New Dextrorotatory Pterocarpan Phytoalexins from Leaflets of Nissolia fruticosa

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Three isoflavonoid phytoalexins produced by the fungus or CuSO₄-treated leaflets of Nissolia fruticosa have been identified as (+)-6aS; 11αS-3,7-dihydroxy-9-methoxypterocarpan (nissicarpin), (+)-6aS; 11αS-7-hydroxy-3,9-dimethoxypterocarpan (fruticarpin) and (+)-6aS; 11αS-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 pterocarpan discovered in papilionate legumes [4], only philenopteran and its 9-O-methyl ether of Lonicocarpus laxiflorus [5] are oxygenated (OCH₃ 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 pterocarpan (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 Cladosporium herbarum Fr. or treated with aqueous CuSO₄.

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 CuSO₄-induced diffusates were chromatographed (Si gel TLC [9]) in CHCl₃-MeOH (50:1) to yield fruticarpin (2), nissolicarpin (3) and nissicarpin (1) as diazotised p-nitroaniline-positive bands at Rf 0.64, 0.37 and 0.16 respectively. Elution (MeOH) and further Si gel TLC (n-pentane-Et₂O-glacial HOAc, 75:25:3) gave compounds (1, Rf 0.26; 2, Rf 0.56; 3, Rf 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 H₂O.

The ¹H NMR spectrum of nissicarpin (1; [M]+ 286) clearly revealed signals attributable to a 6aH-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-d₆ for the A-ring protons of 1 and several 3-hydroxylated pterocarpan including phaseollidin, dolichins A and B [10] and synthetic vesticarp 4.

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Results and Discussion

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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 \( \delta 5.93 \) and \( \delta 6.01 \). 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\(_2\)CO\(_3\) [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>
<thead>
<tr>
<th>Proton</th>
<th>Nissicarpin (1)</th>
<th>Nissolicarpin (3)</th>
<th>Nissolicarpin diacetate (7)</th>
<th>Vesticarpan (4)</th>
<th>3-O-Methylvesticarpan (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-1 (1H)</td>
<td>7.30 d (( J = 8.4 ))</td>
<td>7.00 s</td>
<td>7.17 s</td>
<td>7.34 d (( J = 8.2 ))</td>
<td>7.41 d (( J = 8.4 ))</td>
</tr>
<tr>
<td>H-2 (1H)</td>
<td>6.55 dd (( J = 8.3 ) &amp; 2.2)</td>
<td>–</td>
<td>–</td>
<td>6.56 dd (( J = 8.3 ) &amp; 2.4)</td>
<td>6.63 dd (( J = 8.2 ) &amp; 2.4)</td>
</tr>
<tr>
<td>H-4 (1H)</td>
<td>6.37 d (( J = 2.2 ))</td>
<td>6.40 s</td>
<td>6.66 s</td>
<td>6.37 d (( J = 2.3 ))</td>
<td>6.43 d (( J = 2.6 ))</td>
</tr>
<tr>
<td>H-7 (1H)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>6.76 d (( J = 8.1 ))</td>
<td>6.76 d (( J = 8.4 ))</td>
</tr>
<tr>
<td>H-8 (1H)</td>
<td>5.93 d (( J = 2.0 ))</td>
<td>5.93 d (( J = 1.9 ))</td>
<td>6.28 d (( J = 2.1 ))</td>
<td>6.50 d (( J = 8.1 ))</td>
<td>6.50 d (( J = 8.2 ))</td>
</tr>
<tr>
<td>H-10 (1H)</td>
<td>6.01 d (( J = 2.0 ))</td>
<td>6.01 d (( J = 1.9 ))</td>
<td>6.32 d (( J = 2.2 ))</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>H-6eq (1H)</td>
<td>4.22 m</td>
<td>4.23 m</td>
<td>4.25 m</td>
<td>4.25 m</td>
<td>4.28 m</td>
</tr>
<tr>
<td>H-6ax (2H)</td>
<td>ca. 3.60 m</td>
<td>– (^b)</td>
<td>ca. 3.60 m</td>
<td>ca. 3.60 m</td>
<td>ca. 3.60 m</td>
</tr>
<tr>
<td>OCH(_3) (3H)</td>
<td>3.65 s</td>
<td>3.69 s</td>
<td>3.75 s</td>
<td>3.79 s</td>
<td></td>
</tr>
<tr>
<td>OAc (3H)</td>
<td>–</td>
<td>–</td>
<td>2.33 s</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) All spectra were determined in acetone-d\(_6\), at 80 MHz. Chemical shifts are given as \( \delta \) values (TMS reference). Figures in parentheses refer to coupling constants in Hz.

