Isolation and Identification of Isoflavanone Phytoalexins from Leaflets of *Diphysa robinioides*

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Z. Naturforsch. **38 c**, 899–904 (1983); received July 8, 1983

Leguminosae, Papilionoideae, *Diphysa*, Isoflavonoids, Isoflavanones

A major phytoalexin produced by the fungus-inoculated leaflets of *Diphysa robinioides* has been identified as the new isoflavonoid 5,7,2′,4′-tetrahydroxy-6-(3,3-dimethylallyl)isoflavanone (diphysolone). It is accompanied by smaller quantities of diphysolone-4′ (or 2′)-O-methyl ether (diphysolidone), and the known isoflavonoids 5,7,2′,4′-tetrahydroxy-8-(3,3-dimethylallyl)isoflavanone (kievitone) and 5,7,2′-trihydroxy-4′-methoxyisoflavanone (ferreirin).

Introduction

Over 10 isoflavanone phytoalexins [1, 2] have now been variously obtained from the fungus-inoculated tissues of species belonging to the subfamily Papilionoideae of the Leguminosae. Of these compounds, however, only kievitone **1** [1, 2] and 5-deoxykievitone [3] are known to possess an uncyclised, isoprene-derived (3,3-dimethylallyl) attachment. Apart from 1″,2″-dehydrocyclokievitone [3], all the remainder are simple tri-(7,2′,4′) or tetra-(5,7,2′,4′) oxygenated derivatives containing only OH, or OH and OCH_3, substituents. As part of a systematic survey of phytoalexin formation in the Papilionoideae, we recently examined the response of *Diphysa robinioides* Benth. (tribe Robinieae [4]) to inoculation with the non-pathogenic fungus *Helminthosporium carbonum* Ullstrup. Studies involving other genera of the Robinieae (*Hebestigma*, *Gliricidia*, *Glottidium*, *Robinia* and *Sesbania*) have revealed the widespread formation of both pterocarpan (*e.g.* medicarpin) and isoflavan (*e.g.* vestitol) phytoalexins [5], but *D. robinioides* reacted in an entirely unexpected fashion. Thus, instead of pterocarpans and isoflavans, the leaflets of this shrubby Central American legume produced several isoflavanone phytoalexins including 5,7,2′,4′-tetrahydroxy-8-(3,3-dimethylallyl)isoflavanone (kievitone, **1**) and two previously unrecognised compounds (diphysolone and diphysolidone) each containing a 3,3-dimethylallyl substituent. This paper describes the identification of diphysolone as 5,7,2′,4′-tetrahydroxy-6-(3,3-dimethylallyl)isoflavanone (**2**), and presents evidence to suggest that diphysolidone is probably the corresponding 4′-O-methyl ether (**3**).

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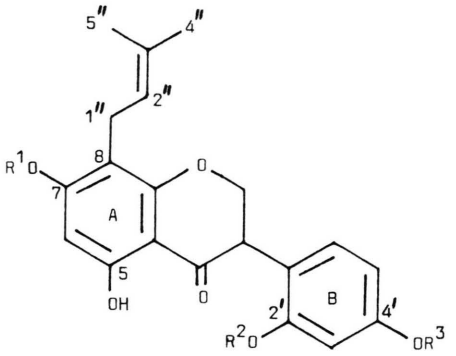
0341-0382/83/1100-0899 $ 01.30/0

Results and Discussion

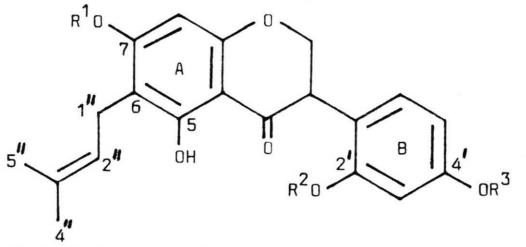
Excised leaflets of *D. robinioides* were inoculated with a spore suspension of *H. carbonum* and the resulting diffusate collected after 48 h incubation [1, 6–8]. Si gel TLC (see Experimental for further details) of the diffusate extract (EtOAc [8]) gave diphysolone (**2**) as the principal isoflavanone, this being accompanied by smaller amounts of diphysolidone (**3**), kievitone (**1**) and 5,7,2′-trihydroxy-4′-methoxyisoflavanone (ferreirin, **4**). Compounds **1** and **4** were identified by direct UV and TLC comparison with authentic material obtained respectively from leaves and stems of *Dolichos biflorus* [9], and heartwood of *Ferreirea spectabilis* [10]. All four isoflavanones proved to be highly fungitoxic when bioassayed (applied level, approx. 20 µg) on Si gel TLC plates [11, 12] against growth of *Cladosporium herbarum* Fr. No antifungal material was ever found in diffusates from *Diphysa* leaflets treated with de-ionised H_2O.

