely depressed bikaverin formation. The inhibitory effect could be alleviated in part by substituting acetic acid for the sodium salt but to obtain sufficient bikaverin (ca. 30 mg) for ^{13}C NMR examination the maximum permissible concentration in the culture was 5 mM. Single additions of (1-^{14}C)acetic acid at different times before and during the accumulation of bikaverin gave relatively low incorporations of radioactivity into the metabolite. Feeding regimens in which the substrate was added in multiple small doses during the growth cycle were more successful and one in which (1-^{14}C)acetic acid was added at 2, 4 and 6 days after

inoculation gave 12-fold molar dilution. This condition was duplicated as closely as possible in the experiments with ^{13}C-labeled acetate.

Assignment of Resonances

Resonances in the ^{1}H NMR spectrum of bikaverin have been assigned by KJAER et al.[7] They were confirmed by nuclear Overhauser and decoupling experiments as follows: δ 7.39 [AB, 2H, H-4 and -2, $\Delta\nu_{AB}$ 9.2, J_{AB} 2.4 Hz; H-2 long range coupled (0.5Hz) to 1-CH_3]; δ 6.86 [S, 1H, H-9]; δ 4.16 [S, 6H, 3- and 8-OCH_3]; δ 3.01 [S, 3H, 1-CH_3]. Nuclear Overhauser effects (irradiated protons in brackets) were: H-2 {1-CH_3} 25%; H-2 {3-OCH_3} 8%; H-4 {3-OCH_3} 28%; H-9 {8-OCH_3} 28%.

The ^{13}C NMR data for bikaverin and two model compounds are given in Table 1. Signals due to the aromatic methyl group, methoxyl groups, and other carbons directly bonded to hydrogen (C-2, C-4, and C-9) were easily identified by their chemical shift values,[13] multiplicities in the high-resolution spectrum, and by an off-resonance decoupling experiment based on the assigned ^{1}H spectrum. The remaining carbons were assigned by comparing spectral data with those for the model compounds lichexanthone (II) and 2,7-dimethoxy-5,8-dihydroxy-1,4-naphthoquinone (III). Carbons 1-4, 4a, 12 and 12a of bikaverin are similar to carbons 1-4, 4a, 10 and 10a of II, while carbons 5a through 11a can be compared with appropriate carbons of III.

Resonances of the model compounds were assigned from chemical shifts, and also from their multiplicities and ^{13}C-^{1}H coupling constants observed in HR and off-resonance decoupled spectra.

Incorporation of ^{13}C-Labeled Methionine

The proton noise decoupled (pnd) ^{13}C NMR spectrum of bikaverin obtained from cultures supplemented with L-(methyl-^{13}C)methionine, when compared with signal intensities for bikaverin at natural abundance, indicated that the methoxyl groups attached to C-3 and C-8 were enriched by about 3% above natural abundance.

Incorporation of ^{13}C-Labeled Acetate

The pnd ^{13}C NMR spectra of bikaverin samples obtained from cultures administered (1-^{13}C)- and (2-^{13}C)acetic acid showed only small differences in signal intensity from those at natural abundance. Since the differences were within the limits of signal intensity variation observed for different natural abundance samples, they did not offer reliable evidence of acetate incorporation into the molecule.

The sample of bikaverin enriched from (1,2-^{13}C)acetic acid yielded a spectrum[10] in which all resonances except those of the methoxyl carbons at δ 58.5 and 57.9, were accompanied by two satellite signals due to one-bond ^{13}C-^{13}C coupling. The satellite resonances at δ 166.4 and 163.0 appeared as an AB quartet partly obscured by solvent peaks. The entire carbon skeleton of bikaverin is, therefore, assembled from intact pairs of carbon atoms derived from acetate. The degree of ^{13}C enrichment at each position was calculated from the intensities of the satellite signals and the central peaks using the formula derived previously.[14] The values (Table 2) indicate uniform incorporation throughout the metabolite, and in the case of C-2, C-4, C-9 and CH_3 agree with those obtained by integrating satellite peak areas in the ^1H NMR spectrum of bikaverin labeled with 2-^{13}C acetate.

The low level of incorporation, and consequent low signal to noise ratio, which introduced uncertainty into the precise location of satellite peak maxima, combined with the close distribution of coupling values due to the similar sp^2 character of the carbon atoms in bikaverin, prevented matching of ^{13}C-^{13}C pairs from satellite spacings in the spectrum. This was accomplished by single-frequency ^{13}C homonuclear decoupling with simultaneous ^{1}H decoupling. To improve the signal-to noise ratio, the large ^{1}H decoupling field was applied without noise modulation in the midst of the H-2, H-6 and H-9 resonances, providing the same effect as broadband decoupling of these nuclei.

