New Dextrorotatory Pterocarpan Phytoalexins from Leaflets of *Nissolia fruticosa*

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Three isoflavonoid phytoalexins produced by the fungus or $CuSO_4$-treated leaflets of *Nissolia fruticosa* have been identified as (+)-6a*S*; 11a*S*-3,7-dihydroxy-9-methoxypterocarpan (nissicarpin), (+)-6a*S*; 11a*S*-7-hydroxy-3,9-dimethoxypterocarpan (fruticarpin) and (+)-6a*S*; 11a*S*-3,7-dihydroxy-2,9-dimethoxypterocarpan (nissolicarpin).

Introduction

Pterocarpan phytoalexins [1, 2] are known to accumulate in the microbially-inoculated tissues of numerous papilionate legumes, and about 40 such compounds have now been described [3, 4]. All are oxygenated at C-3 and C-9 (see structure **1** for pterocarpan ring numbering system) and most are additionally substituted at other molecular sites with the notable exception of C-7. Indeed, of the many induced (phytoalexins) and constitutive pterocarpans discovered in papilionate legumes [4], only philenopteran and its 9-O-methyl ether from *Lonchocarpus laxiflorus* [5] are oxygenated (OCH_3 in both cases) at C-7, but neither of these compounds is currently considered to act as a phytoalexin. Recently, however, we have found that three fungitoxic pterocarpans (nissicarpin **1**, fruticarpin **2**, and nissolicarpin **3**) each possessing a C-7 hydroxyl group are produced when leaflets of *Nissolia fruticosa* Jacq. (Leguminosae-Papilionoideae; tribe Aeschynomeneae; subtribe Ormocarpinae) are inoculated with the fungus *Helminthosporium carbonum* Ullstrup or treated with aqueous $CuSO_4$. In the present communication we describe the identification of these novel *Nissolia* phytoalexins as (+)-3,7-dihydroxy-9-methoxypterocarpan (**1**), (+)-7-hydroxy-3,9-dimethoxypterocarpan (**2**) and (+)-3,7-dihydroxy-2,9-dimethoxypterocarpan (**3**). In TLC plate bioassays [6, 7] all three pterocarpans inhibited growth of *Cladosporium herbarum* Fr. at applied levels greater than 10–15 µg.

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Results and Discussion

During the course of the present study, phytoalexins **1**–**3** were isolated from detached *Nissolia* leaflets using the drop-diffusate procedure [2, 3, 8]. Ethyl acetate extracts of 48 h fungus- or $CuSO_4$-induced diffusates were chromatographed (Si gel TLC [9]) in $CHCl_3$–MeOH (50:1) to yield fruticarpin (**2**), nissolicarpin (**3**) and nissicarpin (**1**) as diazotised *p*-nitroaniline-positive bands at R_F 0.64, 0.37 and 0.16 respectively. Elution (MeOH) and further Si gel TLC (*n*-pentane-Et_2O-glacial HOAc, 75:25:3) gave compounds (**1**, R_F 0.26; **2**, R_F 0.56; **3**, R_F 0.21) which ran as homogeneous spots when chromatographed in several other solvent systems. Nissicarpin, fruticarpin and nissolicarpin were not produced when leaflets of *N. fruticosa* were treated only with de-ionised H_2O.

The ^{1}H NMR spectrum of nissicarpin (**1**; $[M]^+$ 286) clearly revealed signals attributable to a 6a*H*-pterocarpan with oxygenation at the 3, 7 and 9 positions (Table I). A three-proton methoxyl singlet was also apparent (δ 3.65). This latter substituent was assigned to ring D in view of the virtually identical chemical shift values recorded in acetone-d_6 for the A-ring protons of **1** and several 3-hydroxylated pterocarpans including phaseollidin, dolichins A and B [10] and synthetic vesticarpan **4**

(Table I). In contrast, corresponding A-ring signals for 3-O-methylvesticarpan (**5**) were shifted significantly downfield when compared with those of **1** and **4** (Table I). Apart from chemical shift data, the distinct UV maximum at 247 nm in the EtOH + NaOH spectrum of **1** was also strongly indicative of a C-3 hydroxyl group [11].