On TLC plates viewed under long wavelength UV light, diphysolone ($[M]^+$ 356) exhibited a deep blue-black fluorescence quite unlike kievitone and



1: $R^1 = R^2 = R^3 = H$

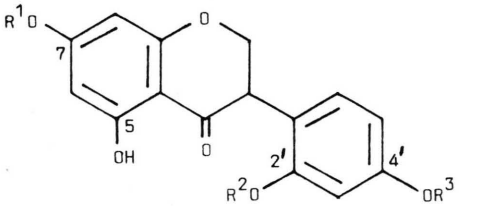
6: $R^1 = R^2 = R^3 = CH_3$



2: $R^1 = R^2 = R^3 = H$

3: $R^1 = R^2 = H$; $R^3 = CH_3$

5: $R^1 = R^2 = R^3 = CH_3$



4: $R^1 = R^2 = H$; $R^3 = CH_3$

7: $R^1 = R^2 = CH_3$; $R^3 = H$

ferreirin which appeared as faint orange-brown fluorescent bands. The UV (MeOH) spectrum of diphysolone (λ_{max}: 293 nm) resembled that of kievitone, and large (38–39 nm) bathochromic shifts were obtained upon addition of NaOH (aromatic OH group) and NaOAc (C-7 OH group [13]). In contrast, $AlCl_3$ had no immediate spectroscopic effect although the maximum at 293 nm shifted bathochromically by 21 nm over a period of 30–40 min, an observation consistent with hydroxylation at C-5 [13]. The *Sophora* isoflavanone, isosophoranone, also reacts very slowly with $AlCl_3$, an effect which Delle Monache *et al.* [14] attribute to the influence of a 3,3-dimethylallyl unit located *ortho* (C-6) to the C-5 hydroxyl group.

As shown in Table I, the ^1H NMR spectrum of diphysolone (acetone-d_6) contained signals at δ1.64, 1.75 (both 3H), 5.25 (1H) and 3.25 (2H) characteristic of a 3,3-dimethylallyl sidechain [15, 16]. In addition, the H-bonded C-5 OH was readily apparent (δ12.67) as also was an ABX system of aromatic protons (δ6.45, 6.33 and 6.94), these being assigned respectively to C-3′, 5′ and 6′ of ring B. Identical, or very closely coincident, chemical shift values in acetone-d_6 have likewise been reported for the B-ring protons of **1**, dalbergioidin (5,7,2′,4′-tetrahydroxyisoflavanone), 5-deoxykievitone and 1″,2″-dehydrocyclokievitone [3, 17]. Finally, the heterocyclic ring protons H-2 (2H) and H-3 (1H) of **2** were evident as a complex series of signals (δ4.21–4.60) in good agreement with corresponding chemical shifts noted for kievitone (δ4.15–4.62 in acetone-d_6 [3, 5]) and related isoflavanones [3]. Since diphysolone can easily be distinguished from kievitone by its long wavelength UV fluorescence and behaviour on Si gel TLC plates developed in Et_2O-*n*-hexane, 3:1 (diphysolone, R_F 0.31; kievitone, R_F 0.27) or $CHCl_3$-MeOH, 20:1 (diphysolone, R_F 0.18; kievitone, R_F 0.13), it follows that the alkyl sidechain and the remaining aromatic proton (δ6.03) must be located at C-6 and C-8 respectively. These assignments are supported by the observation that diphysolone trimethyl ether **5** ($[M]^+$ 398) affords a blue colour with Gibbs reagent [12, 18] whereas the isomeric kievitone trimethyl ether **6** does not [19]. Diphysolone is thus 5,7,2′,4′-tetrahydroxy-6-(3,3-dimethylallyl)isoflavanone (**2**).

