Possible Metabolic Intermediates from IAA to β -Acid in Rice Bran

Haruyasu KINASHI,* Yoshikatsu SUZUKI, Setsuo TAKEUCHI
and Akira KAWARADA

The Institute of Physical and Chemical Research, 2-1 Hirosawa,
Wako-shi, Saitama 351, Japan

Received August 19, 1976

Five new metabolites related to IAA were isolated from aqueous methanol extract of rice bran and their structures were elucidated by spectroscopic methods to be 5-hydroxydioxindole-3-acetic acid (Ia), methyl ester of Ia (Ib), methyl dioxindole-3-acetate (IIb), methyl 5-hydroxyoxindole-3-acetate (IIIb) and methyl oxindole-3-acetate (IVb), respectively. The presence of these metabolites which are likely to be intermediates of oxidation of IAA to β -acid suggests such oxidation pathway operating in rice seed.

In the course of screening study in our laboratory seeking for endogenous plant growth regulators in rice seeds, guanine¹⁾ and nicotinamide²⁾ have been isolated. Further studies revealed the presence of a growth suppressing substance against dwarf rice seedlings, in aqueous methanol extract of rice bran, but this substance was identified as β -acid which had already been isolated from hydrolysate of ethanol extract of rice bran by Sahashi.³⁾ On the other hand, Matsushima *et al.*⁴⁾ have isolated from corn steep liquor zeanic acid which had 4-carboxyquinolone skeleton beside β -acid. Since these two substances were considered to be oxidation products of IAA, we attempted to isolate intermediates of metabolic oxidation route from IAA to β -acid, from rice bran. In this paper we report the isolation and structure elucidation of a series of metabolites related to IAA and discuss possible oxidation pathways of IAA to β -acid.

5-Hydroxydioxindole-3-acetic acid (Ia) and its methyl ester (Ib)

10 kg of rice bran was extracted with 70% aqueous methanol and the filtrate was directly adsorbed on Dowex 50W-X2 (H⁺) column, which was successively eluted with 5% pyridine in 70% methanol and 2 N ammonia in 70% methanol. Each eluate was examined by TLC on silica gel containing fluorescent material employing chloroform-ethyl acetate-formic acid (35:55:10, CEF) as developing solvent and the components were detected under UV light. The ammonia eluate was found to contain two main UV absorbing components at *R_f* 0.22 (Ia) and 0.33 (Ib) and, after concentration, was chromatographed on silica gel column employing CEF. Selected fractions were combined and further purified by preparative TLC on silica gel using the same solvent followed by gel filtration on Sephadex LH20 using methanol. Evaporation of the solvent from fractions gave

* Present address: Mitsubishi-Kasei Institute of Life Sciences, 11-Minamiooya, Machida-shi, Tokyo 194, Japan.

67.8 mg and 253 mg of Ia and Ib.

Ia was amorphous white powder with the molecular formula $C_{10}H_9NO_5$, which was established by high resolution mass spectrometry (M^+ , m/e 223.0504; Calcd. m/e 223.0480). The UV spectrum in methanol [λ_{max} nm(ϵ) 210 (18,800), 264(7360), 314(1740)] was similar to those of dioxindoles⁵⁾ and showed bathochromic shift in alkaline solution suggesting the presence of a phenolic hydroxyl. The IR spectrum exhibited characteristic bands at 3360~3200 (OH, NH) and 1735~1700 cm^{-1} (C=O). The NMR spectrum gave an AA' type triplet at δ 3.00 (2H) which would come from a methylene group adjacent to a carbonyl group. Two broad singlets at δ 6.71 (2H) and δ 6.88(1H) suggested the presence of three aromatic protons. Furthermore, the proton-noise decoupled- and off-resonance decoupled ^{13}C NMR spectra clearly showed the signals of 10 carbons of Ia; two carbonyl carbons (δ 179.8 s, 177.7 s), six aromatic carbons (159.5 s, 132.4 s, 130.8 s, 118.1 d, 113.8 d, 112.2 d), one quaternary carbon (75.6 s) and one methylene carbon (44.3 t). These spectral data indicated Ia as hydroxydioxindole-3-acetic acid, although the position of the phenolic hydroxyl was not known.

