Fungal Metabolism of the Prenylated Isoflavone 2,3-Dehydrokievitone

Satoshi Tahara, Eri Misumi, Junya Mizutani

Department of Agricultural Chemistry, Faculty of Agriculture, Hokkaido University, Kita-ku, Sapporo 060, Japan

and

John L. Ingham

Department of Food Science, Food Studies Building, University of Reading, Whiteknights, P.O. Box 226, Reading RG6 2AP, England

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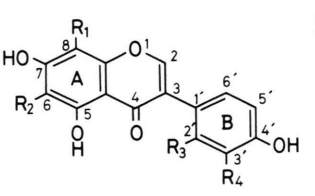
The prenylated isoflavone, 2,3-dehydrokievitone [5,7,2′,4′-tetrahydroxy-8-(3,3-dimethylallyl)-isoflavone, **1**], was metabolized by both *Aspergillus flavus* and *Botrytis cinerea* to yield the same three compounds, a dihydrofurano-isoflavone (DK-M1, **2**), a dihydropyrano-isoflavone (DK-M2, **3**), and a glycol (DK-M3, **4**). Although structure **3** has previously been assigned to lunatone, an isoflavone from $CuCl_2$-stressed lima bean *(Phaseolus lunatus)* seedlings, a comparison of spectroscopic (MS, ^1H NMR) data indicated that the *Phaseolus* compound was not identical with metabolite DK-M2 found in *Aspergillus* and *Botrytis* cultures. Evidence is presented to suggest that lunatone is probably identical with the furano-substituted metabolite DK-M1 (**2**).

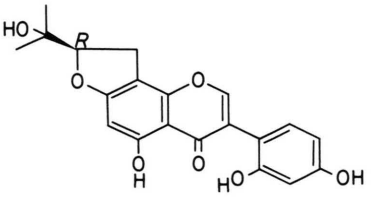
Introduction

In two earlier papers [1, 2] we reported that the 6-prenylated isoflavone luteone (**5**) and wighteone (**6**) were metabolized by the fungus *Aspergillus flavus* to yield the corresponding hydrate derivatives. This fungus, and *Botrytis cinerea,* also converted **5** and **6**, and the 3′-prenylated isoflavones licoisoflavone A (**7**) and 2′-hydroxylupalbigenin (**8**), into various dihydrofurano-isoflavones, dihydropyrano-isoflavones and 2,3-dihydrodihydroxyprenyl-isoflavones [1–4], all of which might arise *via* epoxidation of the unsaturated side-chain in the appropriate substrate. 3′-Side-chain hydration of **7** and **8** was not observed [3, 4].

In contrast to compounds **5**~**8**, the fungal metabolism of isoflavones prenylated at C-8 has not been investigated because of their very limited availability. However, it has recently been found that yellow lupin (*Lupinus luteus* cv. Barpine) roots contain substantial amounts (approx. 40 mg per kg fresh material) of 2,3-dehydrokievitone [5,7,2′,4′-tetrahydroxy-8-(3,3-dimethylallyl)isoflavone, **1**] [5], a substance first isolated as a minor phytoalexin from the *Monilinia fructicola*-inoculated pods of *Phaseolus vulgaris* [6]. The same compound is also produced as a chemically-induced stress metabolite in seedling tissues of *P. lunatus* (lima bean) where it is accompanied by several related isoflavones including



1: $R_1 = CH_2CH=C(CH_3)_2$, $R_2 = R_4 = H$, $R_3 = OH$
5: $R_1 = R_4 = H$, $R_2 = CH_2CH=C(CH_3)_2$, $R_3 = OH$
6: $R_1 = R_3 = R_4 = H$, $R_2 = CH_2CH=C(CH_3)_2$
7: $R_1 = R_2 = H$, $R_3 = OH$, $R_4 = CH_2CH=C(CH_3)_2$
8: $R_1 = H$, $R_2 = R_4 = CH_2CH=C(CH_3)_2$, $R_3 = OH$



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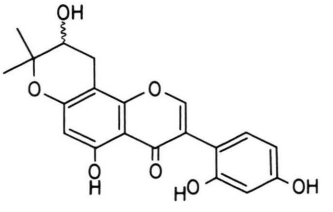

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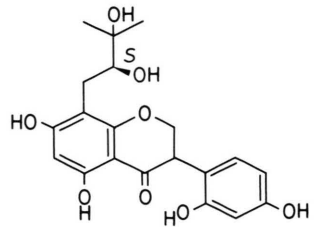