Apart from its occurrence in *D. robinioides*, recent studies have revealed that diphysolone is also produced by the *H. carbonum*-inoculated leaflets of certain other papilionate legumes including the shrubby Old World aeschynomenoid species *Ormocarpum kirkii* and *O. trichocarpum* [5]. The discovery that diphysolone acts as a phytoalexin in two *Ormocarpum* species is of some taxonomic interest because *Diphysa* is considered to have a particularly close affinity with the woody genera of tribe Aeschynomeneae [4]. Indeed, when compared with

Table I. ^{1}H NMR data for diphysolone (**2**), diphysolidone (**3**), ferreirin (**4**) and cajanol (**7**)[a,b].

Proton	Diphysolone	Diphysolidone	Ferreirin	Cajanol
H-2 (2H)	4.43 dd (J = 10.8, 5.4) 4.57 t (J = 10.8)	4.45 dd (J = 10.7, 5.5) 4.59 br t (J = *ca* 10.5)	4.46 dd (J = 11.2, 5.1) 4.58 dd (J = 11.2, 9.1)	4.44 dd (J = 11.2, 5.4) 4.55 t (J = 11.2)
H-3 (1H)	4.23 dd (J = 10.8, 5.4)	4.24 dd (J = 10.3, 5.5)	4.18 dd (J = 9.1, 5.1)	4.28 dd (J = 11.2, 5.4)
H-6 (1H)	–	–	both 5.88 s (2H)	6.03 d (J = 2.4)
H-8 (1H)	6.03 s	6.02 s		6.06 d (J = 2.4)
H-3′ (1H)	6.45 d (J = 2.4)	6.49 d (J = 2.4)	6.49 d (J = 2.7)	6.53 d (J = 2.4)
H-5′ (1H)	6.33 dd (J = 2.4, 8.3)	6.43 dd (J = 2.4, 8.3)	6.41 dd (J = 2.7, 8.5)	6.42 dd (J = 2.4, 8.1)
H-6′ (1H)	6.94 d (J = 8.3)	7.06 d (J = 8.3)	7.07 d (J = 8.5)	6.97 d (J = 8.1)
H-1″ (2H)	3.25 d (J = 6.8)	3.25 d (J = 6.8)	–	–
H-2″ (1H)	5.25 br t (J = *ca* 7.0)	5.25 br t (J = *ca* 7.0)	–	–
4″-CH_3 (3H)	1.64 s	1.64 s	–	–
5″-CH_3 (3H)	1.75 s	1.75 s	–	–
4′-OCH_3 (3H)	–	3.73 s	3.72 s	–
7-OCH_3, 2′-OCH_3 (both 3H)	–	–	–	3.76 s/3.86 s
5-OH (1H)	12.67 s	12.62 s	12.25 br s	12.35 br s

[a] All spectra were determined in acetone-d_6 at 400 MHz. Chemical shifts are expressed as δ values (TMS reference). Data in parentheses refer to coupling constants in Hz.
[b] Authentic samples of ferreirin and cajanol were obtained from *Ferreirea spectabilis* [10] and *Cajanus cajan* [20] respectively.

other Robinieae, the distinctly different phytoalexin response of *D. robinioides* (see Table II for a summary of available data) suggests that the systematic position of *Diphysa* as a whole may need to be reassessed, and one course of action could involve its incorporation into the Aeschynomeneae [4].

Upon methylation with CH_2N_2, the second new *Diphysa* phytoalexin (diphysolidone, **3**; $[M]^+$ 370) yielded a phenolic dimethyl ether indistinguishable (UV; MS; TLC; colour with diazotised *p*-nitroaniline and Gibbs reagent) from the trimethyl ether (**5**) of isoflavanone **2**. As well as a 3,3-dimethylallyl group, assigned to C-6 by analogy with **2**, and all the expected aromatic and heterocyclic ring protons, the ^{1}H NMR spectrum of diphysolidone (Table I) also revealed a three-proton methoxyl singlet at δ3.73. The marked influence of this substituent on the B-ring protons was evident from their downfield shifts of 0.04 ppm (H-3′), 0.10 ppm (H-5′) and 0.12 ppm (H-6′) when compared with diphysolone (Table I). Provisional location of the OCH_3 group at C-4′ instead of C-2′ follows from the fact that diphysolidone and ferreirin (**4**) occur together as phytoalexins in *D. robinioides* and may thus be biosynthetically related (**4** → **3**), and also because chemical shift values recorded for the B-ring protons of both these isoflavanones are essentially identical (*cf.* δ values given in Table I for H-3′ and particularly H-6′ of the 2′-OCH_3/4′-OH substituted isoflavanone, cajanol **7**). As bathochromic UV shifts were obtained with NaOAc (C-7 OH) and $AlCl_3$ (C-5 OH), the structure of diphysolidone would appear to be 5,7,2′-trihydroxy-4′-methoxy-6-(3,3-di-

Table II. Isoflavonoid phytoalexins associated with 6 genera of the tribe Robinieae[a].

