Antioxidant Properties of Benzimidazoles

5-Hydroxybenzimidazoles have been shown to possess marked antioxidant properties. By contrast benzimidazole and its 2-alkyl derivatives have no direct antioxidant activity, but appear to function as retarders of metal-catalyzed autoxidations.

As part of a study of oxidation of heterocyclic molecules related to their biological properties, oxidations of some benzimidazoles have been examined (Cole *et al.*, 1974). Relevant to this work the ability of these compounds to function as antioxidants has been evaluated, leading to the discovery of a novel class of active antioxidants.

EXPERIMENTAL SECTION

Benzimidazole and its 2-alkyl derivatives were prepared by standard methods (Phillips, 1928); 5-hydroxybenzimidazoles were formed by Udenfriend oxidation of the parent compounds (Udenfriend *et al.*, 1954). All compounds were shown to be pure by thin-layer chromatographic (tlc) analysis (Cole *et al.*, 1973). Squalene, vacuum distilled and filtered through grade I alumina under an atmosphere of nitrogen, was used as the substrate, for accelerated oxidations (in triplicate) at 60° under 1 atm pressure of oxygen.

RESULTS AND DISCUSSION

Results are expressed on the basis of time required to reach the arbitrary end point. Oxygen uptake (1% w/w) is shown in Table I.

It will be seen that benzimidazole itself and the 2-alkyl derivatives, while showing no direct antioxidant activity, nevertheless function as retarders of metal-catalyzed autoxidations. On the other hand, 5-hydroxybenzimidazole and 5-hydroxy-2-methylbenzimidazole function effectively as antioxidants.

A possible threefold action for benzimidazoles was envisaged. Thus, apart from direct action, metal scavenging, by complex formation (Goodgame and Cotton, 1962), was expected to counteract heavy metal catalysis of autoxidation. In addition, since chemical hydroxylation of benzimidazoles is known to produce 5-hydroxybenzimidazoles, the possible *in situ* formation of these molecules, leading to more effective antioxidants, was considered.

Present results show no direct antioxidant activity for the benzimidazoles, but demonstrate the expected effective properties of the 5-hydroxy derivatives. Although the metal scavenging effect leads to an observable but weak inhibition of autoxidation, the *in situ* hydroxylation process must be ruled out under the experimental conditions used.

Table I. Antioxidant Activity of Benzimidazoles

Addition[a]	Time to 1% w/v O uptake at 60°, min	Retardation factor[b]
None	135	
Benzimidazole	150	
2-Methyl-	148	
2-Ethyl-	140	
2-Nonyl-	140	
Cobalt stearate	39	
Cobalt stearate + benzimidazole	70	1.8[c]
Cobalt benzimidazole complex	86	2.2[c]
5-Hydroxybenzimidazole (0.025%)	12 hr	5.3
	34 hr	16.6
2-Methyl-	52 hr	23.1

[a] Concentration 0.1% unless otherwise stated. [b] Expressed as ratio of time required with the additive over time required for the control. [c] Relative to the simple cobalt-catalyzed autoxidation.

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Tentative Identification of Gibberellin A_7 in Immature Seeds of Iris

Four gibberellin-like substances were isolated from iris seeds. The least polar of these was tentatively identified as gibberellin A_7, based on chromatographic and fluorescence characteristics. Some properties of the other three are described.

Gibberellin-like activity has previously been detected in Wedgwood iris bulbs by Rodrigues-Pereira (1964). This has been confirmed by Aung *et al.* (1969). No attempt has previously been reported, however, to identify or to characterize the gibberellins involved. This paper reports the isolation and tentative identification of gibberellin A_7 and the presence of three additional polar gibberellin-like substances in iris seeds.