Minor fragments at *m/z* 177 and 164, and *m/z* 147 and 134 in the MS of nissicarpin established that one of the aromatic rings (considered to be ring A from the preceding discussion of ^{1}H NMR and UV data) was monohydroxylated, and that the other (ring D) contained both an OH and an OCH_3 substituent. Assignment of the latter groups to C-7 and C-9 respectively was based on the following spectroscopic and chemical evidence. First, the D-ring protons of **1** (Table I) were evident as two (*meta*-coupled) doublets at δ 5.93 and δ 6.01. Assuming oxygenation at C-9, as in all other reported naturally occurring pterocarpans [4], these signals must be due to H-8 and H-10. Secondly, on TLC plates sprayed with Gibbs reagent/aqueous Na_2CO_3 [7, 12] nissicarpin reacted immediately to afford a deep blue indophenol, an observation which can only be satisfactorily explained if C-7 is hydroxylated. Under similar circumstances **1** gave a predominantly yellow product with diazotised *p*-nitroaniline; apart from providing good evidence for C-9 methoxylation, this result also indirectly supports the proposed C-7 OH group (*cf.* the intense orange diazo derivative typically afforded by C-9 hydroxylated pterocarpans [11]). From the above data, therefore, nissicarpin can be assigned the structure 3,7-dihydroxy-9-methoxypterocarpan (**1**).

The UV (EtOH) spectrum of fruticarpin (**2**; $[M]^+$ 300) resembled that of nissicarpin, and upon diazomethane methylation both pterocarpans yielded the same permethylated product (**6**). Fruticarpin gave a yellow colour with diazotised *p*-nitroaniline and a blue colour with Gibbs reagent, but unlike nissicarpin its alkali (EtOH +

Table I. ^{1}H NMR data for *N. fruticosa* pterocarpans (**1** and **3**) and relevant model compounds[a].

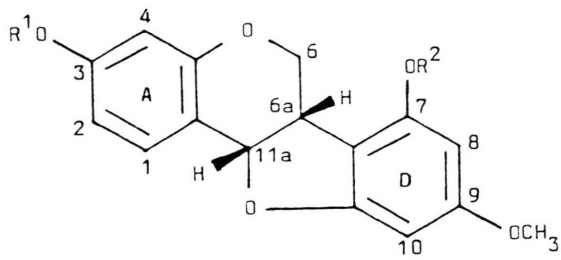
Proton	Nissicarpin (**1**)	Nissolicarpin (**3**)	Nissolicarpin diacetate (**7**)	Vesticarpan (**4**)	3-O-Methyl-vesticarpan (**5**)
H-1 (1H)	7.30 d (J = 8.4)	7.00 s	7.17 s	7.34 d (J = 8.2)	7.41 d (J = 8.4)
H-2 (1H)	6.55 dd (J = 8.3 & 2.2)	–	–	6.56 dd (J = 8.3 & 2.4)	6.63 dd (J = 8.2 & 2.4)
H-4 (1H)	6.37 d (J = 2.2)	6.40 s	6.66 s	6.37 d (J = 2.3)	6.43 d (J = 2.6)
H-7 (1H)	–	–	–	6.76 d (J = 8.1)	6.76 d (J = 8.4)
H-8 (1H)	5.93 d (J = 2.0)	5.93 d (J = 1.9)	6.28 d (J = 2.1)	6.50 d (J = 8.1)	6.50 d (J = 8.2)
H-10 (1H)	6.01 d (J = 2.0)	6.01 d (J = 1.9)	6.32 d (J = 2.2)	–	–
H-6*eq* (1H)	4.22 m	4.23 m	4.25 m	4.25 m	4.28 m
H-6*ax* / H-6a (2H)	*ca.* 3.60 m	–[b]	*ca.* 3.60 m	*ca.* 3.60 m	*ca.* 3.60 m
H-11a (1H)[c]	5.41 d (J = 6.5)	5.42 d (J = 6.0)	5.58 d (J = 6.2)	5.48 d (J = 5.6)	5.49 d (J = 5.5)
OCH_3 (3H)	3.65 s	3.69 s 3.84 s	3.75 s 3.83 s	3.80 s	3.77 s 3.79 s
OAc (3H)	–	–	2.23 s 2.33 s	–	–

