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GROWTH AND GERMINATION INHIBITORS IN RICE HUSKS

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Keyword Index—*Oryza sativa*; Gramineae; rice; germination inhibitors; flavonoids; steroids.

Abstract—A search for growth and germination inhibitors in rice husk (*Oryza sativa* L. cv Koshihikari) revealed four compounds, ineketone, *S*(+)-dehydrovomifoliol, momilactone-C and *p*-coumaric acid, in addition to the previously known momilactones-A and -B. The isolation and structural determination of these inhibitors are reported. The flavonoid tricin and three steroids were also detected in the husk but none showed any inhibitory activity.

INTRODUCTION

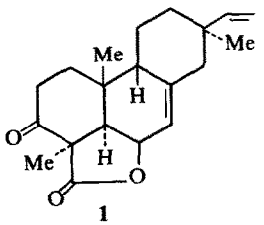
The germination of rice is of great agricultural importance and it has long been known that it is influenced by compounds present in the seed coat (husk) [1–5]. We undertook therefore to investigate the chemistry of the germination inhibitors in the husk of the poorly germinating cultivar Koshihikari [6].

RESULTS

Isolation of growth inhibitors

Husks of koshihikari were immersed in MeOH for several weeks and the MeOH extract concentrated *in vacuo*. The $CHCl_3$-soluble portion was shown to contain germination inhibitors which were not extracted into *n*-hexane. The hexane insoluble material was successively stirred with C_6H_6, ether, CH_2Cl_2 and finally with EtOAc. The most active components were found to be present in the C_6H_6 and ether extracts.

The benzene extract, after removal of acids, was fractionated by silica gel column chromatography yielding two components, momilactone-A (**1**) and -B (**2**) (*ca* 1.1g and 400 mg, respectively) from 300 kg of husk (husk is momi in Japanese)). The structure of these active compounds was determined by NMR and MS [7] and was confirmed by chemical degradation studies which are described elsewhere [8].



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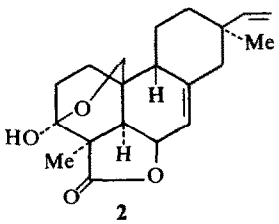

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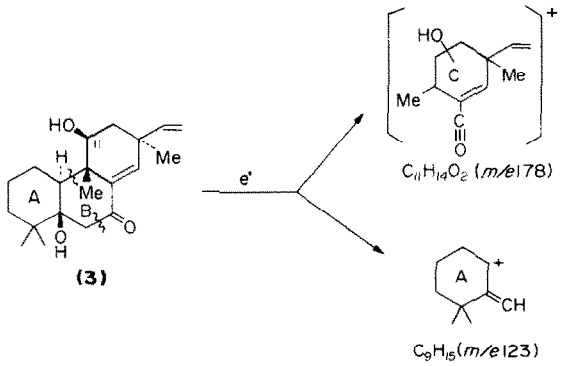
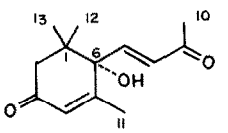


(3)

$C_{11}H_{14}O_2$ (*m/e* 178)

C_9H_{15} (*m/e* 123)

The ether extract was fractionated as above and the resulting active neutral fraction purified by column chromatography, TLC, and HPLC to yield three active compounds. The first (ca 10 mg) was named ineketone (**3**) (rice is called ine in Japanese); the second (13 mg) proved to be identical with *S*(+)-dehydrovomifoliol (**4**); and the third (*ca* 2–3 mg) was named momilactone-C. *p*-Coumaric acid was isolated from the acidic fraction and showed only weak inhibition of germination.