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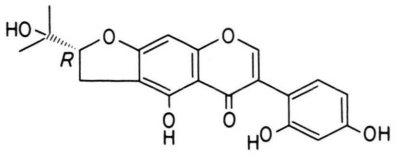

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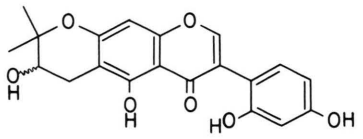

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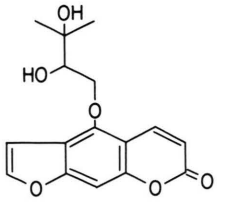

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lunatone [7]. Although lunatone (= cyclo-2,3-dehydrokievitone hydrate) was formulated as a dihydropyrano-isoflavone, ^{1}H NMR data reported [7] for the aliphatic side-structure protons 1″-H and 2″-H are strikingly different from those of previously recognized dihydropyrano-isoflavones such as luteone metabolite BC-2 (**10**) [1] and licoisoflavone A metabolites M-1-1 and M-3-1 [3].

In view of the comparative ease with which 2,3-dehydrokievitone (**1**) can be isolated from *L. luteus*, we decided to investigate if this compound also undergoes metabolism when incubated with either *A. flavus* or *B. cinerea*. Although no products analogous to the hydrates of luteone and wighteone [1, 2] were detected in culture extracts, the results described in this paper clearly show that both fungi convert **1** into the same three compounds. Two of these metabolites have been identified as isomeric dihydrofurano- and dihydropyrano-isoflavones, and a comparison of their spectroscopic (MS, ^{1}H NMR) characteristics with those reported for lunatone [7] leads us to the conclusion that the latter compound possesses a furano, rather than a pyrano, side attachment.

Results and Discussion

2,3-Dehydrokievitone (**1**) was metabolized more slowly than its regio-isomer luteone (**5**) [1] when incubated for 4 days in a shaken liquid medium containing freshly grown cultures of *Aspergillus flavus* or *Botrytis cinerea*. About half the substrate initially administered was recovered unchanged at the conclusion of the experiment. Preparative Si gel TLC (PTLC) of EtOAc extracts of the culture media revealed that **1** was identically converted by both fungi to give three metabolites, designated DK-M1 and DK-M2 with M^+ 370 (= substrate + [O]), and DK-M3 (M^+ 388; = substrate + 2 × [OH]). Yields and chromatographic properties of **1** and the three metabolites are summarized in Table I.

The UV (MeOH) spectrum of all metabolites gave a substrate-like maximum between 265 and 266 nm (*cf.* **1**, 265 nm). However, whilst the MeOH maximum of **1** and DK-M3 was shifted bathochromically upon addition of both $AlCl_3$ (9.5 ~ 10 nm; C-5 OH free) and NaOAc (12 ~ 14 nm; C-7 OH free) [8], that of DK-M1 and DK-M2 was affected only by $AlCl_3$ (11 nm shift). No shift was observed in the presence of NaOAc (C-7 OH derivatized). Apart from a prominent MS fragment (15 ~ 46%) at *m/z* 134 (B-ring ion derived by RDA fission; *cf.* **1**, *m/z* 134 = 20%) [9], all the metabolites exhibited heterocyclic (2-H [10]) and B-ring (3′-H, 5′-H and 6′-H) ^{1}H NMR signals comparable with those recorded for the substrate (Table II). Structural modification of **1** by *A. flavus* and *B. cinerea* can therefore be presumed to directly involve ring A and/or the prenyl side-chain situated at C-8.

In fact, the presence of modified side-structures in compounds DK-M1 ~ DK-M3 was quickly deduced by comparison of their aliphatic ^{1}H NMR signals with those of known dihydrofurano-, dihydropyrano- and dihydrodihydroxyprenyl-substituted isoflavones

Table I. Yields and TLC data for 2,3-dehydrokievitone (**1**) and its fungal metabolites (DK-M1, DK-M2 and DK-M3).

Compound		Yields[a] [mg]	[%]	R_F (TLC[b]) value CAAm	CM
Remaining substrate (**1**)	Asp.[c]	14.0	46.7	0.13	0.21
	Bot.	28.3	47.2		
DK-M1 (**2**)	Asp.	7.8	24.9	0.16	0.20
	Bot.	10.1	16.1		
DK-M2 (**3**)	Asp.	1.2	3.8	0.17	0.17
	Bot.	5.5	8.8		
DK-M3 (**4**)	Asp.	0.6	1.8	0.01	0.06
	Bot.	4.2	6.4		

[a] Yields in mg from 30 mg or 60 mg of substrate incubated respectively with *Aspergillus flavus* or *Botrytis cinerea*; % yield is on a molar basis.
[b] TLC solvent systems are described in the Experimental section.
[c] Asp. = *Aspergillus flavus* cultures; Bot. = *Botrytis cinerea* cultures.