Treatment of Ia with HCl afforded yellow substance, which was identified as β -acid from the comparison with authentic sample. Therefore Ia was determined as 5-hydroxydioxindole-3-acetic acid ($C_{10}H_9NO_5$). The conversion of Ia to β -acid seems to proceed *via* the dicarboxylic acid formed by hydrolysis of the amide bond. The assigned structure was supported

by the fragmentation pattern of Ia depicted in Fig. 1.

The UV spectrum of Ib was almost the same as that of Ia, which suggested 5-hydroxydioxindole nuclei common to both. The NMR spectrum of Ib showed an additional methoxyl signal at δ 3.48. From these spectral data coupled with the molecular ion at m/e 237 in the mass spectrum, Ib seemed to be the methyl ester of Ia ($C_{11}H_{11}NO_5$), which was confirmed by direct comparison.

Other metabolites related to IAA

As Ia is the first known 2,3-dioxy derivative of IAA from nature and considered to be the key intermediate of conversion of the indole nucleus to the quinolone nucleus, the presence of related metabolites situated between IAA and Ia was further sought in the ammonia eluate from Dowex 50 column. Other fractions of the above silica gel chromatography were carefully examined by silica gel TLC using CEF, and three UV absorbing components IIb, IIIb and IVb were found at R_f 0.45, 0.45 and 0.55, respectively (Components IIb and IIIb were separated later by gel filtration). These components were purified by essentially the same procedure as described above, and the yields of IIb, IIIb and IVb obtained from 10 kg of rice bran were 3.5, 56.4 and 2.2 mg, respectively.

The UV spectrum of IIb was characteristic of dioxindoles⁵⁾ but was not altered in alkaline solution, indicating the absence of a phenolic hydroxyl. Furthermore, almost all of the fragment ions of IIb were less than those of Ib by 16 mass units (see Experimental), which corresponds to the replacement of the phenolic hydroxyl in Ib by a hydrogen in IIb. IIb was thus determined to be methyl dioxindole-3-acetate ($C_{11}H_{11}NO_4$). The NMR spectrum showed a two-proton singlet at δ 3.07, a methoxyl singlet at δ 3.47 and a four-proton multiplet at δ 6.85~7.40, which are in accordance with the assigned structure. The three dioxindoles isolated (Ia, Ib and IIb) are all optically active and show very similar ORD curves in methanol (see EXPERIMENTAL), which

FIG. 1. Fragmentation Pattern of Ia.

suggest that their absolute configurations around C-3 are identical.

The UV spectrum of IIIb was characteristic of oxindoles⁵⁾ and was shifted in alkaline solution, indicating the presence of a phenolic hydroxyl. The NMR spectrum showed a methoxyl singlet at δ 3.67 and a three-aromatic-proton multiplet at δ 6.60~6.90. An AB type quartet centered at δ 2.90 might be assigned to aliphatic methylene protons. These spectral data along with the presence of the molecular ion of IIIb at m/e 221 suggested that IIIb was methyl 5-hydroxyoxindole-3-acetate ($C_{11}H_{11}NO_4$). For confirmation of the assigned structure, IIIb was synthesized according to Hinman *et al.*⁶⁾ In the NMR spectrum of IIIb, the methylene protons observed at first as an ABX type signal in deuteromethanol were gradually changed to an AB type quartet. This should be rationalized by the deuterization of the C-3 proton, due to the enolization of the amide carbonyl. That IIIb was isolated as an optically inactive compound suggests that such enolization had already occurred in rice seed.

The UV spectrum of IVb was similar to that of IIIb but was not shifted in alkaline solution. The molecular ion (M^+ , m/e 205) and other fragment ions were smaller by 16 mass units from those of IIIb, which suggested the re-

placement of the phenolic hydroxyl in IIIb by a hydrogen in IVb as in the case of I Ib. The NMR spectrum showed a four-proton multiplet at δ 6.85~7.30 which supported the above interpretation. The identity of IVb was finally confirmed by synthesis of methyl oxindole-3-acetate ($C_{11}H_{11}NO_3$). The methylene protons also give an AB type quartet, and IVb is optically inactive as is IIIb.