(4)

Ineketone. Ineketone (**3**), mp 206–209° (from *n*-hexane–EtOH), $C_{20}H_{30}O_3$ (M^+ 318, M w 318), ORD (MeOH); $[\phi]_{364}$ +1000° $[\phi]_{308}$ −2180° and CD (MeOH); $\Delta\varepsilon$ +1.01 (336 nm). No single large crystals were obtainable so X-ray crystallographic analysis

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could not be performed. IR spectrum ($CHCl_3$) exhibits the presence of –OH (3450 cm^{-1}) and conjugated CO (1680 and 1620 cm^{-2}) groups. The presence of the latter is supported by the UV spectrum, λ_{max} (MeOH) 240 nm (ε 8125) being in good accord with the calculated value for a conjugated ketone having substituents at both α- and and β-positions [9]. The 100 M Hz NMR spectrum* shows, in addition to four tertiary methyls at 0.82, 0.96, 1.02 and 1.09 ppm, the presence of the following groups; vinyl group attached to a tertiary carbon(■–$CH=CH_2$) at 5.03 (1H, *dd*, 10.0 and 1.0 Hz). 5.06 (1H, *dd*, 17.5 and 1.0 Hz), and 5.86 ppm (1H, *dd*, 17.5 and 10.0 Hz), a β-proton of the conjugated ketone at 6.72 ppm (1H, *d*, 1:5 Hz), an isolated methylene (■–CH_2CO–) as AB quartet at 2.33 and 2.56 ppm (1H each, 17.5 Hz) and a proton geminal to a hydroxy group at 3.35 ppm (1H, *m*). The CMR measurements† (Table 1) supports the presence of all the functional groups suggested above. Moreover, a singlet carbon at 74.03 ppm indicates the presence of a tertiary hydroxy group in ineketone.

Table 1. CMR of ineketone

Carbon number	Chemical shifts
C_1, C_2, C_3, C_6 and C_{12}	25.85 (*t*), 27.13 (*t*), 28.19 (*t*), 30.00 (*t*) and 37.13 (*t*)
C_4, C_9, and C_{13}	29.59 (*s*), 38.59 (*s*), and 38.83 (*s*)
C_5	74.03 (*s*)
C_7	201.50 (*s*)
C_8	singlet at 136.77 (*s*)
C_{10}	40.41 (*d*)
C_{11}	77.04 (*d*)
C_{14} and C_{15}	145.78 (*d*) and 146.59 (*d*)
C_{16}	112.63 (*t*)
Four methyls	14.97 (*q*), 16.84 (*q*), 23.80 (*q*), and 27.78 (*q*)

Thus ineketone is a tricarbocyclic diterpene related biogenetically to the momilactones which possess a pimaradiene carbon skeleton. High resolution MS gave two fragments, C_9H_{15} (*m/e* 123) and $C_{11}H_{14}O_2$ (178, base peak), with other prominent peaks at 163 ($C_{10}H_{11}O_2$) and 135 (C_9H_{10}) presumably derived by the elimination of methyl and then CO from 178 fragment ion. This leads to part structure (**3**) for ineketone. The fragment ions at *m/e* 178 and 123 might be formed by the fission of the B ring and their possible structures are depicted in the figure. It is, thus, deduced that the ineketone has a rimuene skeleton. The position of the hydroxy group was deduced as follows. In the NMR, the β proton with respect to the conjugated ketone appeared at 6.72 ppm with a small split of 1.5 Hz, indicating long range coupling. Irradiation at 6.72 ppm caused a change of the signal near 1.6 ppm. Irradiation at 1.6 ppm converted the β proton to a singlet and at the same time simplified the multiplet at 3.35 ppm due to a proton geminal to the hydroxy group, thus suggesting the hydroxy group at C_{11}-position. Although the NMR of ineketone in $CDCl_3$ plus D_2O could not be measured because of its extremely unstable nature toward even a trace of HCl, the coupling mode of the multiplet at 3.35 ppm suggests the hydroxy group to be equatorial.

The stereochemistry at C_9, C_{10}, and C_{13} is presumed on biogenetical grounds to be derived from the momilactone carbon skeleton. Migration of C_{10}–Me to C_9 position of pimaradiene skeleton and concomitant shift of C_5–H to C_{10}–position leads to the ineketone framework. The axial configuration of C_5–OH is more likely since ineketone is easily dehydrated under acidic conditions.

S(+)-Dehydrovomifoliol (**4**). High resolution MS gave no molecular ion and hence molecular formula could not be confirmed but the CMR spectrum showed the presence of thirteen carbon atoms (Table 2). These results as well as the evidence described in the Experimental indicates that the active material is S(+)-dehydrovomifoliol (**4**), which was isolated earlier from the roots of kidney bean (*Phaseolus vulgaris*) [10].