Table II. ^{1}H NMR data (δ values) for 2,3-dehydrokievitone, its metabolites and various related compounds.

Compound Proton	2,3-Dehydro-kievitone (**1**)	DK-M1 (**2**)[b]	DK-M2 (**3**)[b]	DK-M3 (**4**)[c]	Lunatone[d] (250 MHz)	Luteone metabolites[e] BC-1 (**9**) (500 MHz)	BC-2 (**10**)
2-H	8.27 s	8.17 s	8.27 s	8.25 s	8.16 s	8.18 s	8.16 s
5-OH	12.70 s	13.00 s	12.55 s	12.74 s	–	12.96 s	13.13 s
6-H	6.40 s	6.24 s	6.20 s	6.34 s	6.24 s	–	–
8-H	–	–	–	–	–	6.43 s	6.40 s
3′-H	6.49 d*	6.49 d*	6.49 d*	6.49 d*	6.45 d J=2.2	6.49 d J=2.4	6.48 d*
5′-H	6.44 dd*	6.44 dd J=8.8, 2.4	6.45 dd J=8.9, 2.3	6.45 dd*	6.44 dd J=8.3, 2.2	6.45 dd J=7.8, 2.4	6.44 dd*
6′-H	7.15 br. d J=8.8	7.12 br. d J=8.8	7.14 br. d J=8.9	7.15 d J=8.8	7.11 d J=8.3	7.13 d J=7.9	7.13 d J=8.8
1″-H_a	3.47 (2H) br. d J=7.3	3.31 (2H) d-like J=*ca.* 5.7	2.74 dd J=16.7, 6.8	2.76 dd J=14.4, 10.0	3.30 (2H) dd J=7.9, 6.0	3.16 dd J=15.9, 9.8	2.62 dd J=17.1, 7.1
1″-H_b			3.09 dd J=16.7, 5.3	3.29 dd J=14.4, 2.0		3.21 dd J=15.9, 7.9	2.98 dd J=17.1, 5.4
2″-H	5.26 br. t J=7.3	4.88 dd J=9.2, 8.2	3.91 br. q J=*ca.* 5.7	3.69 dd J=10.0, 2.0	4.88 dd J=8.0, 6.0	4.86 dd J=9.8, 7.9	3.87 dd J=7.1, 5.4
4″-H_3	1.81 s	1.30 s	1.40 s	1.30 s	1.30 s	1.30 s	1.40 s
5″-H_3	1.66 s	1.26 s	1.35 s	1.29 s	1.26 s	1.25 s	1.34 s

[a] Except where indicated the spectra were determined at 100 MHz (acetone-d_6; TMS reference). J are in Hz. An asterisk indicates an incomplete signal for which a coupling constant could not be calculated.
[b] OH in the side-structure of **2** appeared at δ 3.83 s, and in **3** at δ 4.44 br. d (J=5.1 Hz).
[c] Comparative ^{1}H NMR data for the side-chain protons of the known isoflavones, luteone glycol [5,7,2′,4′-tetrahydroxy-6-(2,3-dihydroxy-3-methylbutyl)isoflavone] and licoisoflavone A glycol [5,7,2′,4′-tetrahydroxy-3′-(2,3-dihydroxy-3-methylbutyl)isoflavone] are as follows: a) *luteone glycol* [1], δ 1.26 and 1.28 (both 3H, two s, 5″-H_3 and 4″-H_3) 2.62 and 3.25 (both 1H, two dd, J=14.0 & 9.8 Hz, and J=14.0 & 2.0 Hz 1″-H_a and 1″-H_b) and 3.65 (1H, dd, J=9.8 & 2.0 Hz, 2″-H), b) *licoisoflavone A glycol* [3], δ 1.26 (6H, br. s, 5″-H_3 and 4″-H_3), 2.58 and 3.30 (both 1H, two dd, J=14.0 & 9.8 Hz, and J=14.0 & 2.0 Hz, 1″-H_a and 1″-H_b) and 3.63 (1H, dd, J=9.8 & 2.0 Hz, 2″-H).
[d] Isolated from *Phaseolus lunatus* seedlings and assigned structure **3** [7].
[e] See ref. [1].