Table I. Thin-Layer Chromatographic Systems[a] Used

Designation	System
SGG–ECA	Silica gel G; ethyl acetate–chloroform–acetic acid (15:5:1, v/v)
KGG–BPW-2	Kieselguhr G; benzene–propionic acid–water (8:3:5, v/v), a two-phase system: upper, benzene; lower, water phase; equilibrate 2 hr in the vapors of both phases, then separate in the upper phase
KGG–BPW-0	Same system, no equilibration time
SGG–BAW-16	Silica gel G; benzene–acetic acid–water (8:3:5, v/v), a two-phase system: upper, benzene; lower, water; equilibrate 16 hr in the vapors of both phases, then separate in the upper phase

[a] See MacMillan and Suter (1963).

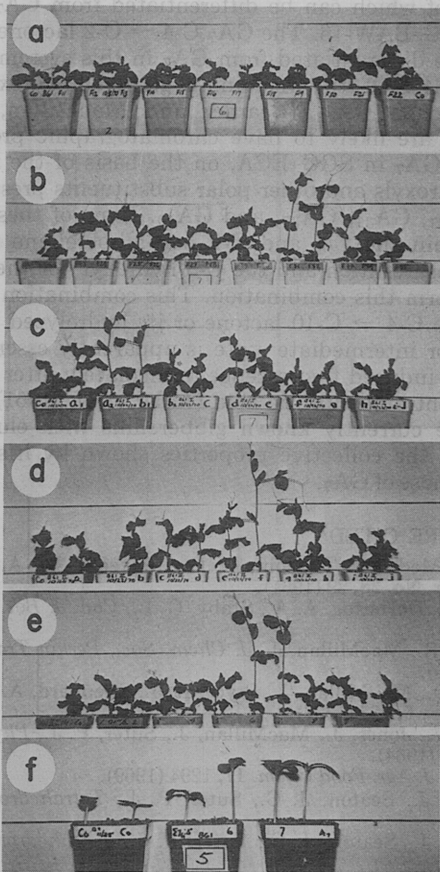

Figure 1. Bioassays of iris seed extract: (a) Dwarf pea plants treated with charcoal–Celite column fractions. Each pot contains two plants. One control plant is located on each end. Two plants showing elongation represent fractions (F) 21 and 22, 6 days after application of solutions. (b) Continuation of part a. Fraction I subfractions (left to right) represented are: control, a_1, a_2, F23 through F35, 7 days after application. (c) Composite fraction I subfractions (left to right) represented are: control, a_1, a_2, b_1, b_2, c, d, e, f, g, h, i + j, at 7 days. (d) Composite fraction II subfractions (left to right) represented are: control, a, b, c, d, e, f, g, h, i, j, at 7 days. (e) Composite IIefg subfractions (left to right): control, 1–10, representing R_f 0–0.1, 0.1–0.2, 0.2–0.3 . . . to 1.0, at 6 days. Separation was made on SGG–BAW-16. Active material is in subfractions 6 and 7. (f) Cucumber bioassay of above subfractions. Left to right: control, control, subfractions 5, 6, 7, and GA_7 (0.2 μg at 5 days after treatment).

EXPERIMENTAL SECTION

Seeds of the Dutch iris, Lemogul, gathered 30 days after flower wilt, shelled and frozen (1.4 kg), were homogenized and extracted three times with a total of 6.8 l. of redistilled ACS grade methanol to obtain 3.1 g of the crude gum residue by evaporation under reduced pressure. The gum was adsorbed on 5 g of silicic acid, placed atop a 2.5 cm diameter × 58 cm charcoal–Celite (1:2, w/w) column, eluted with increasing concentration of acetone in water as described by MacMillan *et al.* (1960, 1962), and collected in 95-ml fractions. Each fraction was evaporated under reduced pressure at <40°. The residue was dissolved in methanol and adjusted to a final volume of 2 ml. Twenty microliters of each fraction was bioassayed on dwarf peas for the presence of gibberellins. Biological activity in the form of internode elongation was detected in fractions 21–26 and 29–33 (Figure 1a,b). These subsequently were combined and designated as composites I and II, respectively. In a trial separation, a 60-μl aliquot of the column fraction 23 was streaked and chromatographed on SGG–ECA (Table I). Biological activity of the scrapings indicated that the active material had an R_f value of 0–0.1, an R_f much lower than that of gibberellin A_3 (GA_3), and the gibberellin A_8 (GA_8) reference markers. Similar treatment of fraction 31 indicated that the active material in this fraction was located in a zone adjacent to that occupied by gibberellin A_7 reference chromatographed on the same plate.