The resonances of 1-CH_3, C-2, C-4 and C-9 were readily assignable (Table 1). Their ^{13}C-^{13}C coupled partners were found by irradiating other resonances in turn and looking for homonuclear decoupling of the assigned peaks, this procedure being adopted because of the lower signal/noise of the unassigned resonances. Peaks for the partners of 1-CH_3 and C-4 were immediately identifiable, but C-2 and C-9 were decoupled together when two nearly-overlapping resonances at δ 172.3 and 172.5 were irradiated. Comparison of $^{1}J_{cc}$ values resolved the ambiguity. Thus the resonances of C-1, C-3 and C-4a were assigned as the only possible partners to 1-CH_3, C-2 and C-4. The resonance at δ 172.5, which was coupled to C-9, appeared as a doublet in the HR spectrum. On this basis it was assigned to C-10 since C-8 would be expected to give a quartet due to coupling with the methoxyl protons. The signal at δ 163.0, a quartet, could then be assigned to C-8, and together with C-7 formed the AB quartet noted above.

Similar observations located the pairs C-12a, C-12 and C-10a, C-11; C-12a and C-10a were distinguished by their long range couplings to protons in the HR spectrum. (Table 1: C-12a a multiplet, $^{3}J_{CH}$ to H-2, H-4 and CH_3; C-10a a doublet, $^{3}J_{CH}$ to H-9).

None of the four remaining carbons (C-5a, -6, -6a, -11) could be assigned on the basis of coupling with protons. However the chemical shift of C-6 cannot be significantly affected by a carbonyl group at C-11a and so must have a chemical shift similar to C-1 and C-8 of the model compound III. On the other hand, C-5a cannot occur at a lower field than the corresponding carbon in lichexanthone because of the presence of an adjacent hydroxyl group. Thus C-6 must be at lower field than C-5a, and this, together with the pairing information, permits the signals for the remaining carbons (C-6a, C-11a) to be identified. Coupling constants ($^{1}J_{CC}$, Hz) for the matched pairs are: CH_3, C-1, 41.8; C-2, C-3, 63.4; C-4, 74.2, C-4a, 76 ± 2; C-5a, 68.7, C-11a, 68.0; C-6, 64 ± 2, C-6a, 62 ± 2; C-9, 66.0, C-10, 65.5; C-10a, 65 ± 1, C-11, 66 ± 1; C-12, C-12a, 68.0; C-7, C-8 (AB quartet) J_{AB} = 65 ± 2. All evidence from ^{13}C-^{13}C coupling supported the provisional assignments based on chemical shifts, direct and long range coupling to hydrogen, and comparison with model compounds. Since assignments based only on evidence from model compounds are prone to error, confirmation from homonuclear ^{13}C decoupling of bonded pairs is a significant advantage of the technique. However, the most noteworthy result is the direct evidence that bikaverin is biosynthesized by condensation of acetate units according to pattern (A) in Fig. 1. Pattern (B) and all other possibilities are excluded.

Discussion

Isotopic enrichment of metabolites from precursors singly labeled with ^{13}C can provide valuable biosynthetic information. However, its usefulness is limited to situations where adequate isotopic incorporation occurs, since the degree of enrichment must be estimated from peak height differences between spectra of labeled and natural abundance specimens. For several reasons[12] signal intensities are subject to variation, and differences in intensity caused by enrichments below 0.5% are difficult to measure, although relaxation reagents[15] may help in some cases. The low enrichment encountered during the work on bikaverin stemmed mainly from the toxic effects of acetate on F. oxysporum, and was exacerbated by the number of low intensity signals associated with quaternary carbons in the molecule. A further complication was the low solubility of the metabolite in suitable solvents. It dissolved in trifluoracetic acid (TFA), but we could not obtain a satisfactory lock and many critical resonances were obscured by the solvent signals. A mixture of $CDCl_3$ and TFA was a satisfactory compromise although some resonance frequencies varied slightly with solvent composition. Attempts to prepare a more soluble derivative in high yield were unsuccessful.

In these circumstances the use of a doubly labeled precursor can often prove invaluable. Signals due to coupling of adjacent ^{13}C pairs are present in natural abundance spectra but at such low intensity (i.e. 1.1% of natural abundance signals) that they are not readily distinguished from background noise. Levels of enrichment that would increase the natural abundance signal by 0.1% would cause a tenfold

increase in ^{13}C-^{13}C satellite intensity. Detection and measurement of such signals is limited only by the signal-to-noise ratio of the instrument. Incorporation of (1,2-^{13}C)acetate generated satellites at every resonance associated with the carbon skeleton of bikaverin, and so established that the molecule is formed by condensation of acetate units. The uniformity of labeling calculated from signal intensities is not unexpected in an experiment where substrate was added several times during the biosynthetic phase to maintain a uniform precursor pool in the mycelium.