[1–4]. As shown in Table II, whilst the aliphatic proton signals of DK-M1 [δ 1.26 and 1.30 (both 3H, two s, 5″-H_3 and 4″-H_3), 3.31 (2H, d-like, J = ca. 8.3 Hz, 1″-H_2) and 4.88 (1H, dd, J = 9.2 & 8.2 Hz, 2″-H)] and DK-M2 [δ 1.35 and 1.40 (both 3H, two s, 5″-H_3 and 4″-H_3), 2.74 and 3.09 (both 1H, two dd, J = 16.7 & 6.8 Hz, and 16.7 & 5.3 Hz, 1″-H_a and 1″-H_b) and 3.91 (1H, br. q, J = ca. 5.7 Hz, 2″-H coupled with C-2″-OH)] are clearly different from each other, they closely resemble chemical shift values respectively obtained for luteone metabolites BC-1 (**9**) and BC-2 (**10**) [1]. Together with the MS (M^+ 370 = substrate + [O]) and UV data (C-7-OH derivatized), the ^{1}H NMR results indicate that DK-M1 and DK-M2 are isomers, with the former possessing a dihydrofurano attachment, and the latter a dihydropyrano substituent. Fungal metabolites DK-M1 and DK-M2 can thus be represented by structures **2** and **3** respectively (Fig.1).

The second metabolite, DK-M2 (**3**) has a structure identical with that earlier assigned to lunatone, an inducible isoflavone produced by chemically-stressed lima bean *(Phaseolus lunatus)* seedlings [7]. However, the ^{1}H NMR data recorded for lunatone (acetone-d_6, 270 MHz; see Table II) more closely resemble those of the furano compounds **2** and **9** rather than the pyrano derivatives **3** or **10**. As described in our previous papers [1, 11], the structures of **9** and **10** were unambiguously established using a combination of chemical (dehydration and acetylation) and spectroscopic procedures. In contrast, the structure of lunatone was inferred only from spectroscopic data [UV, MS, ^{1}H NMR (δ values, coupling constants, and an NOE experiment)] which in our view do not firmly exclude the isomeric structure **2**.

Apart from obvious differences between **3** and lunatone with respect to their aliphatic ^{1}H NMR sig-

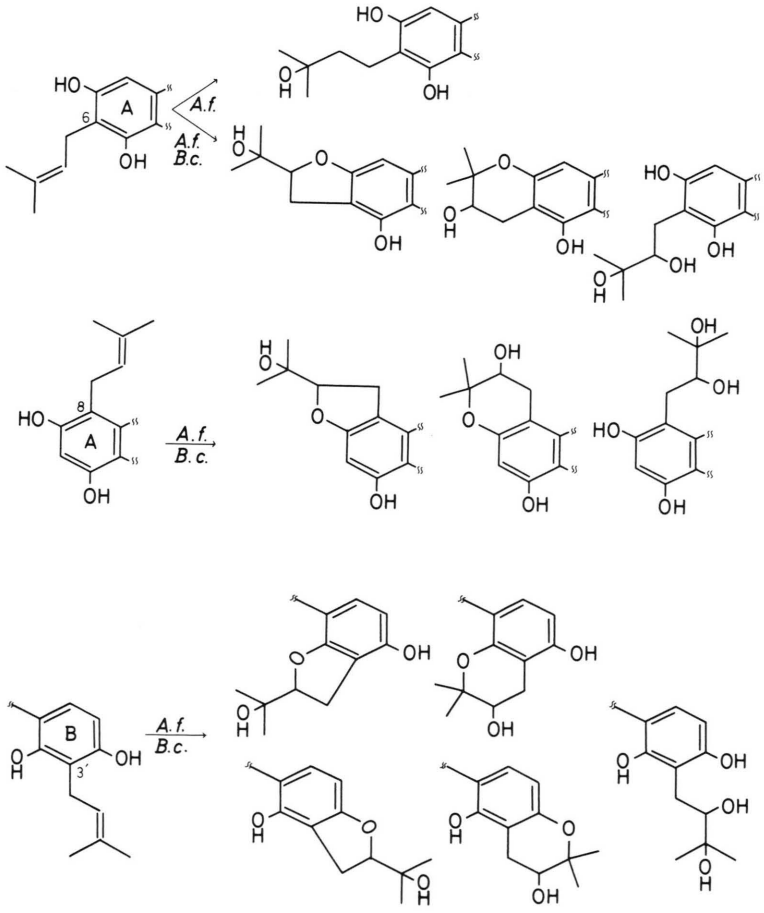

Fig. 1. Substrate specificity in fungal metabolism of prenylated isoflavones *(A. f. = Aspergillus flavus; B. c. = Botrytis cinerea)*.