Table 2. CMR of S(+)-dehydrovomifoliol

Carbon number	Chemical shifts
C_1	41.26 (*s*)
C_2	49.33 (*t*)
C_3	196.46 (*s*)
C_4	127.11 (*d*)
C_5	146.16 (*s*)
C_6	78.87 (*s*)
C_7	144.40 (*d*)
C_8	129.78 (*d*)
C_9	196.03 (*s*)
C_{10}	28.21 (*q*)
C_{11}	18.63 (*q*)
C_{12}	24.21 (*q*)
C_{13}	22.87 (*q*)

Other compounds. *p*-Coumaric acid and 5, 7, 4′-trihydroxy-3′, 5′-dimethoxyflavone (tricin), and the three common plant steroids (sitosterol, stigmasterol, and campesterol) were identified. Momilactone-C gave single crystals and its structure is now under investigation.

Physiological activities

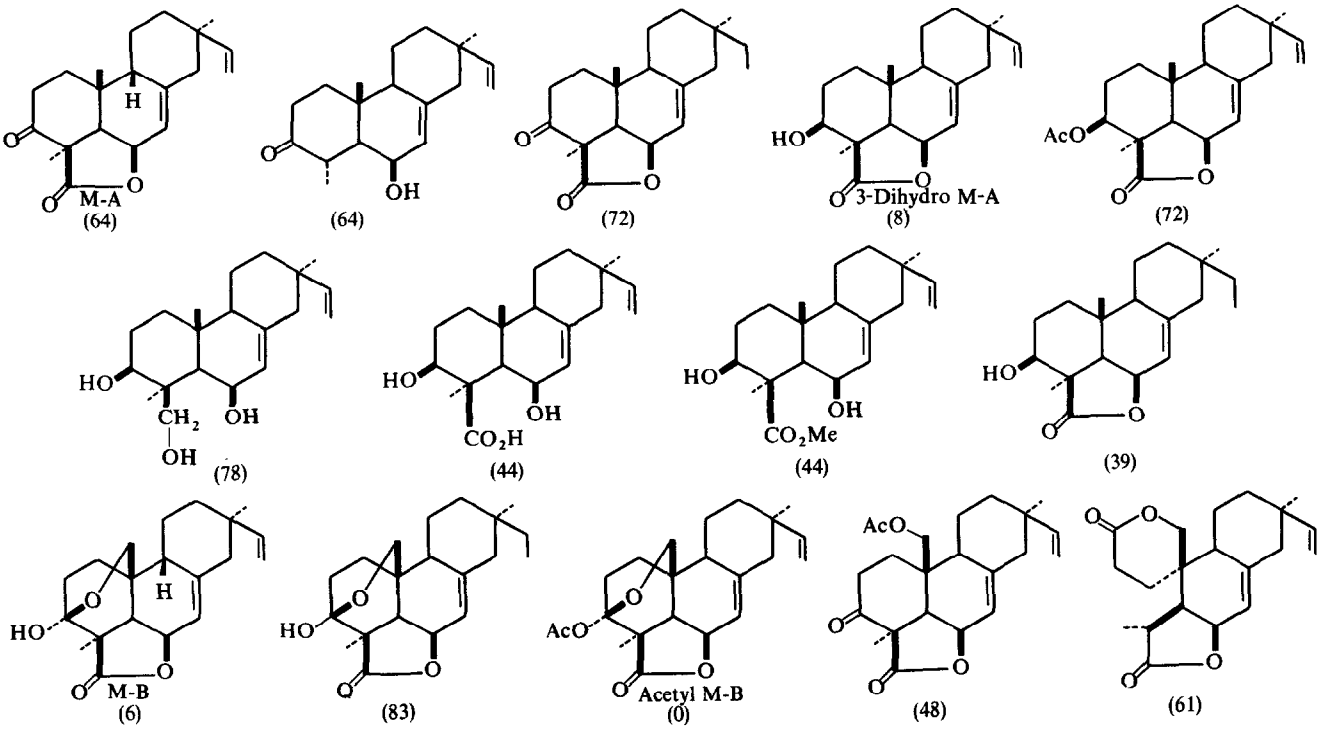
Momilactones–A and –B, especially the latter, inhibit the germination of lettuce seeds and growth of roots of rice. In order to establish the functional groups in the momilactones necessary for activity, several derivatives were prepared [8] and assayed for inhibitory activity (Table 3). It was found that all derivatives which inhibited lettuce seed germination had activity against the growth of rice roots. The inhibitory activity was strongest with 3-dihydro momilactone-A and acetyl momilactone-B (Figs. 1 and 2), while reduction of the vinyl group on ring C diminished the activity.