In addition we further isolated IAA in the ammonia eluate from Dowex 50 column, which also has been isolated from rice bran along with Ia and IIIa, in our laboratory using a IAA-induced ethylene production assay. The details of the assay and isolation procedure will be presented elsewhere.⁷⁾ In summary a series of metabolites related to IAA were successfully isolated from aqueous methanol extract of rice bran. However, when the ion exchange method in the above purification process was substituted by carbon adsorption method using ammoniacal 70% aqueous acetone as eluant, there were obtained 69.0 and 4.2 mg of Ia and Ib, respectively from 2.2 kg of rice bran. The relative ratio of Ia to Ib is very much larger in this case than that in the ion exchange method, which suggests that at least a part of Ia had been esterified during the isolation procedure with cation resin. This can be also said for other compounds isolated here in methyl ester.

FIG. 2. Effects of Compounds Ia to IVb on the Elongation of *Avena* Coleoptile Sections.

○—○, IAA-Me; □—□, I Ib; △—△, IVb.

○—○, 5HIAA-Me; ■—■, Ia; □—□, Ib; △—△, IIIb.

Biological activities

Effects of compounds Ia to IVb on the elongation of *Avena* coleoptile sections are shown in Fig. 2. All of the compounds isolated here proved inactive in this test. That IVb was inactive too indicates that the oxidation of methyl IAA at C-2 results in a total loss of elongating activity. On the other hand, methyl 5-hydroxyindole-3-acetate (5HIAA-Me) added to the list as a reference was active, although much less so than methyl IAA. Despite these results, compounds Ia and IIIa have been shown active in a test for synergistic activity in IAA-induced ethylene production from mungbean hypocotyl sections,⁷⁾ and studies of other types of biological activities of these compounds may prove interesting.

Oxidation pathway of IAA to β -acid

The degradation pathway of IAA by horseradish peroxidase has been extensively studied, and that by Hinman *et al.*⁸⁾ according to which IAA is successively oxidized to 3-perhydroxy-IAA, oxindole-3-carbinol and to 3-methyleneoxindole, is generally accepted. The present isolation of a series of probable oxidation products of IAA from rice bran, however, suggests possibility of another oxidation pathway to β -acid operating in rice seed, which is shown in Fig. 3 as a tentative scheme.

In this scheme, while oxidation of IAA to oxindole (IVa) and then to dioxindole (IIa) is easily conceivable, it is not at all certain which of these three is preferentially hydroxylated at

C-5. Although 5HIAA was not isolated in this study, a possibility can be also considered that 5-hydroxy compounds Ia and IIIa are derived from 5-hydroxytryptophan *via* 5HIAA. As the immediate precursor to β -acid, Ia looks a plausible one. If this reaction by dehydration and recyclization is taken for granted, also IIa seems to have a comparable opportunity to undergo same type of reaction to form 4-carboxyquinolone, though this compound was not isolated. It is also interesting to consider if 3-methyleneoxindole, a final oxidation product of IAA according to Hinman *et al.*, could not be formed *via* IIa by decarboxylation and dehydration. Furthermore, that we have also isolated the amide and ethanolamide of β -acid from the same source⁹⁾ suggests subsequent conversion of β -acid to these and other derivatives. The rough scheme in Fig. 3 is thus no more than a possibility at this stage and needs confirmation by tracer experiments with labeled intermediates. Isolation of zeanic acid from corn steep liquor⁴⁾ may be also taken as an indirect evidence of a similar oxidation pathway of IAA in corn plant.

EXPERIMENTAL

Melting points were determined on a hot-stage and were uncorrected. UV spectra were recorded on a Shimadzu double beam spectrophotometer UV-200. IR spectra were measured on a JASCO IRA-1 grating infrared spectrophotometer. ¹H NMR spectra were obtained on a JEOL 4H-100 spectrometer using CD₃OD as solvent and TMS as an internal standard.

FIG. 3. Possible Oxidation Pathways of IAA to β -Acid.

^{13}C NMR spectra were obtained on a JEOL FX-60 spectrometer using 2 N $\text{ND}_3\text{-D}_2\text{O}$ as solvent and dioxane (δ 67.4) as an internal standard. Low resolution mass spectra were obtained on a Hitachi RMU-6L mass spectrometer and, high resolution mass measurement, on a JEOL 01-SG double focussing spectrometer. ORD curves were recorded on a JASCO J-20 automatic recording spectrometer. Thin-layer chromatography (TLC) was conducted by use of Silica gel GF₂₅₄ Type 60 (Merck), and preparative TLC was carried out on plates of the same material (25×25 cm, 0.5 mm thickness). Column chromatography was performed by use of Silicic acid AR 100 Mesh (Mallinckrodt).

