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(+)-5-HYDROXY-DIOXINDOLE-3-ACETIC ACID, A SYNERGIST FROM RICE BRAN OF AUXIN-INDUCED ETHYLENE PRODUCTION IN PLANT TISSUE

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Key Word Index—*Oryza sativa*; Gramineae; rice bran; synergist; 5-hydroxy-dioxindole-3-acetic acid; 5-hydroxy-oxindole-3-acetic acid; IAA.

Abstract—(+)-5-Hydroxy-dioxindole-3-acetic acid (**1**) was isolated from rice bran as a substance synergistic with auxin in the auxin induced ethylene production by etiolated mungbean hypocotyl segments. 5-Hydroxy-oxindole-3-acetic acid (**4**) and IAA were also obtained. The importance of a hydroxyl group in the 5-position in the two compounds was suggested since synthesized (±)-dioxindole-3-acetic acid (**6**) was inactive.

INTRODUCTION

Ethylene, which is a plant hormone having widely varied physiological actions, is produced by bacteria and fungi as well as by higher plants. Production of ethylene is a variety of plant tissues is known to be enhanced by natural and synthetic auxins, and, in some, by kinetin. Despite the supposed importance of such ethylene regulators in plant growth, studies on this subject are relatively few [1–3]. During the course of study on plant growth regulators in rice seeds, we found that methanol extracts of rice bran contained components that markedly stimulate auxin-induced ethylene production, and we attempted to isolate the active principles [4]. The present paper describes the isolation of (+)-5-hydroxy-dioxindole-3-acetic acid (**1**) and IAA as such stimulants, and of 5-hydroxy-oxindole-3-acetic acid (**4**) which is probably one of the proximate metabolites of **1**, and also the synthesis of **1**, **4** and dioxindole-3-acetic acid (**6**). Their biological activities are also reported.

RESULTS AND DISCUSSION

The active principles were isolated on the basis of their property to enhance ethylene production in etiolated mungbean hypocotyl segments [5]. The detailed method of the bioassay and of the isolation are described in Experimental. Three active substances, **1**, **2** and **3**, and a related compound **4** were obtained from the methanol extracts of rice bran. **3** and **4** were finally isolated as their Me ester derivatives.

The active substance **1***, $C_{10}H_9O_5N_1$, showed the presence of phenolic OH groups from the UV spectrum and of carbonyl groups from the IR spectrum. In the PMR spectrum peaks at δ3.02 (2H, *q*), due to methylene protons adjacent to a carbonyl group and at δ6.72 (2H, broad *s*), and δ6.88 ppm (1H, *t*), due to aromatic protons were observed. Treatment of **1** with 12N HCl yielded a β-acid [6]. Based on these data the structure of **1** was assumed to be either 5-hydroxy-dioxindole-3-acetic acid or 3,4-dihydro-2,4,6-trihydroxy-4-carboxyquinoline. The MS of **1** supported the former structure because of the presence of the fragment peaks, M^+—CH_2COOH and M^+—COOH—OH.

The second active substance **2** resembled **1** in IR, UV and PMR spectra. Especially, the MS of Me esters of **1** and **2** were completely identical. **2** also afforded a β-acid by acid treatment. Gel filtration of **2** on Sephadex G-15 using dilute HCl yielded **1** and, on the contrary, PLC of **1** gave **2**. These results suggested **2** probably to be a complex of **1** with a metallic component introduced during the isolation procedure.

The third active substance **3** was identified as IAA Me ester by comparing its UV and MS, R_f value on TLC and GLC R_t with those of a synthetic specimen. This is the first evidence of the presence of IAA in rice seeds.

The compound **4** had a UV spectrum similar to that of **1**. The MS of **4** revealed a M^+ corresponding to $C_{11}H_{11}O_4N_1$ and the fragment corresponding to M^+—CH_2COOMe. Thus, the structure of **4** was assigned as Me 5-hydroxy-oxindole-3-acetate.

The final structural confirmation of **1** and **4** was done by chemical syntheses. 5-Hydroxy IAA (**7**) was acetylated and the acetate **8** was treated with *N*-bromosuccinimide (NBS) [7] to afford the oxindole **10** in 80% yield. **10** was oxidized with equimolar selenium dioxide. The desired compound **11** was obtained although in low yield with unexpected deacetylation. This substance was identical with the natural one in all respects, including biological activity. Thus, the structure of **1** was demonstrated as **11**, although the absolute configuration at C_3 is not known.