The inhibitory activity of ineketone and dehydrovomifoliol toward the germination of lettuce seeds is shown in Fig. 3. As can be seen, neither is as effective as the momilactone derivatives and it is of interest to note that ineketone promotes the growth of roots of rice at concentrations, below 500 ppm (Fig. 4).

* PMR spectra were measured with a Varian HA-100. Chemical shifts are in ppm from TMS. The coupling constants were obtained by first order analyses. The symbol ■ refers to a carbon bearing no hydrogen.

† CMR spectra were measured with JEOL FX-100. Chemical shifts were in ppm down field from TMS. Multiplicity shows *s* = singlet, *d* = doublet, *t* = triplet and *q* = quartet, respectively.

Table 3. Germination inhibition of lettuce seeds by momilactones and the derivatives.



Values in parentheses show the germination ratio at 1000 ppm.

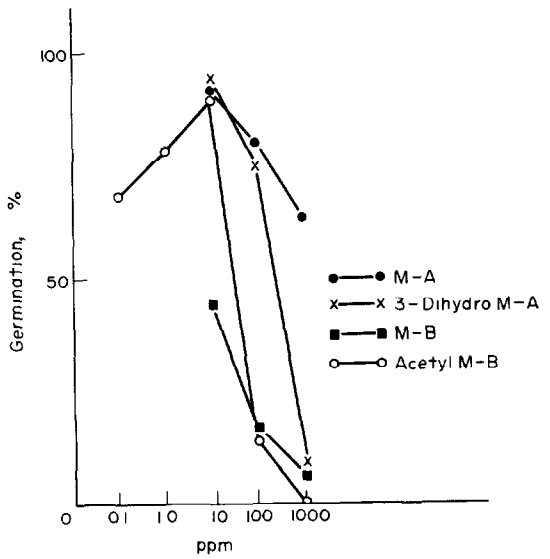


Fig. 1.

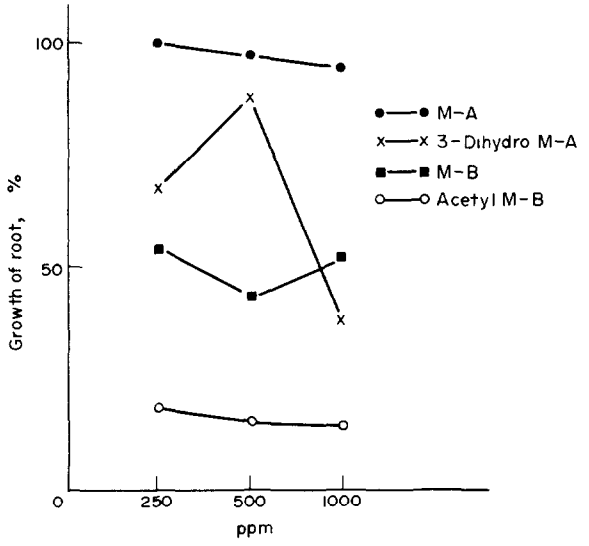


Fig. 2.

EXPERIMENTAL

Bioassay with lettuce seeds. Each compound at the requisite concentration in acetone was absorbed on filter paper and its solvent removed. The paper was saturated with aqueous tween-80 (100 ppm) and 50 seeds of lettuce (Ferry Morse Seed Co. Mountain View, Cal.) were placed on each filter paper in a Petri dish and kept in the dark at 20° for 24 and 48 hr.