nals, the latter compound also exhibits an MS feature, namely the presence of a prominent fragment at $M^+ -59$, but the absence of one at $M^+ -71$, which strongly suggests the possession of a dihydrofurano side attachment. In line with an earlier report [4], we found that MS fragments at *m/z* $M^+ -59$ and *m/z* 59 [characteristic of a 2-(1-hydroxy-1-methylethyl)-2,3-dihydrofurano residue], and *m/z* $M^+ -71$ (typical of a 2,3-dihydro-3-hydroxy-2,2-dimethylpyrano residue) were also given by DK-M1 [**2**; *m/z* 311 (93%) and *m/z* 59 (88%)] and DK-M2 [**3**; *m/z* 299 (70%)] respectively. Significantly, whilst the MS of lunatone showed an intense fragment at *m/z* 311 ($M^+ -59$; 62%), no peak at *m/z* 299 ($M^+ -71$) was apparently observed [7]. The ^{1}H NMR and MS data recorded for lunatone are therefore inconsistent with the proposed dihydropyrano-substituted structure (**3**), and we believe that revision to **2** is necessary for this compound. If the structure of lunatone is incorrect, it seems likely that cyclo-kievitone hydrate from the black gram *(Phaseolus mungo)* [12] has similarly been misidentified. This compound, which has been assigned an isoflavanone possessing a dihydropyrano side-attachment as in **3** [12], actually exhibits a set of aliphatic ^{1}H NMR signals [δ 1.28 (6H, s, $2 \times CH_3$), 3.05 (2H, m) and 4.76 (1H, m)] more closely resembling those associated with the dihydrofurano substituent found in metabolite DK-M2 (**2**).

The MS of metabolite DK-M3 gave the molecular ion at *m/z* 388 (corresponding to **1** + 2 × [OH]), and major fragments at *m/z* 370 ($M^+ - H_2O$; 18%), 329 ($M^+ -59$; 64%), 299 ($M^+ -89$; 100%), 165 (main A-ring fragment; 48%) and 134 (main B-ring fragment; 15%). Losses of 18 and particularly 89 mass units from the molecular ion, together with data mentioned earlier showing (^{1}H NMR; Table II) an intact 2,3-dehydrokievitone nucleus and (UV) underivatized OH groups at C-5 and C-7, established that DK-M3 possessed a dihydrodihydroxy side-chain. The exact identify of this side-chain as a 2,3-dihydroxy-3-methylbutyl residue was subsequently determined by comparing the MS and ^{1}H NMR (see Table II, and footnote c) spectra of DK-M3 with those of luteone metabolite AF-2/BC-3 = luteone glycol [M^+ 388; $M^+ -18$ (4%) and $M^+ -89$ (100%)] [1], and licoisoflavone A metabolite M-3-2 = licoisoflavone A glycol [M^+ 388; $M^+ -18$ (8%), $M^+ -89$ (100%)] [3]. Metabolite DK-M3 can therefore be assigned structure **4**.

Luteone and wighteone with prenyl groups at C-6 in ring A are already known to be rapidly converted into the corresponding hydrates by *A. flavus* [1, 2]. A degree of substrate preference is clearly evident, however, as *A. flavus* lacks the ability to similarly transform licoisoflavone A (prenyl at C-3′ on ring B) [3]. The present study confirms this C-6 specificity since TLC examination of extracts of *A. flavus* cultures has provided no evidence to suggest that 2,3-dehydrokievitone (**1**; prenyl at C-8) is metabolized to give a hydrate derivative. In contrast, *Fusarium oxysporum* is less specific in its action, catalyzing hydration of the prenyl side-chain at both C-8 and C-10 (≡ C-3′) of kievitone (isoflavanone) and phaseollidin (pterocarpan) respectively [13]. Unlike *A. flavus*, the fungus *B. cinerea* appears unable to form hydrated isoflavone metabolites regardless of whether the prenyl group is sited at C-6, 8 or 3′ [1–3, and this study]. Substrate specificity, and the various structures encountered as metabolites during our studies on the metabolism of prenylated isoflavones, are summarized in Fig. 1.