5-Hydroxy-oxindole-3-acetic acid (**9**) was also prepared by oxidation of **7** with NBS. Deacetylation of **10** by methanolic ammonia was unsuccessful. The Me ester of **9** was found identical with that of **4** by MS and UV spectra, and by R_f values on TLC.

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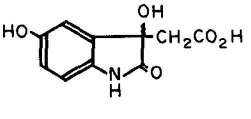
* Compound 1 was isolated as a metabolic intermediate leading to β-acid from IAA, from rice bran using UV spectroscopic detection in our laboratory.

Table 1. Effect of 1 and its related substances on ethylene production induced by 1 ppm of auxin in etiolated mungbean hypocotyl segments

substance (ppm)	Ethylene production % of control			
	1	**4**	**6**	**5**
100	162	129	106	101
50	160	123	95	100
10	149	109	106	100
1	118	107	106	98
0.1	117	92	105	102
None (control)	100(0.066)*	100(0.055)*	100(0.083)*	100(0.088)*

Mean values from parallel experiments in triplicate. Ethylene was measured by GLC after 18 hr incubation at 25°.

* µl/18 hr/10 sections.



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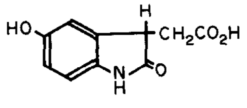

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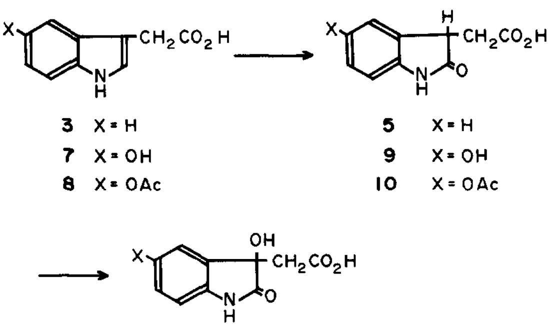

3 X = H
7 X = OH
8 X = OAc

5 X = H
9 X = OH
10 X = OAc

6 X = H
11 X = OH

Similarly, dioxindole-3-acetic acid (**6**), which is considered to be a possible intermediate in the enzymatic oxidation of IAA leading to methyleneoxindole [8], was also prepared from IAA via oxindole-3-acetic acid (**5**), in order to test its synergistic activity with IAA on the ethylene production. The structure of this synthesized material (**6**) was confirmed by its spectral data.

The biological activity of **1** on ethylene production primarily induced by 1 ppm auxin are shown in Table 1, together with those of the above synthetic oxindoles related to **1**. Compound **4** also exhibited fairly strong synergistic action, whereas, unexpectedly, either **5** or **6** were virtually inactive. These results suggest that the presence of the 5-OH group in **1** or **4** is indispensable for the appearance of the synergistic action. In Table 2, the effect of compound **1** on ethylene production at various concentrations of auxin is shown. The ratio of the increase of the amount of ethylene produced in the presence of **1** and IAA was shown to be much higher in low concentration ranges of IAA, than in the high concentration range. In the absence of auxin, however, no stimulative action was observed, which confirmed that **1** is a synergist with auxin. This action was recognized even in the presence of highly concentrated auxin (100 ppm), which is interesting considering the fact that GA and kinetin showed no synergistic action under similar conditions [9].

Compound **1** is the first natural indole product found having a 2,3-dioxy group and might be, from biogenetic consideration, derived from IAA via 5-hydroxy-oxindole-3-acetic acid (**4**). Isolation of β-acid from acid hydrolysate of rice bran has been reported [6], but it is probable that β-acid was an artifact of acid treatment of our compound **1** because we have so far succeeded in isolating the amide and the ethanol-amide of β-acid, from a methanol extract prepared under less drastic conditions.†

The studies on the absolute configuration at C_3 and the physiological significance of **1** are now in progress.

EXPERIMENTAL

PMR spectra were recorded at 100 MHz, using CD_3OD as solvent and TMS as internal standard. MS were measured at 70 eV by direct inlet. Pre-coated Si gel F_{254} plates (0.25 mm thickness) for TLC and Si gel PF_{254} for PLC (0.5 mm thickness) were used. MeOH was used for elution of developed PLC plates.