[a] All spectra were determined in acetone-d_6 at 80 MHz. Chemical shifts are given as δ values (TMS reference). Figures in parentheses refer to coupling constants in Hz.
[b] Signals obscured by H_2O signal at *ca.* 3.40 ppm.
[c] Doublet shows some variation in the extent of its resolution.

NaOH) UV spectrum lacked the low wavelength (approx. 250 nm) maximum characteristic of a 3-hydroxylated pterocarpan [11]. This suggested that **2** was nissicarpin-3-O-methyl ether, a view confirmed by selective methylation of the 3-OH group of **1** to afford a monomethyl ether identical (UV, MS, TLC and colour reactions) with fruticarpin obtained from *N. fruticosa* leaflets.

The third *Nissolia* pterocarpan (nissolicarpin **3**; $[M]^+$ 316) exhibited an alkali UV maximum at 246 nm (C-3 OH [11]) and, like **1** and **2**, gave a stable blue colour on chromatograms treated with Gibbs reagent. The ^1H NMR spectrum of **3** (Table I) revealed two OCH_3 groups (δ 3.69 and δ 3.84) and also established that ring A was 2,3-dioxygenated because in contrast to nissicarpin the H-1 and H-4 signals appeared as singlets (*cf.* relevant ^1H NMR data for lathycarpin [9]) with H-1 additionally showing a significant (0.30 ppm) upfield shift as the result of *ortho*-oxygenation [13]. Chemical shift values for the two *meta*-coupled D-ring protons were identical with those measured for nissicarpin (Table I). The only diagnostic low intensity fragments in the MS of **3** occurred at *m/z* 177 and 164, and this suggested that both aromatic rings possessed an OH as well as an OCH_3 substituent. Monohydroxylation of rings A and D was subsequently confirmed by acetylation (Py–Ac_2O) to afford a diacetate derivative (**7**; $[M]^+$ 400) in which the signals due to H-1, 4, 8 and 10 were all shifted downfield relative to those of **3** (Table I) [14]. 2-Methoxy-3-hydroxy substitution in the A ring was also confirmed by this means as upon acetylation of **3** the H-1 signal displayed a smaller downfield shift (0.17 ppm) than did that of H-4 (0.26 ppm). This effect, which has been exploited in the identification of other 6a*H*-pterocarpans [15, 16], establishes that the A-ring OH group is located at C-3 (*i.e. ortho* to H-4 and *meta* to H-1). Finally, as in pterocarpans **1** and **2**, the OH and OCH_3 substituents of ring D were respectively assigned to C-7 and C-9 from colours obtained with Gibbs reagent (deep blue) and diazotised *p*-nitroaniline (yellow [11]), and also from the unequal chemical shifts of the H-8 and H-10 signals observed after acetylation (Table I). Nissolicarpin (**3**) is therefore 3,7-dihydroxy-2,9-dimethoxypterocarpan (2-methoxynissicarpin).

