Metabolism of the Prenylated Pterocarpan Edunol by *Aspergillus flavus*

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Z. Naturforsch. **42c,** 1050–1054 (1987); received June 10, 1986

Isoflavonoid, Prenylated pterocarpan, Edunol, Fungal metabolism, *Aspergillus flavus*

When incubated in liquid culture with *Aspergillus flavus,* the prenylated pterocarpan (−)-edunol [2-(3,3-dimethylallyl)-3-hydroxy-8,9-methylenedioxypterocarpan (**1**)] was converted into a dihydrofurano-pterocarpan (**2**), a dihydropyrano-pterocarpan (**3**), and a 2,3-dihydro-dihydroxyprenyl-substituted pterocarpan (**4**).

Introduction

In our previous papers, we reported that isoflavones with a 3,3-dimethylallyl (prenyl) substituent at C-6, C-8 (ring A) or C-3′ (ring B) were variously metabolized by the fungus *Aspergillus flavus* to give hydrates (luteone [1] and wighteone [2]), and derivatives possessing dihydrofurano, dihydropyrano or 2,3-dihydrodihydroxyprenyl side-attachments (luteone [1], wighteone [2], 2,3-dehydrokievitone [3], licoisoflavone A [4], and 2′-hydroxylupalbigenin [5]). Studies involving the plant pathogenic fungus *Fusarium oxysporum* f. sp. *phaseoli* have shown that two other prenylated isoflavonoids, kievitone [5,7,2′,4′-tetrahydroxy-8-(3,3-dimethylallyl)isoflavanone] and phaseollidin [3,9-dihydroxy-10-(3,3-dimethylallyl)pterocarpan] are also metabolized *in vitro* to give the corresponding hydrates [6, 7]. In kievitone and phaseollidin, the prenyl groups are located on different aromatic rings, and their hydration by *F. oxysporum* f. sp. *phaseoli* suggests that the enzyme responsible (kievitone hydratase [8]) is relatively non-specific in its action, or the fungus contains two hydratases differing in the substrate specificity [9].

In contrast, *A. flavus* exhibits a higher degree of substrate preference bringing about hydration of prenyl groups at C-6 on ring A of isoflavones (luteone and wighteone [1, 2], but not those located at C-8 (also ring A; 2,3-dehydrokievitone [3]) or C-3′ (ring B; licoisoflavone A and 2′-hydroxylupalbigenin [4, 5]). The present study involving (−)-edunol [2-(3,3-dimethylallyl)-3-hydroxy-8,9-methylenedioxypterocarpan (**1**)], a fungitoxic [10] isoflavonoid from the root bark of *Neorautanenia edulis* (Leguminosae) [11], was undertaken to determine if *A. flavus* could: a) metabolize an isoflavonoid of a type different from the isoflavones previously tested, and b) convert the prenyl group at C-2 (≡ C-6 of isoflavones) into a hydrated sidechain analogous with that encountered in the hydrates of luteone and wighteone [1, 2].

Results and Discussion

When shaken for 4 days (25 °C) in a liquid medium with *A. flavus,* (−)-edunol (**1**) was gradually metabolized to give three laevorotatory products designated ED-AF-1, ED-AF-2 (both M^+ 368), and ED-AF-3 (M^+ 386). These compounds, together with unchanged edunol (M^+ 352), were extracted from the medium with EtOAc, and then were separated by preparative Si gel TLC (PTLC) as outlined in the Experimental section. Their characterization as pterocarpans **2**–**4** is described in this report. Yields and comparative R_F values for each metabolite, and for the substrate, are shown in Table I. Surprisingly, no evidence was obtained to indicate that edunol could be converted to a hydrate derivative (expected M^+ 370) despite the fact that *A. flavus* has been found to readily hydrate the isoflavones luteone and wighteone [1, 2], each of which is prenylated at the position (C-6) equivalent to C-2 in **1**.

The UV (MeOH) spectrum of metabolite ED-AF-1 (M^+ 368; = substrate + [O]) was unaffected by NaOMe indicating the absence of a phenolic OH

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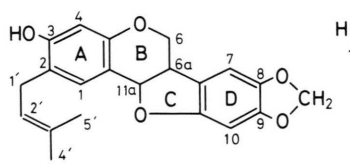
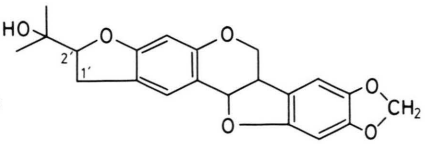
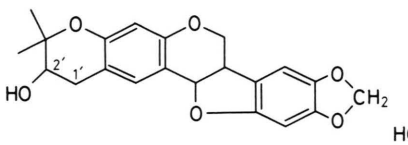
0341–0382/87/0900–1050 $ 01.30/0







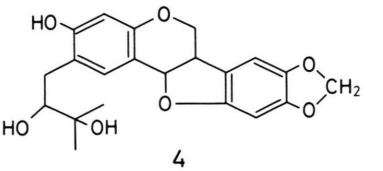


Table I. Chromatographic properties and yields of edunol and its metabolites.