Information about the pattern of acetate-polymalonate condensation and the mode of cyclization is also contained in the ^{13}C NMR spectrum of molecules biosynthesized from (1,2-^{13}C)acetate. By matching coupling constants ^{13}C-^{13}C pairs can be identified and their arrangement deduced. In molecules where coupling constants are sufficiently dissimilar and incorporation (or solubility) is high enough to give sharp satellite peaks the ^{13}C-^{13}C pairs can be identified by accurately measuring satellite spacings in the pnd ^{13}C NMR spectrum. Where, as with bikaverin, bonded pairs cannot be recognized by direct measurements, the information can be acquired by homonuclear ^{13}C decoupling with simultaneous proton broadband decoupling. This procedure also provided necessary confirmation of ^{13}C spectral assignments.

Of the five pathways known for naphthoquinone biosynthesis[16] the acetate-polymalonate condensation is most commonly encountered in secondary metabolites of fungi. Clearly the carbon skeleton of bikaverin can be derived from nine acetate units by such a pathway to give the observed pattern. There are, however, several ways in which the structure could

be assembled. The most plausible of these are: (i) condensation to a single polyketide chain which is then folded and cross-linked to form the carbon skeleton of the metabolite (Fig. 1, route a); (ii) initial formation of orsellinic acid by acetate-trimalonate condensation and subsequent extension of an orsellinate starter unit (Fig. 1. route b); (iii) initial formation of orsellinic acid and naphthalenic intermediates, followed by their condensation to a benzoxanthone structure (Fig. 1, route c). The napthalene ring could be formed by folding a polyketide chain in several different ways; (iv) initial folding of a single polyketide chain to form a naphthacenic intermediate, followed by ring scission and recyclization to generate the benzoxanthone structure (Fig. 1, route d).

Because the skeletal carbon atoms in bikaverin were uniformly labeled it was not possible to distinguish whether chain-building intermediates occurred during synthesis of the benzoxanthone structure, or whether the molecule was formed by appropriately folding a single polyketide chain. However, any mechanism which involves a symmetrical naphthoquinone intermediate can be discounted and since no orsellinate could be detected in the culture medium, a single chain polyketide intermediate is favoured. Further experiments using pulse labeling might strengthen the evidence but will be fruitful only if the extent of acetate incorporation into the metabolite can be increased.

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Table 1. ^{13}C NMR data for bikaverin and model compounds in trifluoroacetic acid -d- chloroform-d (1:1, v/v)

	δ_cTMS [$^nJ_{CH}$ Hz]*				
	Lichexanthone (II)		Bikaverin (I)	2,7-Dimethoxy-5,8-dihydroxy-1,4-naphthoquinone (III)	
C-1	145.4 q [^2J 5.8]	C-1	146.7 q [^2J 6.4]		
C-2	120.4 ddq [^1J 164.7, ^3J 7.0 (H-4), ^3J 4.8 (CH_3)]	C-2	124.2 dm‡ [^1J 167]		
C-3	171.2 q [^3J 4.0]	C-3	172.3 m		
C-4	101.4 dd [^1J 166.6, ^3J 4.9]	C-4	100.6 dd [^1J 170.5, ^3J 4.2]		
C-4a	159.3‡	C-4a	163.1‡		
C-5a	159.3‡	C-5a	157.5 s	C-7	160.3 q [^3J 3.7]
		C-6	167.1 s	C-8	168.4 d [^3J 7.8]
		C-6a	113.6 s	C-8a	111.7 s
C-6	95.2 dd [^1J 168.8, ^3J 4.6]	C-7	166.4 d [^3J 7.8]	C-1	168.4 d [^3J 7.8]
C-7	169.7 q [^3J 3.4]	C-8	163.0 q‡ [^2J ~ 4.8]	C-2	160.3 q [^3J 3.7]
C-8	99.2 dd [^1J 167.3, ^3J 4.3]	C-9	109.8 d [^1J 167.2]	C-3	110.0 d [^1J 165.1]
C-9	162.4 m	C-10	172.5 d [^2J 4.0]	C-4	174.7 bd [^2J ~ 3]
C-9a	100.8 dd [^3J 4.8, 5.7]	C-10a	104.3 d [^3J 5.3]	C-4a	104.3 t [^3J 4.5]