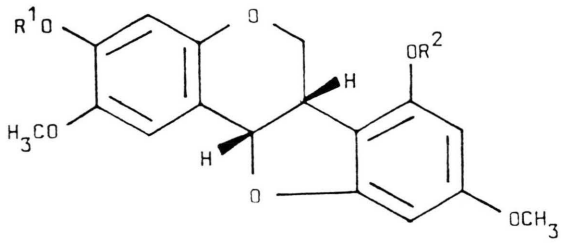
It is interesting to note that all three *Nissolia* pterocarpans are strongly dextrorotatory and thus



1 : $R^1 = R^2 = H$ (Nissicarpin)

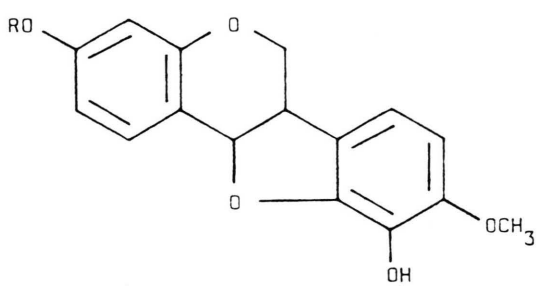
2 : $R^1 = CH_3$; $R^2 = H$ (Fruticarpin)

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



3 : $R^1 = R^2 = H$ (Nissolicarpin)

7 : $R^1 = R^2 = Ac$



4 : R = H (Vesticarpan)

5 : $R = CH_3$

possess the 6a*S*; 11a*S* absolute configuration shown in structures **1**–**3**. Optical rotation measurements have now been made for a considerable number of 6a*H*-pterocarpan phytoalexins and most are laevorotatory [3], the only reported exceptions apart from compounds **1**–**3** being (+)-medicarpin and (+)-maackiain which accumulate in the *H. carbonum*-inoculated leaflets of the temperate leguminous tree *Sophora japonica* [3, 17].

Experimental

Plant material

Seeds of *Nissolia fruticosa* Jacq. (obtained from the University of Coimbra Botanic Garden, Portugal) were sown in John Innes No. 1 compost and the resulting plants grown as described elsewhere [18]. Leaflets for phytoalexin experiments were harvested at intervals of 15–20 weeks over a 2 year period.

Induction, isolation and quantification of nissicarpin (**1**), *fruticarpin* (**2**) *and nissolicarpin* (**3**)

Phytoalexins were isolated from detached *N. fruticosa* leaflets using the drop-diffusate technique [2, 8]. The leaflets were normally inoculated with droplets of a conidial suspension of *H. carbonum*, but an aqueous solution of $CuSO_4$ [9] was also occasionally employed to stimulate phytoalexin accumulation. Drops of de-ionised H_2O served as the control. After incubation for 48 h [19] the diffusate was collected, shaken (×3) with equal volumes of EtOAc, and the organic fractions pooled and reduced to dryness *in vacuo* (40°). Si gel TLC of the residue from *H. carbonum* or $CuSO_4$-induced diffusate extracts (see Results and Discussion) yielded the pure phytoalexins in varying but always readily detectable quantities. Compounds **1**–**3** were never produced by the control leaflets. Typical yields from 10 ml of *H. carbonum*-induced diffusate were as follows: nissicarpin 170–200 µg, fruticarpin 40–80 µg (values given for **1** and **2** are based on $\varepsilon = 3700$ at 288 nm for vesticarpan [20]) and nissolicarpin 300–350 µg (based on $\varepsilon = 6750$ at 294 nm for mucronucarpan [21]). Diffusates from *Nissolia* leaflets treated with aqueous $CuSO_4$ also contained measurable quantities of nissicarpin (4–7 µg/ml), fruticarpin (1–3 µg/ml) and nissolicarpin (13–16 µg/ml).