Compound	R_F (TLC) value[a]		Yield[b]	
	CM (25:1)	CAAm (35:30:1)	[mg]	[%]
Edunol (**1**)	0.86	0.82	7.4	24.7
ED-AF-1 (**2**)	0.85	0.78	3.0	9.6
ED-AF-2 (**3**)	0.80	0.78	8.4	26.8
ED-AF-3 (**4**)	0.26	0.27	5.9	17.9

[a] Solvent system abbrv., CM = $CHCl_3$-MeOH; CAAm = $CHCl_3$-acetone-conc. aqueous NH_3.
[b] Yield in mg from 30 mg of substrate (**1**); % yield is on a molar basis.

group (*cf.* the alkaline spectrum of **1** with a C-3 hydroxyl substituent). This observation, and the difference of 16 atomic mass units between the molecular weight of **1** and ED-AF-1, can be explained by the fungal-mediated formation of an ether ring side-attachment involving the substrate prenyl (C-2) and the *ortho* (C-3)-located OH group.

In the ^{1}H NMR spectrum of ED-AF-1, this side-attachment afforded a set of signals [δ 1.21 and 1.24 (both 3H, two s, 4′- and 5′-H_3), 3.07 and 3.26 (both 1H, two dd, J = 16.3 & 9.3 Hz, and 16.3 & 8.3 Hz, 1′-H_a and 1′-H_b), and 4.64 (1H, dd, J = 9.8 & 8.3 Hz, 2′-H)] with chemical shift values closely resembling those given by the 2-(1-hydroxy-1-methylethyl)-2,3-dihydrofuran substituent previously found in several isoflavones including luteone metabolite BC-1 [1] and lupinisoflavones B-F [12]. Prominent MS fragments at *m/z* 309 (M^+ −59; 11%) and 59 (24%) were also consistent with the presence of such a side-attachment [1]. ^{1}H NMR signals attributable to the aromatic (A/D) and heterocyclic (B/C) ring protons, and the O-C$\underline{H}_2$-O group, of edunol (Table II) were similarly evident in the spectrum of ED-AF-1, thereby permitting the metabolite to be formulated as shown in **2**. This structure has already been assigned to "neoplanol", a racemic dihydrofurano-pterocarpan produced by chemical modification of edunol [13], but not as yet reported to be a natural product. MS and ^{1}H NMR data obtained for ED-AF-1 were in good agreement with those published [13] for synthetic "neoplanol" (see Table II, and the Experimental section for comparative details).

The ^{1}H NMR spectrum of ED-AF-2 (M^+ 368; = substrate + [O]) clearly revealed that this major, non-phenolic *Aspergillus* metabolite differed from **2** only with respect to the nature of the A-ring side-attachment (Table II). Thus, instead of a dihydrofurano substituent, a set of aliphatic proton signals at δ 1.25 and 1.33 (both 3H, two s, 4′- and 5′-$\underline{H}_3$), 2.62 and 3.01 (both 1H, two dd, J = 16.5 & 7.3 Hz, and 16.5 & 5.4 Hz, 1′-H_a and 1′-H_b) and 3.79 (1H, ddd, J = 7.3, 5.4 & 5.1 Hz, 2′-H coupled to 2′-O$\underline{H}$ with J = 5.1 Hz) defined the presence of a 2,3-dihydro-3-hydroxy-2,2-dimethylpyrano side-attachment as in luteone metabolite BC-2 [1], 2,3-dehydrokievitone metabolite DK-M2 [3], and licoisoflavone A metabolites M-1-1 and M-3-1 [4]. As with these four metabolites, the MS of ED-AF-2 afforded a characteristic fragment at M^+ −71 (*m/z* 297; 98%) [1, 3, 4]. Metabolite ED-AF-2 must therefore have structure **3**. Neorautanol from *Neorautanenia amboensis* has also been formulated as **3**. MS and ^{1}H NMR data

Table II. ^{1}H NMR data (δ values) for edunol and its *Aspergillus flavus* metabolites[a]