DK-M1 (**2**) from *B. cinerea* cultures was found to be laevorotatory ($[\alpha]_D^{23} -76°$), a feature indicative of the *R*-configuration at C-2″ in the side-attachment (*cf.* luteone metabolite BC-1, **9**, $[\alpha]_D^{23} -100°$ of established *R*-stereochemistry [11]). Although luteone glycol and licoisoflavone A glycol [1, 3] have only been isolated in optically inactive forms, metabolite DK-M3 (**4**) produced by *B. cinerea* proved to be slightly laevorotatory ($[\alpha]_D^{23} -15°$). The C-2″ configuration in **4** was deduced as being *S* because the osmate ester-pyridine complex gave a negative CD Cotton effect at approx. 480 nm ($[\theta]_{480}^{23} -380$; see ref. [11]). The relationship between the signal observed for a CD Cotton effect of an osmate ester-pyridine complex around 470–480 nm, and the absolute configuration of a 1,2-glycol (C-2 chiral), was established by Sakota *et al.* as: Cotton effect negative = *S*, and Cotton effect positive = *R* [14]. The stereochemistry of DK-M3 (laevorotatory, *S*-configuration) is also in accord with data determined for certain natural coumarins (*e.g.* oxypeucedanin hydrate, **11**) containing a single chiral centre in the 2,3-dihydroxy-3-methylbutyl side-chain where (−) indicates the *S*-configuration, and (+) the *R* [15, 16].

Lastly, the *S* absolute configuration of DK-M3 is further supported by the stereochemical mechanism proposed for the metabolism of prenylated isoflavones. As described in an earlier paper [11], if a dihydrofurano-isoflavone (*e.g.* DK-M1) with the *R*-

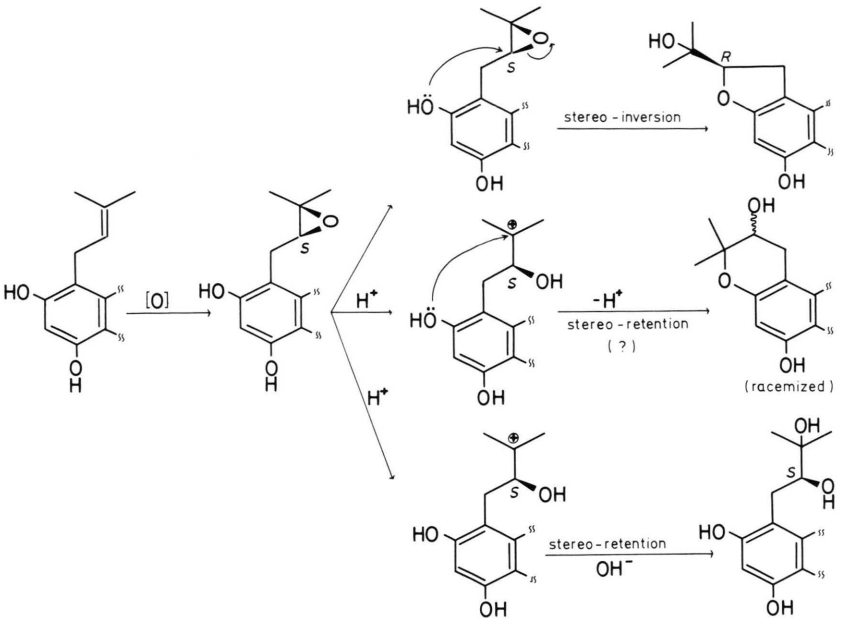

Fig. 2. Stereochemical explanation for the mechanism of prenylated isoflavone metabolism by *Botrytis cinerea*.

configuration at C-2″ is produced *via* the corresponding epoxide, formation of the dihydrofurano ring and associated stereo-inversion requires an *S*-configuration at C-2″ in the intermediate epoxide (Fig. 2). Similarly, if glycol **4** is produced, with accompanying stereo-retention, by hydrolysis of the epoxide in the acidic (approx. pH 4) culture medium, it must possess the same configuration (*S*) as the intermediate epoxide.

Experimental

General procedures (*e.g.* UV, MS, ^{1}H NMR and $[\alpha]_D$ measurements, silica gel PTLC, and the Gibbs reaction) were carried out using the equipment and conditions described in our earlier papers [3, 17]. Melting points (m.p.) were determined by the micro hot-plate method and are uncorrected. Si gel PTLC was performed using the following two solvent systems: CAAm = $CHCl_3$-acetone-conc. aq. NH_3 (35:30:1), and CM = $CHCl_3$-MeOH (25:1).