3,7-Dihydroxy-9-methoxypterocarpan **1** *(nissicarpin)*

Colour on Si gel TLC plates sprayed with diazotised *p*-nitroaniline reagent, yellow; corresponding colour with Gibbs reagent, immediate deep blue. UV: λ_{max}, nm: EtOH 212 (100%), 234 sh (42%), 277 sh (15%), 281 (16%), 287 (14%); λ_{max}, nm: EtOH + NaOH 214, 247, 290, 298 sh. MS: $[M]^+$ 286 (100), *m/z* 285 (24), 272 (9), 271 ($M^+ - 15$; 46), 177 (12), 164 (10), 149 (8), 147 (6), 143 (13), 137 (5), 134 (5). ^1H NMR: see Table I. $[\alpha]_{589\,nm} + 200°$ (approx. 450 µg, based on $\varepsilon = 3700$ at 288 nm for vesticarpan [20], in 1 ml of MeOH). *Dimethyl ether* **6** (CH_2N_2; R_F 0.83 in $CHCl_3$). UV: λ_{max}, nm: EtOH 212 (100%), 234 sh (63%), 276 sh (25%), 280 (26%), 286 (23%). MS: $[M]^+$ 314 (100), *m/z* 313 (22), 300 (6), 299 ($M^+ - 15$; 29), 191 (8), 178 (22), 161 (15), 157 (13), 148 (16).

7-Hydroxy-3,9-dimethoxypterocarpan **2** *(fruticarpin)*

Colours with diazotised *p*-nitroaniline and Gibbs reagent, as given for **1**. UV: λ_{max}, nm: EtOH 212 (100%), 234 sh (45%), 277 sh (15%), 280 (16%), 286 (14%); λ_{max}, nm: EtOH + NaOH 218, 280, 286. MS: $[M]^+$ 300 (100), *m/z* 299 (20), 286 (5), 285 ($M^+ - 15$; 24), 177 (18), 176 (6), 164 (16), 161 (9), 150 (8), 149 (5), 148 (15), 137 (9). $[\alpha]_{589\,nm} + 190°$ (approx. 160 µg, based on $\varepsilon = 3700$ at 288 nm for vesticarpan [20], in 1 ml of MeOH). *Monomethyl ether* **6** (CH_2N_2). UV, MS and TLC data as given for the dimethyl ether of **1**.

Preparation of fruticarpin by selective (C-3) methylation of nissicarpin

CH_2N_2 was slowly bubbled through a solution of nissicarpin (**1**; approx. 250 µg) in MeOH (4 ml) and CH_2Cl_2 (1 ml). The passage of CH_2N_2 was stopped at 30 s intervals, and the gradual formation of **2** was monitored by Si gel TLC of a small portion (0.1 ml) of the reaction mixture. After a total of 2 min treatment with CH_2N_2 the solvent was removed *in vacuo* (40°), and the residue immediately chromatographed (Si gel TLC, $CHCl_3$–MeOH, 50:1) to afford a product (R_F 0.64; approx. 200 µg) indistinguishable (UV, MS, TLC and colour reactions) from a *Nissolia*-derived sample of fruticarpin.

3,7-Dihydroxy-2,9-dimethoxypterocarpan **3** *(nissolicarpin)*

Colours with diazotised *p*-nitroaniline and Gibbs reagent, as given for **1**. UV: λ_{max}, nm: EtOH 213 (100%), 234 sh (47%), 281 sh (15%), 293 (21%), 298 sh (19%), 303 sh (15%); λ_{max}, nm: EtOH + NaOH 214, 246, 287 sh, 300. MS: $[M]^+$ 316 (100), *m/z* 315

(7), 302 (7), 301 ($M^+ - 15$; 35), 283 (7), 231 (6), 177 (8), 164 (9), 158 (7). ^{1}H NMR: see Table I. $[\alpha]_{589\,nm} + 350°$ (approx. 850 µg, based on $\varepsilon = 6750$ at 294 nm for mucronucarpan [21], in 1 ml of MeOH). *Diacetate* **7** (Py–Ac_2O; R_F 0.46 in $CHCl_3$). UV: λ_{max}, nm: EtOH 208 (100%), 230 sh (27%), 287 (14%), 303 sh (9%). MS: $[M]^+$ 400 (48), *m/z* 359 (18), 358 ($M^+ - 42$; 89), 317 (17), 316 ($M^+ - 42 - 42$; 100), 315 (74), 301 (19), 177 (37), 164 (39), 153 (14), 149 (12), 134 (11). ^{1}H NMR: see Table I.

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