Composites I and II each were streaked on thin-layer chromatographic (tlc) plates for separation in the solvent system SGG–ECA. Composite I plate was divided into ten equal strips designated Ia, Ib, Ic . . . Ij, representing R_f 0.0–0.1, 0.1–0.2, 0.2–0.3, etc., respectively. Strips Ia and Ib were divided further into a_1, a_2, b_1, and b_2 in ascending R_f value. Composite II was also divided into equal strips designated IIa–IIj in increasing R_f values. Each strip was extracted four times with 10-ml portions of methanol. The combined extracts of four extractions were evaporated. The residue was redissolved in 2 ml of methanol. A 20-μl aliquot was used for pea bioassay. Strong biological activity was found in Ia_2b_1, Ide (Figure 1c), and IIefg (Figure 1d). The three apparent centers of biological activity were

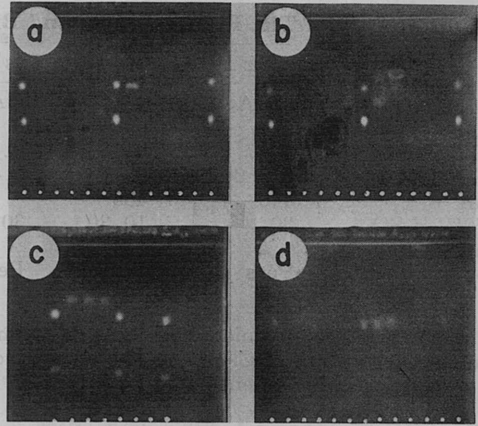

Figure 2. Thin-layer chromatograms of iris seed extract. (a) Composite fraction II subfractions a–j chromatographed in the system SGG–ECA, sprayed with 70% sulfuric acid and illuminated from below with long-wavelength ultraviolet. The two bright spots on the left side, center, and the right side of the plates are GA_3 (lower) and GA_7 (upper). IIa to IIe are located to the left, and IIf to IIj are located to the right of the center markers. The bright fluorescence to the right of the center GA_7 marker is from IIf. (b) Same plate as above after a total of 30 min at 120°. (c) Chromatoplate was separated in system KGG–BPW-2. Left to right: markers (GA_1, GA_3, GA_8), subfractions of Icde: 4, 5, 6, markers, subfractions of Ia_2b_1; 6, 7, and markers, shown after sulfuric acid spraying and 30 min of heating at 120°. Icde: 4, 5, and 6 contain iris II [adjacent to GA_1 (top) marker]. Ia_2b_1: 6 and 7 contain iris III adjacent to the GA_8 (bottom) marker. (d) Plate showing subfractions of the composite IIefg described under Figure 1e after 70% sulfuric acid spraying. The single spots on each side are GA_7 markers. Three spots near the center are, left to right, GA_7 marker, subfraction 6, and subfraction 7.

Table II. Summary of Tlc, Fluorescence, and Biological Properties of Iris Gibberellins I–IV

	Iris I	Iris II	Iris III	Iris IV	GA_3	GA_7	GA_8
Group[a]	<GA_8	GA_3	GA_3	GA_7			
R_f, SGG–BAW-16[b]	0	0	0	0.53	0	0.53	0
R_f, KGG–BPW-2[b]	0.27	0.68	0.55	1.00	0.60	1.00	0.27
R_f, SGG–ECA[b]	0.0–0.1	0.45	0.45	0.60	0.45	0.61	0.33
FEP,[c] nm	440	435		472	463[e]	472	463[e]
FDC[d]: 70% H_2SO_4 & min at 120°	30	10–30	30	0	0	0	10
Active on	Peas	Peas	Peas	Peas, cucumber	Peas, cucumber	Peas, cucumber	Inactive