Extraction of husk. 30 × 10 kg of dried husk of cv. Koshihikari, was immersed in 60 l of 80% MeOH for 2 months. The MeOH extract was concentrated and shaken twice with 0.5 Vol $CHCl_3$. The $CHCl_3$ soluble extractives (400 g) were stirred at room temp with *n*-hexane (50 g/l) and the solvent decanted. The insoluble fraction was successively stirred with an equal vol. of benzene, Et_2O, CH_2Cl_2 and finally with EtOAc. Evaporation of the solvents afforded 10, 15, 4, 15, and 3 g, respectively. Each mixture was dissolved in the corresponding solvent and shaken successively with aq $NaHCO_3$, 0.1 N-NaOH and then H_2O. Evaporation of the solvent afforded the corresponding neutral fraction.

Isolation of p-*coumaric acid from the ether soluble fraction.* The acid fraction from the $NaHCO_3$ extract of the ether solubles (5 g) was passed through a charcoal column (100 g) and eluted

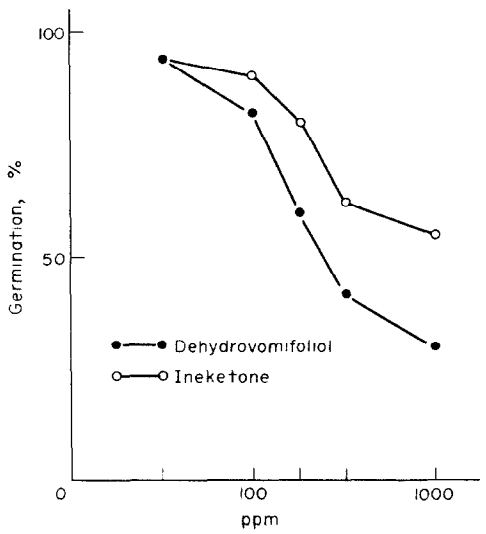


Fig. 3.

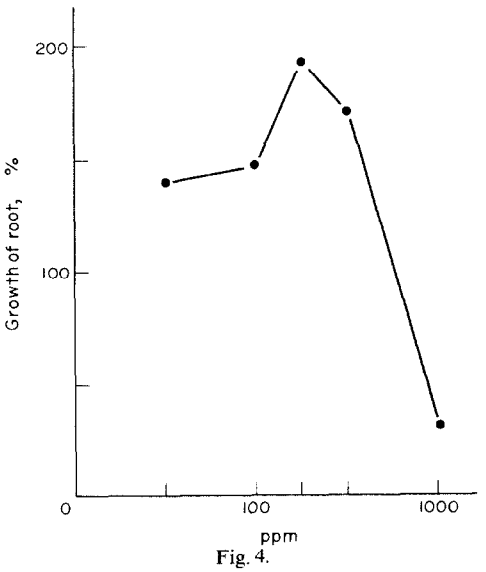


Fig. 4.

with Me_2CO-H_2O (1:1) to obtain *p*-coumaric acid (120 mg) as the active material, (UV, IR, mmp.).

Separation of momilactones from benzene soluble fraction. The neutrals (23 g) from benzene soluble extracts were chromatographed over SiO_2 (900 g) and eluted with C_6H_6, C_6H_6–EtOAC, hexane–EtOAc, EtOAc and MeOH. The active compounds were contained in the hexane–EtOAC fractions. Recrystallization of the early fractions from benzene and rechromatography of the mother liquid afforded total amounts of 1.08 g of momilactone-A. The next fraction gave similary 0.38 g of momilactone-B. Other active material(s) were also present but were not examined further due to the difficulty of separation. Momilactone-A (1), mp 235-236, $C_{20}H_{26}O_3$ (M^+ 314.1891, mol wt 314.1880) (Found C, 76.47; H, 8.37 $C_{20}H_{26}O_3$ requires C, 76.40; H, 8.34%). $[\alpha]_D^{CHCl_3}$ −277°, $[\phi]$ −50 600° (228 nm), −10 530 (317 nm). IR ($CHCl_3$) 1770, 1700, 1665, and 1640 cm^{-1}. PMR ($CDCl_3$) δ 0.90, 1.00, 1.52 ppm (each 3H, *s*), 2.03 and 2.22 (each 1H, *d*, 12.5 Hz), 2.32 (1H, *d*, 5.0 Hz), 4.83 (1H, *t*, 5.0 Hz), 4.93 (1H, *dd*, 10.8 and 1.2 Hz), 4.95 (1H, *dd*, 17 and 1.2 Hz), 5.70 (1H, *d*, 5.0 Hz), and 5.85 ppm (1H, *dd*, 17 and 10.8 Hz). CMR $CDCl_3$) 34.9 (*t*, C_1), 31.3 (*t*, C_2), 205.0 (*s*, C_3), 53.8 (*s*, C_4), 46.7 (*d*, C_5), 73.2 (*d*, C_6), 114.1 (*d*, C_7), 148.0 (*s*, C_8), 50.2 (*d*, C_9), 32.5 (*s*, C_{10}), 24.0 (*t*, C_{11}), 37.3 (*t*, C_{12}), 40.2 (*s*, C_{13}), 47.6 (*t*, C_{14}), 149.0 (*d*, C_{15}), 110.1 (*t*, C_{16}), 22.0 (*q*, C_{17}), 21.4 (*q*, C_{18}), 174.3 (*s*, C_{19}), and 21.8 ppm (*q*, C_{20}).