Substrate

2,3-Dehydrokievitone (**1**) used in the present investigation was isolated from the roots of yellow lupin (*Lupinus luteus* L. cv. Barpine, or cv. Topaz) as previously reported [5]. **1**: Pale yellow columnar crystals from EtOAc-hexane, m.p. 145–146 °C. Gibbs test: (+), rapid, purple-blue. $UV_{(365\ nm)}$ fluorescence on thin-layer plates: dark purple. MS (rel. int. %): *m/z* 355 (M^+ +1; 23), 354 (M^+; 100), 339 (19), 312 (18), 311 ($M^+ - C_3H_7$; 94), 300 (16), 299 ($M^+ - C_4H_7$; 85), 298 (17), 219 (11), 205 (15), 177 (16), 165 (39), 134 (20), 69 (14). UV: λ_{max} nm: MeOH 219 sh, 265, 285 sh, 335–340 sh; + NaOMe 230 sh, 282, 335 sh; + $AlCl_3$ 225 sh, 275, 317, 376; + NaOAc 279, 330–335 sh (addition of H_3BO_3 regenerated the MeOH spectrum).

Metabolic experiments and the isolation of metabolites DK-M1, DK-M2 and DK-M3

Cultures of *Aspergillus flavus* AHU 7049 and *Botrytis cinerea* AHU 9424 were shaken at 25 °C for 4 days in 500 ml shaking flasks containing a liquid medium composed of glucose (5 g), peptone (1 g), yeast extract (0.1 g) and H_2O (100 ml) [1]. Each flask contained 100 ml of medium. A solution of 2,3-dehydrokievitone (**1**; 5 mg in 1 ml of EtOH) was then added, and after a further 4 days incubation, the metabolic process was halted by addition of acetone (100 ml) to each flask. The contents were filtered by suction and the retained mycelial mass was washed

(× 3) with 10 ml of 50% acetone. After combination, the filtrate and washings were concentrated *in vacuo* (30 °C) to remove the acetone. The concentrate was acidified to pH 3 (2 N HCl) and shaken (× 3) with EtOAc, the pooled EtOAc fractions then being washed first with 5% aq. $NaHCO_3$, and finally with a saturated solution of NaCl. Evaporation of the EtOAc gave an oily residue from which the unchanged substrate (**1**), and the *A. flavus*- or *B. cinerea*-metabolites were isolated by PTLC (see Table I for comparative R_F values). Cultures of both fungi contained the same three metabolites denoted DK-M1 to DK-M3. The concentrated EtOAc extract of each fungus culture was first chromatographed (PTLC) in CAAm to give DK-M1 + DK-M2 (upper band), unchanged substrate (**1**; middle band), and DK-M3 (lower band). After elution with EtOAc, the constituents of the upper band were separated by multi-development (× 3) PTLC in CM (DK-M1 = top zone, and DK-M2 = bottom zone). The substrate (**1**) and metabolite DK-M3 in eluates from the CAAm chromatogram were also purified by further PTLC in CM. The yields of each metabolite, and the amount of **1** recovered from the culture media, are shown in Table I.

Metabolite DK-M1 (**2**)

Pale yellow needles from acetone, m.p. 201–203 °C. Gibbs test: (+), rapid, purple-blue. $UV_{(365\ nm)}$ fluorescence on thin-layer plates, dark purple. $[\alpha]_D^{23}$ −76° ($c = 0.105$, MeOH). UV: λ_{max}, nm: MeOH 221 sh, 265, approx. 300 sh (br.), approx. 330 sh (br.); + NaOMe 264 sh, 277, 320 sh; + $AlCl_3$ 217 sh, 237 sh, 276, 315, 366; + NaOAc, unchanged. MS (rel. int. %): *m/z* 371 (M^+ +1; 34), 370 (M^+; 100), 338 (12), 337 (38), 313 (18), 312 (64), 311 ($M^+ - C_3H_7O$; 93), 237 (14), 203 (19), 179 (28), 178 (20), 177 (33), 176 (20), 165 (13), 150 (22), 149 (14), 135 (16), 134 (26), 77 (11), 69 (18), 65 (11), 59 ($C_3H_7O^+$; 88).

Metabolite DK-M2 (**3**)

Pale yellow needles from acetone, m.p. 185–187 °C. Gibbs test: (+), rapid, purple-blue. $UV_{(365\ nm)}$ fluorescence on thin-layer plates, dark purple. $[\alpha]_D^{23}$ 0° ($c = 0.105$, MeOH). UV: λ_{max}, nm: MeOH 221 sh, 266, 286 sh, 330 sh (br.); + NaOMe 265 sh, 277.5, 320 sh (br.); + $AlCl_3$ 228 sh, 277, 313, 363; + NaOAc, unchanged. MS (rel. int. %): *m/z* 371 (M^+ +1; 24), 370 (M^+; 100), 300 (17), 299 ($M^+ - 71$; 70), 298 (23), 281 (14), 237 (41), 166 (12), 165 (70), 149 (16), 135 (12), 134 (46), 123 (12), 83 (16), 81 (12), 75 (15), 73 (10), 71 (14), 69 (22), 59 (25).