Genus	Phytoalexin								
	Isoflavanone				Isoflavan[b]			Pterocarpan[b]	
	Kievitone (1)	Diphysolone (2)	Diphysolidone (3)	Ferreirin (4)	Vestitol	Isovestitol	Sativan	Medicarpin	Maackiain
Diphysa (1 sp.)[c]	+	+	+	(+)	−	−	−	−	−
Gliricidia (1 sp.)	−	−	−	−	+	−	+	−	−
Glottidium (1 sp.)	−	−	−	−	+	−	−	+	(+)
Hebestigma (1 sp.)	−	−	−	−	−	+	−	−	−
Robinia (6 spp.)	−	−	−	−	+	+	+	+	−
Sesbania (15 spp.)	−	−	−	−	+	−	−	+	(+)

Key: + = Present; − = Absent; (+) = Detected only as a minor phytoalexin.

[a] As constituted by Polhill [4].
[b] *Vestitol* = 7,2′-dihydroxy-4′-methoxyisoflavan; *Isovestitol* = 7,4′-dihydroxy-2′-methoxyisoflavan; *Sativan* = 7-hydroxy-2′,4′-dimethoxyisoflavan; *Medicarpin* = 3-hydroxy-9-methoxypterocarpan; *Maackiain* = 3-hydroxy-8,9-methylenedioxypterocarpan.
[c] Number of species currently examined in each genus.

methylallyl)isoflavanone (**3**) although the alternative 2′-methoxy-4′-hydroxy arrangement of ring B cannot be definitely excluded from the available data.

After approx. 48 h incubation, diffusates from *H. carbonum*-treated *Diphysa* leaflets typically contained isoflavanones **1**–**4** at the following concentrations: diphysolone 40 µg/ml, diphysolidone 17 µg/ml, kievitone 9 µg/ml (all values were determined spectrophotometrically using $\log \varepsilon = 4.22$ at 293 nm for **1** [19]) and ferreirin 4 µg/ml (based on $\log \varepsilon = 4.31$ at 288 nm for 5,7,2′,4′-tetrahydroxyisoflavanone [17]). In leaf tissues immediately beneath the inoculum droplets, diphysolone, diphysolidone, kievitone and ferreirin accumulated to levels of about 450, 120, 80 and 25 µg/g fr. wt. respectively.

Experimental

Plant material

Seeds of *Diphysa robinioides* Benth. (originally obtained from Costa Rica, and held in the B. A. Krukoff legume seed collection at the Royal Botanic Gardens, Kew, England) were kindly supplied by Mr. G. P. Lewis. They were sown in John Innes No. 1 compost and the resulting plants maintained as reported elsewhere [15]. Leaflets for phytoalexin induction experiments were first removed when the plants were 12 weeks old, and further material was collected at 6–8 week intervals thereafter.

Induction, extraction and purification of Diphysa phytoalexins

In a typical experiment, between 400 and 500 excised *Diphysa* leaflets were each inoculated with 1 or 2 drops (approx. 30 µl) of an *H. carbonum* spore suspension [1, 6–8] and incubated as previously described [8] for 48 h. The diffusate (approx. 10–12 ml [1, 6]) was then collected and extracted (×3) with EtOAc. After removing the EtOAc *in vacuo* (40°), the residue was chromatographed (TLC; Merck Si gel 60, F-254, layer thickness 0.25 mm) in $CHCl_3$–MeOH (20:1) to yield diphysolidone **3** (R_F 0.62), ferreirin **4** (R_F 0.39), diphysolone **2** (R_F 0.18) and kievitone **1** (R_F 0.13) as well separated bands. All four isoflavanones were eluted (MeOH) and additionally chromatographed in either *n*-pentane-Et_2O-glacial HOAc (PEA), 75:25:3 (compounds **3**, R_F 0.26 and **4**, R_F 0.15) or PEA 75:25:6, ×3 (compounds **1** and **2**). Isoflavanones **1**–**4** did not accumulate when *Diphysa* leaflets were treated with droplets of de-ionised H_2O. Leaf tissues immediately beneath the *H. carbonum* inoculation droplets were also excised (No. 1 cork

borer) and exhaustively extracted with EtOH [20]. Si gel TLC ($CHCl_3$–MeOH, 100:8) of the extract gave **1** (R_F 0.30), **2** (R_F 0.34), **3** (R_F 0.71) and **4** (R_F 0.52) which were eluted with MeOH and purified using the PEA solvent systems referred to above.