\(^b\) Signals obscured by H:0 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 (\(\delta 3.69\) and \(\delta 3.84\)) 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\(_2\)O) 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-\(H\)-pterocarpan phytoalexins [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 pterocarpons 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 pterocarpons are strongly dextrorotatory and thus possess the 6aS; 11aS absolute configuration shown in structures 1–3. Optical rotation measurements have now been made for a considerable number of 6aH-pterocarpan phytoalexins and most are laevo-rotatory [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 CuSO₄ [9] was also occasionally employed to stimulate phytoalexin accumulation. Drops of de-ionised H₂O served as the control. After incubation for 48 h [19] the diffusate was collected, shaken (x3) 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 CuSO₄-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 ε = 3700 at 288 nm for vesticarpan [20], 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. [z]₃₈⁹nm + 200° (approx. 450 μg, based on ε = 3700 at 288 nm for vesticarpan [20], in 1 ml of MeOH). Dimethyl ether 6 (CH₂N₂; R₆ 0.83 in CHCl₃). UV: λmax, 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).

3,7-Dihydroxy-3,9-dimethoxypterocarpan 1 (nissicarpin)

Colour on Si gel TLC plates sprayed with diazotised p-nitroaniline reagent, yellow; corresponding colour with Gibbs reagent, immediate deep blue. UV: λmax, nm: EtOH 212 (100%), 234 sh (42%), 277 sh (15%), 281 (16%), 287 (14%); λmax, 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. [z]₃₈⁹nm + 200° (approx. 450 μg, based on ε = 3700 at 288 nm for vesticarpan [20], in 1 ml of MeOH). Dimethyl ether 6 (CH₂N₂; R₆ 0.83 in CHCl₃). UV: λmax, 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: λmax, nm: EtOH 212 (100%), 234 sh (45%), 277 sh (15%), 280 (16%), 286 (14%); λmax, 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). [z]₃₈⁹nm + 190° (approx. 160 μg, based on ε = 3700 at 288 nm for vesticarpan [20], in 1 ml of MeOH). Monomethyl ether 6 (CH₂N₂). UV, MS and TLC data as given for the dimethyl ether of 1.

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

CH₂N₂ was slowly bubbled through a solution of nissicarpin (1; approx. 250 μg) in MeOH (4 ml) and CH₂Cl₂ (1 ml). The passage of CH₂N₂ 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 CH₂N₂ the solvent was removed in vacuo (40°), and the residue immediately chromatographed (Si gel TLC, CHCl₃–MeOH, 50:1) to afford a product (Rf 0.64; approx. 200 μg) indistinguishable (UV, MS, TLC and colour reactions) from a Nissolia-derived sample of fruticarpin.

3,7-Dihydroxy-9-methoxypterocarpan 3 (nissolicarpin)

Colours with diazotised p-nitroaniline and Gibbs reagent, as given for 1. UV: λmax, nm: EtOH 213 (100%), 234 sh (47%), 281 sh (15%), 293 (21%), 298 sh (19%), 303 sh (15%); λmax, 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). 1H NMR: see Table I. 

$[\gamma]_{350}^{\text{nm}} = +350^\circ$ (approx. 850 ng, based on $c = 6750$ at 294 nm for mucronucarpan [21], in 1 ml of 

MeOH). 

Diacetate 7 (Py−Ac$_2$O; $R_F$ 0.46 in CHCl$_3$). 

UV: $\lambda_{\text{max}}$, 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). 1H NMR: see Table I. 

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