Compound Proton	Edunol (**1**)	ED-AF-1 (**2**)[b]	ED-AF-2 (**3**)[b]	ED-AF-3 (**4**)
1-H	7.16 s	7.22 s	7.15 s	7.22 s
4-H	6.38 s	6.21 s	6.23 s	6.34 s
7-H	6.88 s	6.89 s	6.90 s	6.89 s
10-H	6.40 s	6.40 s	6.39 s	6.40 s
6-H_{eq}	4.24 m	4.27 m	4.26 m	4.27 m
6-H_{ax} 6a-H	3.55 m (2H)	3.58 m (2H)	3.60 m (2H)	3.57 m (2H)
11a-H	5.46 br. d $J=6.4$	5.50 br. d $J=6.4$	5.49 br. d $J=6.1$	5.47 br. d $J=6.1$
O-CH_2-O	5.90 d $J=1.0$ 5.93 d $J=1.0$	5.91 d $J=1.0$ 5.94 d $J=1.0$	5.91 d $J=1.0$ 5.94 d $J=1.0$	5.91 d $J=1.0$ 5.93 d $J=1.0$
1′-H_a	3.29 br. d (2H) $J=7.3$	3.07 dd $J=16.3, 9.3$	2.69 dd $J=16.6, 7.3$	2.62 dd $J=14.5, 9.5$
1′-H_b		3.26 dd $J=16.3, 8.3$	3.01 dd $J=16.6, 5.4$	2.93 dd $J=14.5, 2.3$
2′-H	5.35 br. t $J=7.3$	4.64 dd $J=9.3, 8.3$	3.79 ddd $J=7.3, 5.4, 5.1$	3.64 dd $J=9.4, 2.3$
4′-CH_3	1.73 s (3H)	1.21 s (3H)	1.25 s (3H)	1.25 s (3H)
5′-CH_3	1.74 s (3H)	1.24 s (3H)	1.33 s (3H)	1.27 s (3H)
2′-OH	–	–	4.23 d $J=5.1$	–
3′-OH	–	3.62 s	–	–

[a] All spectra were determined in acetone-d_6 at 100 MHz (TMS reference). Coupling constants (*J*) are in Hz. For multiplets, the δ value indicates the centre of the signal.

[b] ^{1}H NMR chemical shift values for synthetic "neoplanol" (= **2**) and natural neorautanol (= **3**) in $CDCl_3$ (80 MHz) are reported in ref. [13].

obtained for ED-AF-2 agreed reasonably with those of neorautanol [13].

The third metabolite, ED-AF-3 (M^+ 386; = substrate + 2 × [OH]), ran well below **2** and **3** on thin-layer chromatograms developed in both CM and CAAm (Table I). This increased polarity relative to the other *Aspergillus* metabolites, coupled with the detection of an intense MS ion at $M^+ - 89$ (*m/z* 297; 100%), suggested that ED-AF-3 contained a 2,3-dihydrodihydroxyprenyl side-chain [1, 3, 4]. An underivatized C-3 OH group was apparent from the UV (MeOH + NaOMe) maximum at 251 nm [14].

Structure **4** for ED-AF-3 was confirmed by the ^{1}H NMR spectrum which exhibited signals typical of a 1,2-glycol type side-chain at δ 1.25 and 1.27 (both 3H, two s, 4′- and 5′-H_3), 2.62 and 2.93 (both 1H, two dd, $J=14.5$ & 9.4 Hz, and 14.5 & 2.3 Hz, 1′-H_a and 1′-H_b) and 3.64 (1H, dd, $J=9.4$ & 2.3 Hz, 2′-H). In all other respects, the ^{1}H NMR spectrum closely resembled that of edunol (Table II). Apart from its appearance in ED-AF-3, the 2,3-dihydrodihydroxyprenyl (2,3-dihydroxy-3-methylbutyl) side-chain has also been encountered in luteone glycol [1], wighteone glycol [2], 2,3-dehydrokievitone glycol [3] and

licoisoflavone A glycol [4], all of which are produced by *A. flavus* from the corresponding isoflavone substrates.

Experimental

General procedures (*e.g.* silica gel PTLC, and m.p., UV, MS and ^{1}H NMR measurements) were undertaken using the equipment and conditions previously described [4, 12]. The substrate pterocarpan (**1**) was isolated from the shredded root bark of *Neorautanenia edulis* [11].