Isolation of Ia and Ib

10 kg of rice bran was agitated in 70 liters of 70% aqueous methanol for 3 days. The filtrate was passed through a Dowex 50W-X2(H^+) column (8×50 cm) and the column was washed with 15 liters of 70% aqueous methanol. It was eluted first with 15 liters of 5% pyridine in 70% aqueous methanol and then with 15 liters of 2 N ammonia in 70% aqueous methanol. Each eluate was concentrated *in vacuo* to give brown residues weighing 52.1 and 15.6 g, respectively. The ammonia eluate (15.6 g) containing two main UV absorbing components was chromatographed on silica gel column (4.5×62 cm) employing CEF. Appropriate fractions were combined and further purified by preparative TLC on silica gel using the same solvent followed by gel filtration on Sephadex LH20 (3×80 cm) employing methanol. Thus 67.8 and 253 mg of Ia and Ib were obtained respectively. Ib was further crystallized from hot ether to afford colorless needles.

Isolation of IIb, IIIb and IVb

The early fractions of the eluate at the above silica gel chromatography were combined and rechromatographed on silica gel plates using CEF, and further purified by gel filtration on Sephadex LH20 (3×80 cm) using methanol. If necessary, the preparative TLC and gel filtration were repeated again and 3.5, 56.4 and 2.2 mg of IIb, IIIb and IVb were obtained respectively. IVb was further purified by crystallization from hot hexane to afford fine white needles.

An alternative isolation procedure for Ia and Ib

Seventy liters of 70% aqueous methanol extract of 10 kg of rice bran were concentrated to remove methanol. To the filtrate 1 kg of active carbon was added and stirred for 2 hr and then filtered. The carbon bed was washed with 20 liters of water and 10 liters of acetone, and then stirred in 15 liters of 70% aqueous acetone containing 200 ml of 28% ammonia water for 1 hr. The filtrate was evaporated to dryness to afford 36.1 g of brownish powder, of which 7.9 g was successively subjected to column chromatography, preparative TLC and gel filtration as described

above to give 69.0 and 4.2 mg of Ia and Ib respectively.

Physico-chemical properties of compounds Ia-IVb

Ia: white amorphous powder; UV λ_{max} nm (ϵ) 210 (18,800), 264 (7360), 314 (1740) in MeOH, 211 (74,100), 280(7980), 333 (1760) in 0.03 N NaOH-MeOH; ^1H NMR δ 3.00 (2H, t, $J=17$ Hz), 6.71 (2H, br. s), 6.88 (1H, br. s); MS m/e (%) 223 (M^+ , 76), 205 (49), 177 (58), 164 (80), 161 (100), 136 (47), 135 (68), 133 (71), 108 (42), 107 (41); IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} 3360~3200, 1735~1700; ORD $[\phi]_{252\text{nm}} = -9980$, $[\phi]_{270} = 1120$.

Ib: colorless needles; mp $78 \sim 80^\circ\text{C}$; UV λ_{max} nm (ϵ) 210 (17,700), 264 (6770), 315 (1470) in MeOH, 210 (44,500), 281 (7560), 337 (1630) in 0.03 N NaOH-MeOH; ^1H NMR δ 2.98 (2H, s), 3.48 (3H, s), 6.68 (2H, br. s), 6.84 (1H, br. s); MS m/e (%) 237 (M^+ , 89), 177 (81), 164 (100), 162 (24), 149 (14), 136 (26), 135 (42), 121 (11), 108 (17), 107 (15); IR $\lambda_{\text{max}}^{\text{KBr}}$ cm^{-1} 3400~3200, 1735~1700; ORD $[\phi]_{252\text{nm}} = -12,300$, $[\phi]_{270} = 663$.

IIb: white amorphous powder; UV λ_{max} nm (ϵ) 209 (22,500), 254 (5080), 293 (1380) in MeOH, 211 (74,700), 253 (5110) in 0.03 N NaOH-MeOH; ^1H NMR δ 3.07 (2H, s), 3.47 (3H, s), 6.85~7.40 (4H, m); MS m/e (%) 211 (M^+ , 62), 161 (89), 148 (100), 146 (30), 133 (23), 120 (64), 119 (42); IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} 3320~3180, 1740~1720, 1626; ORD $[\phi]_{251\text{nm}} = -7820$, $[\phi]_{270} = 1180$.