Momilactone-B (2) mp 242 (decom.), $C_{20}H_{26}O_4$ (M^+ 330.1834, mol wt 330.1830), (Found C, 72.70; H, 7.93. $C_{20}H_{26}O_4$ requires C, 72.55; H, 8.07%). $[\alpha]_D^{CHCl_3}$ −185°, $[\phi]_{max}$ −735 10° (217 nm). IR ($CHCl_3$) 3500, 1750, 1663, and 1638 cm^{-1}. PMR ($CDCl_3$) 0.87 and 1.40 (each 3H, *s*), 2.20 (1H, *dd*, 7.1 and 2.1 Hz), 3.55 (1H, *dd*, 9.0 and 2.1 Hz), 4.07 (1H, *bd*, 9.0 Hz), 4.92 (1H, *dd*, 10.5 and 1.2 Hz), 4.94 (1H, *dd*, 7.1 and 4.5 Hz), 4.95 (1H, *dd*, 17.6 and 1.2 Hz), 5.68 (1H, *d*, 4.5 Mz) and 5.83 ppm (1H, *dd*, 17.6 and 10.5 Hz). CMR ($CDCl_3$) 28.8 (*t*, C_1), 26.5 (*t*, C_2), 96.6 (*s*, C_3), 50.4 (*s*, C_4), 43.0 (*d*, C_5), 73.8 (*d*, C_6), 114.0 (*d*, C_7), 146.7 (*s*, C_8), 44.7 (*d*, C_9), 30.8 (*s*, C_{10}), 24.8 (*t*, C_{11}), 37.3 (*t*, C_{12}), 40.0 (*s*, C_{13}), 47.4 (*t*, C_{14}), 148.8 (*d*, C_{15}), 110.2 (*t*, C_{16}), 21.9 (*q*, C_{17}), 19.0 (*q*, C_{18}), 180.5 (*s*, C_{19}) and 72.7 ppm (*t*, C_{20}).

Separation of active compounds from ether soluble fraction. 8 g of the neutrals of ether soluble fraction were column chromatographed over SiO_2 (600 g) and eluted successively with C_6H_6-EtOAc (10:1), C_6H_6–EtOAc (3:1) and finally with EtOAc. The first solvent gave 200 mg of a mixture of steroids (sitosterol, stigmasterol, campesterol), methyl *p*-coumarate (150 mg) and a mixture of momilactone-A and -B (100 mg). The C_6H_6–EtOAc fraction afforded tricin (20 mg), $C_{17}H_{14}O_7$, mp 288–289° (from MeOH), IR (KBr) 3300, 1650, 1610, 1500, and 830 cm^{-1}; NMR (pyridine-D_5) 13.65 (1H, *bs*), 7.34 (2H, *s*), 6.90 (1H, *s*), 6.78 (1H, *d*, 1.5 Ha), 6.67 (1H, *d*, 1.5 Hz) and 3.83 (6H, *s*); UV λ_{max}^{MeOH} 242, 270 and 352 nm, λ_{max}^{NaOH} 420 nm. Triacetate (M^+ 458) [cf. 11]. EtOAc fraction (1.9 g), after column chromatography on SiO_2 (190 g) and preparative TLC (SiO_2) with the C_6H_6–EtOAc (4:1), CH_2Cl_2–MeOH (60:1), and then $CHCl_3$–EtOH (50:1), afforded the active mixture which was again chromatographed on SiO_2 with C_6H_6–EtOAc (4:1) to elute ineketone (ca 10 mg). The active mixture was again chromatographed on SiO_2 and then preparative TLC, yielded two active fractions, I (50 mg) and II (50 mg). The first was separated with HPLC (column; Hitachi gel, solvent, $CHCl_3$ + 2% EtOH) to obtain momilactone-C [3 mg, mp 227–228° (*n*-hexane–$CHCl_3$ (1:1)].