Bioassay procedure. The assay for biological activities was carried out as described in ref. [5]; Mungbean seeds (*Phaseolus aureus* Roxb.) were washed with H_2O, placed on a 0.4 % agar-gel bed and grown in a moist chamber at 25° in darkness. After 72 hr, when the hypocotyl length reached 30–35 mm, a 6 mm-long hypocotyl segment below the cotyledonary hook was excised. 10 segments were placed in 0.5 ml of an incubation medium consisting of 50 mM KPi buffer (pH 6.8) containing 50 ppm chloramphenicol and various concns of the auxin and the test sample. IAA (K salt) was used as the auxin. The vial was closed with a rubber cap and incubated at 30° in darkness.

Table 2. Effect of 1 on ethylene production induced by various concentrations of auxin in etiolated mungbean hypocotyl segments

1 (ppm)	Ethylene production % of control IAA concentration (ppm)				
	0.0*	0.1	1.0	10	100
100		176	162	146	117
50	97	186	160	151	126
10	104	151	149	121	117
1		119	118	105	102
None (control)	100(0.001)†	100(0.005)†	100(0.066)†	100(0.241)†	100(0.880)†

* 20 segments/vial. † µl/18 hr/10 sections.

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† Unpublished result. In the two, the amide of β-acid may be an artifact derived from 1.

After 18 hr, 2 ml gas was withdrawn and its C_2H_4 content was measured by FID GLC Al_2O_3 column: 2 m × 3 mm, oven temp 75°, N_2 30 ml/min, R_t 15 sec. Assay results were expressed as the ratio of C_2H_4 content by means of measuring peak heights of chromatogram, relative to control expts without sample substances. Each expt was run in triplicate.

Isolation of **1**, **2**, **3** *and IAA.* 10 kg of rice bran (var. Sasanishiki) was extracted in 40 l. of 70% aq. MeOH with stirring for 3 days. The filtrate was passed through a Dowex 50W-X2 (H^+) column. The column was eluted successively by 2N Py and then by 2N NH_4OH in 70% aq. MeOH (each 5 l.), after washing with aq. MeOH (2 l.). The Py eluate was concentrated to dryness, and the residue (*ca* 30 g) was placed on a Si gel column (500 g), and developed with *iso*-PrOH-7N NH_4OH (5:1, Solvent A). The active fraction which contained **1**, **2** and **4** was concentrated, and the residue (*ca* 1.5 g) was chromatographed on Sephadex LH-20 using MeOH, followed by Si gel PLC developing with $CHCl_3$–EtOAc–HCO_2H (7:11:2, Solvent B). The bands at R_f 0.2 (active zone) and 0.34 which were visible under UV were eluted with MeOH and concentrated to give a mixture of **1** and **2** (200 mg) and **4** (10 mg), respectively. The former was rechromatographed on Sephadex LH-20 using MeOH, affording a white powder, **1** (40 mg) and a pale yellow powder, **2** (110 mg). The latter was methylated with CH_2N_2 in MeOH and then purified on Si gel PLC using Solvent B, affording a colorless oil, the Me ester of **4** (2.2 mg).

The NH_4OH eluate (*ca* 10 g) was purified as described above. The active substance (10 mg), which had R_f 0.57 in Solvent B, was methylated with CH_2N_2 in EtOAc and purified by Si gel PLC. The oil obtained (2.4 mg) was characterized as IAA Me ester, by comparison of UV and MS, R_f values (0.73) on Si gel TLC with 5% MeOH–$CHCl_3$, and GLC R_t on a 1.5% OV-17 column (2 m × 3 mm, oven temp. 190°, N_2 30 ml/min, R_t 10 min), with a synthetic specimen.

Active substance 1. **1** was $[\alpha]_D^{21}$ +6.0° (MeOH, *c* 1.0); UV λ_{max}^{MeOH} nm (log ε): 313 (3.2), 263 (3.86), 213 (4.24), $\lambda_{max}^{0.03N\ NaOH-MeOH}$ nm: 335, 275, 213; MS *m/e* (rel. int.): 223.0504 (M^+, $C_{10}H_9O_5N_1$; 68), 205.0345 (M^+—H_2O; 100), 177.0395 (M^+—COOH—H; 52), 164.0371 (M^+—CH_2COOH; 80), 161.0444 (M^+—COOH—OH; 48), PMR: δ3.02 (2H, AA′ type of *q*, J = 15 and 16 Hz), 6.72 (2H, *br s*), 6.88 (1H, *t*, J = 1.5 Hz); IR ν_{max}^{KBr} cm^{-1}: 1700, 3200. Si gel PLC of **1** (10 mg) with Solvent A afforded a pale yellow powder (8 mg), identical with **2** by PMR.