IIIb: white amorphous powder; UV λ_{max} nm (ϵ) 207 (13,000), 258 (5930), 305 (1320) in MeOH, 210 (34,700), 276 (5930), 321 (1390) in 0.03 N NaOH-MeOH; ^1H NMR δ 2.90 (2H, ABq, $J=17$ Hz), 3.67 (3H, s), 6.60~6.90 (3H, m); MS m/e (%) 221 (M^+ , 37), 189 (5), 161 (100), 148 (11), 144 (12), 133 (30); IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} 3280~3160, 1736, 1690, 1600.

IVb: fine white needles; mp $170 \sim 172^\circ\text{C}$; UV λ_{max} nm (ϵ) 207 (25,300), 249 (8960), 279 (1460) in MeOH, 210 (53,800), 249 (8820) in 0.03 N NaOH-MeOH; ^1H NMR δ 2.94 (2H, ABq, $J=17$ Hz), 3.65 (3H, s), 6.85~7.30 (4H, m); MS m/e (%) 205 (M^+ , 24), 173 (10), 145 (100), 132 (11), 128 (12), 117 (40); IR $\nu_{\text{max}}^{\text{KBr}}$ cm^{-1} 3150, 3100, 1736, 1708, 1622

Acid treatment of Ia

A suspension of 5 mg of Ia in 1 ml of 4 N HCl was heated to 120°C overnight in a sealed tube. Then the solution was cooled in refrigerator to afford 4.1 mg of yellow crystalline precipitate, which was identified as β -acid from the comparison with authentic specimen.

Syntheses of IIIb and IVb

To a solution of 30.9 mg of methyl IAA in 1 ml of 95% aqueous *t*-butanol, 32.1 mg of N-bromosuccinimide (NBS) was added and stirred for 10 min at room temperature. The reaction mixture was concentrated *in vacuo* and chromatographed on silica gel plates using CEF, followed by gel filtration on Sephadex LH20 (2×50 cm) employing methanol. Thus 13.1 mg (39%) of IVb was obtained, which was further crystallized

from hot hexane to afford fine white needles.

By a similar procedure using 33.6 mg of methyl 5HIAA and 36.0 mg of NBS, 9.9 mg (27%) of IIIb was obtained as white amorphous powder.

Bioassay

Dehusked oat seeds (*Avena sativa* var. Victory) were placed on the surface of 0.5% agar layer and grown for 48 hr at 27°C under red light and then for 20 hr in darkness. Sections of 5 mm length were cut from coleoptile at 5 mm below the tip, and 10 sections per each dish were floated on 2 ml of aqueous solution of the test substance and 2% sucrose. After 18 hr growth in the dark the length was measured and compared with that of control.

Acknowledgement. The authors wish to express their thanks to Mr. K. Furihata of Institute of Applied Microbiology, The University of Tokyo for ¹H NMR measurements. We also thank Mr. J. Uzawa for ¹³C NMR measurements, Ms. R. Sakurazawa for her help of bioassay and Dr. K. Tsuji of this institute for reviewing the manuscript. This work was supported in part by a grant from Science and Technology

Agency of Japan.

REFERENCES

- 1) S. Takeuchi and A. Kawarada, *Proceedings of 8th International Conference on Plant Growth Substances*, **1973**, 157.
- 2) S. Takeuchi, Y. Kono, A. Kawarada, Y. Ota and M. Nakayama, *Agr. Biol. Chem.*, **39**, 859 (1975).
- 3) Y. Sahashi, *Biochem. Zeitschrift*, **159**, 221 (1925); **168**, 69 (1926); **189**, 208 (1927).
- 4) H. Matsushima, H. Fukumi and K. Arima, *Agr. Biol. Chem.*, **37**, 1865 (1973); H. Matsushima and K. Arima, *ibid.*, **37**, 1873 (1973).
- 5) R. L. Hinman and C. P. Bauman, *J. Org. Chem.*, **29**, 2341 (1964).
- 6) R. L. Hinman and C. P. Bauman, *ibid.*, **29**, 1206 (1964).
- 7) Y. Suzuki, H. Kinashi, S. Takeuchi and A. Kawarada, *Phytochem.*, submitted for publication.
- 8) R. L. Hinman and J. Lang, *Biochem.*, **4**, 144 (1965).
- 9) Unpublished data.