*5,7,2',4'-tetrahydroxy-8-(3,3-dimethylallyl)isoflavanone (**1**) (kievitone)*

Colour with diazotised *p*-nitroaniline reagent, orange; colour with Gibbs reagent, immediate purple-blue. Fluorescence on Si gel TLC plates viewed under long wavelength UV light, orange-brown. UV: λ_{max}, nm: MeOH 293, 346 sh; + NaOH 332; + NaOAc 332 (addition of solid boric acid regenerated the MeOH spectrum); + $AlCl_3$ 313 (shift observed immediately).

*5,7,2',4'-tetrahydroxy-6-(3,3-dimethylallyl)isoflavanone (**2**) (diphysolone)*

Colour with diazotised *p*-nitroaniline reagent, orange; colour with Gibbs reagent, immediate purple-blue. Long wavelength UV fluorescence, blue-black (*cf.* kievitone, **1**). UV: λ_{max}, nm: MeOH 208, 232 sh, 293, 346 sh; + NaOH 331; + NaOAc 332 (addition of solid boric acid regenerated the MeOH spectrum); + $AlCl_3$ 314 (shift occurred gradually over a 30–40 min period). MS: $[M]^+$ 356 (12), *m/z* 221 (18; A-ring derived fragment), 194 (36), 179 (26), 165 (34), 139 (100), 136 (10; B-ring derived fragment). ^{1}H NMR: see Table I. *Trimethyl ether* (**5**) (CH_2N_2; R_F 0.86 in $CHCl_3$–CCl_4, 3:1). Colour with diazotised *p*-nitroaniline reagent, pale orange; colour with Gibbs reagent, blue (colour intensifies upon warming with a hair-dryer). UV: λ_{max}, nm: MeOH 208, 232 sh, 290, 342 sh. MS: $[M]^+$ 398 (44), *m/z* 383 (M^+–15; 7), 355 (M^+–43; 10), 344 (9), 343 (M^+–55; 20), 260 (11), 245 (18), 234 (28; A-ring derived fragment), 179 (43), 165 (21), 164 (100; B-ring derived fragment), 151 (79), 149 (42), 121 (41).

*Diphysolone-4'(or 2')-O-methyl ether (**3**) (diphysolidone)*

Colour with diazotised *p*-nitroaniline reagent, yellow; colour with Gibbs reagent, immediate deep blue (*cf.* the slow reaction of cajanol **7** [21]). Long wavelength UV fluorescence as given for **2** (*cf.* ferreirin, **4**). UV: λ_{max}, nm: MeOH 208, 232 sh, 294, 342 sh; + NaOH 331; + NaOAc 332 (addition of solid boric acid regenerated the MeOH spectrum); + $AlCl_3$ 315 (shift occurred gradually over a 30–40 min period). MS: $[M]^+$ 370 (15), *m/z* 221 (21; A-ring derived fragment), 194 (46), 179 (35), 177 (20), 176 (79), 165 (39), 151 (13), 150 (27; B-ring derived fragment), 148 (82), 139 (100), 138 (18), 137 (12). ^{1}H NMR: see Table I. *Dimethyl ether* (**5**) (CH_2N_2). R_F, UV and MS data, and colour with diazotised *p*-nitroaniline reagent and Gibbs reagent, as given for the trimethyl ether of **2**.

*5,7,2'-trihydroxy-4'-methoxyisoflavanone (**4**) (ferreirin)*

Colour with diazotised *p*-nitroaniline reagent, yellow; colour with Gibbs reagent, immediate deep blue. Long wavelength UV fluorescence as given for **1**. UV: λ_{max}, nm: MeOH 288, 333 sh; + NaOH 324; + NaOAc 326 (addition of solid boric acid regenerated the MeOH spectrum); + $AlCl_3$ 311 (shift observed immediately).

Acknowledgements

We thank Mr. R. W. Butters and Miss Y. Atsuta for determination of mass spectra, Mr. M. Ikura for obtaining ^{1}H NMR data, and Dr. T. J. King for supplying a generous sample of authentic ferreirin. Financial assistance (to J. L. I.) was provided by the S.E.R.C.

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