Active substance 2. **2** was $[\alpha]_D^{21}$ +4.5° (MeOH, *c* 1.0); UV λ_{max}^{MeOH} nm (log ε): 310 (3.11), 263 (3.76), 214 (4.05); PMR: δ2.63 (2H, AB type of *q*, J = 15 and 27 Hz), 6.68 (2H, *br s*), 6.97 (1H, *br s*); IR ν_{max}^{KBr} cm^{-1}: 1700, 3200. Its MS was not obtained because of its low vapor pressure. Gel filtration of **2** (10 mg) on Sephadex G-15 using 0.04N HCl afforded a white powder (6 mg), identical with **1** by PMR. Its Me ester, MS *m/e* (rel. int.): 237 (M^+; 74) 219 (42), 186 (29), 177 (89), 164 (100), 149 (82), was identical with the Me ester of **1**.

HCl treatment of 1 *and* 2. 1 mg of **1** (or **2**) dissolved in conc HCl (1 ml) was treated in sealed tube at 100° for 1 hr and then concentrated to give yellow needles, identical with authentic β-acid by UV and R_f values on Si gel TLC.

Me ester of 4. UV λ_{max}^{MeOH} nm: 304, 257, 208; MS *m/e* (rel. int.): 221 (M^+; 35), 162 (41), 161 (100). 148 (13), 145 (13), 133 (28).

Synthesis of (±)-*5-hydroxy-dioxindole-3-acetic acid* (**11**). 5-Hydroxy IAA (**7**, 100 mg) was acetylated with Ac_2O and Py by stirring at 0° for 1 hr. Concn of the reaction mixture at room temp. afforded the acetate **8**. A mixture of **8** and NBS (94 mg) in 95% aq. *t*-BuOH (60 ml) was stirred at 21° for 2 hr. The soln was concentrated at room temp., diluted with H_2O, and extracted with EtOAc (20 ml × 3). The solvent extract was washed with H_2O, dried and then concentrated. The residue was purified by Si gel PLC using Solvent B. The band at R_f 0.41 gave a pale yellow powder (**10**, 80 mg), MS *m/e* (rel. int.): 249 (M^+; 20), 207 (73), 163 (70), 162 (100), 161 (63); UV λ_{max}^{MeOH} nm (log ε): 280 (3.03), 250 (3.8) 206 (4.13); PMR: δ2.13 (3H, *s*), 2.93 (2H, ABX type of splitting, J = 16, 7 and 4 Hz), 3.92 (1H, *m*), 6.9 (2H, *br s*), 7.06 (1H, *br s*). Measuring the PMR spectrum of the same sample after 24 hr, the signal at δ2.93 was observed as that of AB of *q*, and the signal at δ3.92 disappeared because of deuterization of the methine proton of C_3 by the solvent. **10** (60 mg) was dissolved in HOAc (1.5 ml). To this soln SeO_2 (27 mg) was added and refluxed for 10 min. The reaction mixture was concentrated to dryness. The residue was purified by Sephadex LH-20 using MeOH, followed by Si gel PLC using Solvent A. The band at R_f 0.2 afforded a pale yellow powder (6.8 mg), which was identical with **2** in all respects.

Synthesis of (±)-*5-hydroxy-oxindole-3-acetic acid* (9) *and* (±)-*dioxindole-3-acetic acid* (6). Both compounds were prepared as described above. **9**, white powder, UV λ_{max}^{MeOH} nm (log ε): 304 (3.23), 257 (3.9), 208 (4.12); PMR: δ2.96 (2H, AB type of *q*, J = 15 and 32 Hz), 6.76 (2H, *br s*), 6.86 (1H. *br s*). The Me ester of **9** was identical with that of **4** by MS and UV and R_f values on Si gel TLC using Solvent B. **6**, white powder, UV λ_{max}^{MeOH} nm (log ε): 290 (3.23), 257 (3.89), 208 (4.38); MS *m/e* (rel. int.): 207.0546 (M^+; $C_{10}H_9O_4N_1$; 95), 162.0544 (M^+—COOH; 34), 161.0496 (M^+—COOH—H; 87), 149.0480 (M^+—CHCOOH; 33), 148.0413 (M^+—CH_2COOH: 100) ; PMR: δ3.06 (2H, *s*), 6.8 ~ 7.9 (4H, *m*).

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