The second (II) was repeatedly recycled in HPLC (column; μ-porasil, solvent; 1.6% EtOH in $CHCl_3$) to yield 13.4 mg of S(+)-dehydrovomifoliol (4), and two other compounds, $C_{15}H_{20}O_3$ (4.8 mg) and $C_{19}H_{26}O_3$ (5.9 mg). The structure of the latter two materials was not investigated.

S(+)-Dehydrovomifoliol (4), $C_{13}H_{18}O_3$ oil, $[\alpha]_D^{25}$ +142.7° (c 0.782, MeOH), IR ($CDCl_3$) 3450, 1660, 1620, 982, and 872 cm^{-1}, PMR ($CDCl_3$) δ 1.01 and 1.10 (each 3H, *s*), 1.86 (3H, *d*, 1.0 Hz), 2.26 (3H, *s*), 2.24 and 2.56 (each 1H, ABq, 17 Hz), 5.92 (1H, *q*, 1 Hz), 6.39 and 6.84 (each 1H, AB*q*, 15.5 Hz). Mass *m/e* 166 (M-56), 124. UV λ_{max}^{MeOH} 238 nm (ε 14500), ORD (MeOH): $[\phi]_{260}$ +650°, CD (MeOH) $\Delta\varepsilon$ −2.86 (325 nm), +34.7 (243 nm).

Ineketone (3), mp 206–209° (*n*-hexane + EtOH) (Found: C, 75.65. H, 9.32 $C_{20}H_{30}O_3$ requires C, 75.43, H, 9.50%)

REFERENCES

1. Roberts, E. H. (1961) *J. Exp. Botany* **12**, 430.
2. Ishizumi, K. (1959) *Text book of Rice Cultivars*. p. 52. Fukui Nojyo Kankobu.
3. Mikkelsen, D. S. and Sinah, M. N. (1961) *Crop. Sci* **1**, 332; Mikkelsen, D. S. and Glazweskii, A. T. (1966) The 11th Pacific Sci. Congr., Tokyo.
4. Ota, Y. (1966) The 11th Pacific Sci. Cong., Tokyo.
5. Dutta, A. K. (1973) *Chem. Abst.* **78**, 155375.
6. Takahashi, N. (1968) *Rep. Inst. Res. Tohoku Univ.*, **19**, 1; Uotani, K. Umezu, T., Meguro, H., Tsuzimura K. and Takahashi, N. (1972) *Tohoku J. Agr. Res* **23**, 58.
7. Kato, T., Kabuto, C., Sasaki, N., Tsunakawa, M., Aizawa, H., Fujita, K., Kato, Y., Kitahara, Y. and Takahashi, N. (1973) *Tetrahedron Letters* 3861.
8. Kato, T., Aizawa, H., Tsunakawa, M., Sasaki, N., Kitahara, Y. and Takahashi, N. (1976) *J. Chem Soc. C*, to be published
9. Scott, A. I. (1964) *Interpretation of the Ultraviolet Spectra of Natural Products*, p. 46. Pergamon Press.
10. Takasugi, M., Anetai, M., Katsui, N. and Mesamune, T. (1973) *Chem. Letters* 245.
11. Kuwatsuka, S (1961) *Agr. Chem. (Japan)*, **35**, 67.