Edunol (**1**) *[2-(3,3-dimethylallyl)-3-hydroxy-8,9-methylenedioxypterocarpan]*

Colourless needles, m.p. 151–152 °C; $[\alpha]_D^{23}$ −325° (c = 0.088, MeOH). UV: λ_{max}, nm: MeOH 232 sh, 288 sh, 294, 310; + NaOMe, 251, 304. MS (rel. int. %): *m/z* 353 (M^+ +1; 25), 352 (M^+; 100), 298 (14), 297 (M^+ −55; 71), 296 (31), 176 (12), 175 (21), 148 (16), 147 (13), 71 (12), 69 (12), 57 (21).

Metabolic experiments

Aspergillus flavus (isolate AHU 7049) was cultured for 4 days in a shaking liquid medium consisting of glucose (5 g), peptone (1 g), yeast extract (0.1 g) and H_2O (100 ml). A solution of **1** (5 mg in 1 ml of EtOH) was then added, and after a further 4 days incubation the metabolites, and any remaining substrate, were extracted from the medium with EtOAc (see ref. [1] for exact details).

Isolation and purification of edunol metabolites

An EtOAc extract of the combined *Aspergillus* culture medium (600 ml, initially containing 30 mg of **1**) from 6 flasks was washed with 5% aqueous $NaHCO_3$, and then with a saturated solution of NaCl. After removal of the EtOAc *in vacuo* (40 °C), the residue was chromatographed (Si gel preparative TLC) in $CHCl_3$-acetone-conc. aqueous NH_3 (CAAm; 35:30:1) to afford unchanged edunol (upper band), a mixture of ED-AF-1 and ED-AF-2 (middle band), and ED-AF-3 (lower band). See Table I for R_F values. After elution with EtOAc, edunol and ED-AF-3 were rechromatographed in $CHCl_3$-MeOH (CM; 25:1) to give the pure pterocarpans. After concentration, the eluate of the middle band (CAAm chromatogram) deposited colourless plates of ED-AF-2. The mother liquor was then chromatographed (Si gel PTLC in CM, × 3) to afford ED-AF-1 (upper zone) and a further quantity of ED-AF-2 (lower zone). The yields of each metabolite are given in Table I.

Metabolite ED-AF-1 (**2**; = *"neopranol"* [13])

Colourless fine rods, m.p. 204–205 °C (subliming at >200 °C); $[\alpha]_D^{23}$ − 340° (c = 0.096, MeOH). UV: λ_{max}, nm: MeOH 232 sh, 291 sh, 296 sh, 302, 308 sh; + NaOMe, no change. MS (rel. int. %): *m/z* 369 (M^+ +1; 24), 368 (M^+; 100), 335 (16), 310 (18), 309 (M^+ −59, 11), 297 (27), 296 (12), 175 (18), 162 (25), 160 (13), 147 (13), 71 (16), 59 (24), 57 (29). MS data published for synthetic "neopranol" [13] are: *m/z* 368 (M^+; 100), 353 (2), 335 (14), 310 (16), 297 (25), 175 (19), 162 (29), 160 (10), 151 (12), 148 (15).

Metabolite ED-AF-2 (**3**; = *neorautanol* [13])

Colourless plates, m.p. 207–209 °C (subliming at >195 °C; neorautanol [13], m.p. 93–95 °C); $[\alpha]_D^{23}$ − 271° (c = 0.136, MeOH). UV: λ_{max}, nm: MeOH 232 sh, 285 sh, 289 sh, 295, 310; + NaOMe, no change. MS (rel. int. %): *m/z* 369 (M^+ +1; 24), 368 (M^+; 100), 335 (8), 298 (22), 297 (M^+ −71; 98), 296 (27), 267 (9), 175 (9), 162 (17), 85 (8), 71 (16), 69 (12), 57 (27).

Metabolite ED-AF-3 (**4**)

Colourless fine rods, m.p. 195–197 °C (subliming at >185 °C); $[\alpha]_D^{23}$ − 277° (c = 0.188, MeOH). UV: λ_{max}, nm: MeOH 227 sh, 281 sh, 287 sh, 291.5, 310.5; + NaOMe, 251, 303.5. MS (rel. int. %): *m/z* 387 (M^+ +1; 22), 386 (M^+; 92), 368 (M^+ −18; 34), 298 (23), 297 (M^+ −89; 100), 296 (20), 175 (11), 162 (15), 151 (14), 85 (11), 71 (21), 69 (16), 59 (13), 57 (38).

Acknowledgements

We thank Professor S. Takao for kindly supplying the *Aspergillus* culture, and Miss S. Endo (^{1}H NMR) and Mr. K. Watanabe and Miss Y. Atsuta (MS) for spectroscopic measurements. Financial support (to S. T.) by a grant for scientific research (No. 61560130) from the Ministry of Education, Science and Culture of Japan is also gratefully acknowledged.

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