Metabolite DK-M3 (**4**)

Colourless gum. Gibbs test: (+), rapid, purple-blue. $UV_{(365\ nm)}$ fluorescence on thin-layer plates, dark purple. $[\alpha]_D^{23}$ −15° ($c = 0.145$, MeOH). UV: λ_{max}, nm: MeOH 216 sh, 265, 288 sh, approx. 335 sh (br.); + NaOMe 279, 325 sh; + $AlCl_3$ 236 sh, 274.5, 315, 363; + NaOAc, 277, 322 (br.) (addition of H_3BO_3 regenerated the MeOH spectrum). MS (rel. int. %): *m/z* 388 (M^+; 20), 370 ($M^+ - H_2O$; 18), 330 (20), 329 ($M^+ - C_3H_7O$; 64), 300 (34), 299 ($M^+ - C_4H_9O_2$; 100), 298 (12), 281 (12), 167 (12), 165 (48), 134 (15), 123 (12), 69 (11), 59 (15).

CD determination of the osmate ester-pyridine complex of DK-M3

Dry DK-M3 (**4**, 1.5 μmol) was dissolved in a mixture of CH_2Cl_2 (73 μl) and pyridine (30 μmol) containing OsO_4 (1.5 μmol) [18]. After being kept at 23 °C for 40 min, the reaction mixture was diluted with more CH_2Cl_2 to give a final volume of 2.8 ml. The CD spectrum of this solution was recorded at 23 °C over the range 350–650 nm using a Model J-20A Automatic Recording Spectropolarimeter (Japan Spectroscopic Co. Ltd.): $[\theta]_{380\ nm}$ 0, $[\theta]_{480\ nm}$ −380 and $[\theta]_{610\ nm}$ 0.

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[1] S. Tahara, S. Nakahara, J. Mizutani, and J. L. Ingham, Agric. Biol. Chem. **48,** 1471 (1984).
[2] S. Tahara, S. Nakahara, J. L. Ingham, and J. Mizutani, Nippon Nōgeikagaku Kaishi **59,** 1039 (1985).
[3] S. Tahara, S. Nakahara, J. Mizutani, and J. L. Ingham, Agric. Biol. Chem. **49,** 2605 (1985).
[4] S. Nakahara, S. Tahara, J. Mizutani, and J. L. Ingham, Agric. Biol. Chem. **50,** 863 (1986).
[5] Y. Hashidoko, S. Tahara, and J. Mizutani, Agric. Biol. Chem. **50,** 1797 (1986).
[6] M. D. Woodward, Phytochemistry **18,** 2007 (1979).
[7] M. J. O'Neill, S. A. Adesanya, M. F. Roberts, and I. R. Pantry, Phytochemistry **25,** 1315 (1986).
[8] T. J. Mabry, K. R. Markham, and M. B. Thomas, The Systematic Identification of Flavonoids, p. 169, Springer, Berlin 1970.
[9] K. R. Markham and T. J. Mabry, The Flavonoids (J. B. Harborne, T. J. Mabry, and H. Mabry, eds.), p. 45, Chapman and Hall, London 1975.
[10] T. J. Mabry and K. R. Markham, The Flavonoids (J. B. Harborne, T. J. Mabry, and H. Mabry, eds.), p. 97, Chapman and Hall, London 1975.
[11] S. Tahara, J. L. Ingham, and J. Mizutani, Agric. Biol. Chem. **51,** 211 (1987).
[12] S. A. Adesanya, M. J. O'Neill, and M. F. Roberts, Z. Naturforsch. **39c,** 888 (1984).
[13] P. J. Kuhn and D. A. Smith, Physiol. Plant Path. **14,** 179 (1979).
[14] N. Sakota, S. Tanaka, K. Okita, and N. Koine, Nippon Kagaku Zasshi **91,** 265 (1970).
[15] B. E. Nielsen and J. Lemmich, Acta Chem. Scand. **18,** 1379 (1964).
[16] B. E. Nielsen and J. Lemmich. Acta Chem. Scand. **23,** 962 (1969).
[17] S. Tahara, J. L. Ingham, S. Nakahara, J. Mizutani, and J. B. Harborne, Phytochemistry **23,** 1889 (1984).
[18] J. L. Ingham, N. T. Keen, K. R. Markham, and L. J. Mulheirn, Phytochemistry **20,** 807 (1981).