Table 1 (con't)

δ_cTMS [$^nJ_{CH}$ Hz]*					
Lichexanthone (II)		Bikaverin (I)		2,7-Dimethoxy-5,8-dihydroxy-1,4-naphthoquinone (III)	
		C-11	178.3 s	C-5	174.7 bd [^2J ~ 3]
		C-11a	112.5 s	C-6	110.0 d [^1J 165.1]
C-10	177.4 s	C-12	180.0 s		
C-10a	108.7 m	C-12a	112.4 m‡		
1-CH_3	24.3 dq [^1J 130.0, ^3J 6.7]	1-CH_3	23.9 dq [^1J 130.7, ^3J 5.2]		
3-OCH_3	57.3 q [^1J 147.0]	3-OCH_3	58.5 q [^1J 148.8]		
		8-OCH_3	57.9 q [^1J 147.5]	2-OCH_3	57.6 q [^1J 147.1]
7-OCH_3	57.0 q [^1J 146.8]			7-OCH_3	57.6 q [^1J 147.1]

*Pulse Fourier transform at 25.16 MHz, 5kHz width,32K transform, data accuracy ± 0.15 Hz. Multiplicities (high resolution spectra) are given by b = broad, d = doublet, m = multiplet, q = quartet, s = singlet, t = triplet. For comparison data for rings A and B of II are listed opposite the equivalent positions in I, and data for III opposite that for rings C and D in I. Data for ring C in II cannot be compared with data for I.

‡The multiplicity of these signals was not clear, due either to a low S/N, or overlap by solvent peaks.

Table 2. ^{13}C Enrichments* in bikaverin estimated (a) from ^{13}C-^{13}C satellite intensities in the ^{13}C NMR spectrum after labelling with sodium (1,2-^{13}C)acetate and (b) from ^{13}C-H satellite intensities in the ^{1}H NMR spectrum after labelling with sodium (2-^{13}C)acetate

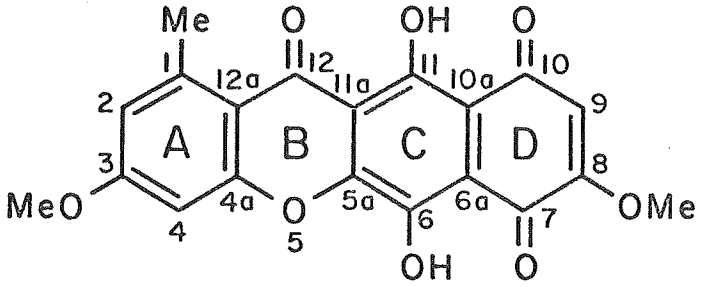
	(a)+	(b)
CH_3	0.40	0.4
C-1	0.61	
C-2	0.45	0.3
C-3	0.45	
C-4		0.3
C-4a	0.47	
C-5a	0.49	
C-6	~0.7	
C-6a	~0.3	
C-8	~0.55	
C-9	0.38	0.2
C-10	0.35	
C-10a	0.46	
C-11	0.32	
C-11a	0.53	
C-12	0.46	
C-12a	0.42	

*As atom percent above natural abundance. Where no value is reported the peaks were obscured or no satellite was present.

+The average enrichment was 0.46 ± 0.10. An earlier figure of 0.40 ± 0.07 was based on less data.[10)]

Caption for Figure 1

Possible routes and associated labelling patterns of the biosynthesis of bikaverin from acetate (⟶● = CH_3COOH).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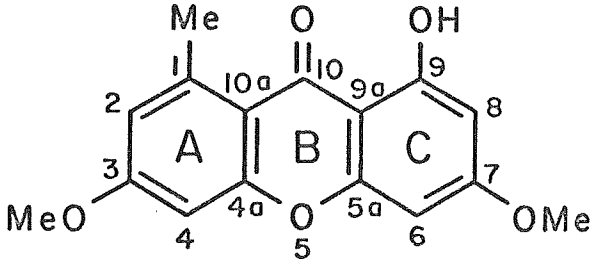
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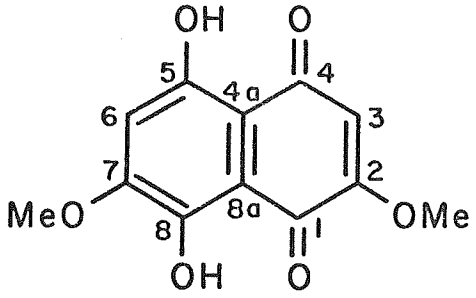
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III

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AUTHOR(S) McInnes, Walter, Smith, Wright and Vining

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