[a] Group refers to the convenient classification (Kimura, 1969) of a gibberellin by its R_f proximity to gibberellins A_8, A_3, A_7, and A_9 in the system SGG–ECA. [b] See Table I. [c] Fluorescence emission peak, corrected for tlc background; activated at 365 nm. [d] Fluorescence development conditions, on tlc. [e] Published values, Elson *et al.* (1964), obtained with heating.

associated with gibberellins of the <A_8 group (Ia_2b_1), A_3 group (Ide), and A_7 group (IIefg), by tlc R_f classification (Kimura, 1969) on SGG–ECA. Examination by tlc of composite I fractions at this stage of the separation in the system SGG–ECA (Table I) revealed nothing significant. However, tlc of composite II fractions in the same system revealed, after sulfuric acid spraying, what later was recognized as GA_7 (Figure 2a). Heating of the plate (120°) resulted in the appearance of overlapping compounds (Figure 2b).

RESULTS AND DISCUSSION

Purification by repetitive tlc, followed in each case by bioassay, indicated that biologically active composite fraction Ia_2b_1 contained an extremely polar gibberellin (iris I) that fluoresced violet under 365-nm ultraviolet after 70% sulfuric acid spraying and heating. The R_f value in the system SGG–ECA was 0.0–0.1. In KGG–BPW-0 (Table I), the R_f was 0.55. The GA_8 and GA_3 reference markers on the same plate gave R_f values of 0.65 and 0.82, respectively. Other values are listed in Table II.

Composite fraction Ide contained two gibberellins, one characterized by a violet fluorescence (iris II), and the other by a barely discernible fluorescence but strong biological activity (iris III). The former migrated with GA_1 in the system KGG–BPW-2 (Table I, Figure 2c). The latter migrated slightly behind GA_3 in the same system. The violet fluorescing iris II was biologically active on dwarf peas but inactive on cucumber. Fluorescence was first visible on tlc plate after sulfuric acid (70%) spraying and 10 min of 120° heating. The fluorescence intensified as the length of heating increased to 30 min. The emission peak was at 435 nm.

A similar technique of repetitive tlc was used on composite fraction IIefg in isolating a gibberellin (iris IV) with the properties of GA_7. Iris IV was visible as a blue-green spot under long-wavelength ultraviolet immediately after spraying with sulfuric acid, a property of GA_7 described by MacMillan and Suter (1963). The fluorescence emission spectrum was identical with that of GA_7 (Table II). Tlc in systems SGG-BAW-16 and SGG–ECA showed that the R_f values were identical with that of GA_7 (Figure 2d). Bioassays of the purified material on dwarf peas and cucumber both showed strong activity similar to GA_7 (Figure 1e,f).

The tlc data of Cavell *et al.* (1967) showed that of 17 gibberellins (GA_{1-15}, GA_{18}, GA_{19}) those migrating in the vicinity of GA_7 in SGG–ECA are GA_4, GA_5, GA_6, and GA_{14}, all of which can be differentiated from GA_7 in the system SGG–BAW-16. The GA_7 C-4 → C-2 lactone isomer can also be differentiated from GA_7 in this system (Elson *et al.*, 1964). Of the remaining gibberellins (excluding GA_{39}) through GA_{42} (Bearder and MacMillan, 1973), those that are likely to have chromatographic properties similar to GA_7 in SGG–ECA, on the basis of the number of free hydroxyls and other polar substituents present, are GA_{20}, GA_{21}, GA_{31}, GA_{37}, and GA_{40}. None of these, however, contain the C-1 and C-2 (*ent*-gibberellane number system) unsaturation and the C-3 hydroxyl nor the ability to easily form this combination. This combination together with the C-4 → C-10 lactone or its hydrolyzed and dehydrated or intermediate state is apparently essential for ultraviolet-induced fluorescence immediately after contact with concentrated sulfuric acid (Adler *et al.*, 1961). Thus, among the currently known gibberellins with elucidated structures, the collective properties shown by iris IV are uniquely those of GA_7.

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