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ASTRACICERAN: A NEW ISOFLAVAN PHYTOALEXIN FROM *ASTRAGALUS CICER*

JOHN L. INGHAM* and PAUL M. DEWICK†

* Phytochemical Unit, Department of Botany, University of Reading, Reading, RG6 2AS, U.K. and † Department of Pharmacy, University of Nottingham, Nottingham, NG7 2RD, U.K.

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Key Word Index—*Astragalus cicer;* Galegeae; Leguminosae; isoflavonoids; isoflavans; phytoalexins; antifungal activity; phenolic compounds; synthesis.

Abstract—A new phytoalexin isolated from the fungus-inoculated leaflets of *Astragalus cicer* has been identified as 7-hydroxy-2′-methoxy-4′,5′-methylenedioxyisoflavan (astraciceran). The synthesis of astraciceran and its 3′,4′-methylenedioxy analogue is described.

INTRODUCTION

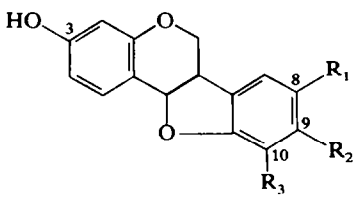
The phytoalexin medicarpin (3-hydroxy-9-methoxypterocarpan, **1**) accumulates in leaf tissues of many papilionate legumes following challenge-inoculation with the fungus, *Helminthosporium carbonum* Ullstrup [1–3]. Although **1** appears to be the most common isoflavonoid phytoalexin, it frequently co-occurs with related compounds such as the pterocarpan maackiain (3-hydroxy-8,9-methylenedioxypterocarpan, **2**) and the isoflavan vestitol (7,2′-dihydroxy-4′-methoxyisoflavan, **3**) [1, 3, 4]. Whilst maackiain and other pterocarpans (e.g. 4-methoxymaackiain and pisatin) possessing an 8,9-methylenedioxy group are regularly encountered as phytoalexins [1, 3, 5, 6], studies undertaken in these and other laboratories have failed to reveal the presence of simple isoflavans with a similar B-ring (4′, 5′) oxygenation pattern; this apparent absence of induced methylenedioxy isoflavans is particularly remarkable in species such as *Trifolium arvense* and *T. hybridum* which produce substantial quantities of **1**, **2** and **3** (medicarpinisoflavan) but, for reasons which are unclear, no detectable maackiainisoflavan (7,2′-dihydroxy-4′,5′-methylenedioxyisoflavan, **4**) [1]. Our studies on legume phytoalexins have recently encompassed the largely north-temperate tribe Galegeae—an amalgam of Hutchinson's Astragaleae, Coluteae and Galegeae [7, 8]—which consists of *ca* 20 genera including *Astragalus*, an immense group of between 1500 and 2500 species. Detailed examination of *Astragalus cicer* L. has shown that fungus-inoculated leaflets produce mucronulatol (7,3′-dihydroxy-2′,4′-dimethoxyisoflavan, **5**), a phytoalexin previously obtained from *A. gummifer* [9]; in *A. cicer*, **5** is also accompanied by a second hitherto undescribed isoflavan (7-hydroxy-2′-methoxy-4′,5′-methylenedioxyisoflavan, **6**) for which the common name astraciceran is proposed. This paper outlines the isolation and purification of astraciceran and describes the total synthesis of both **6** and its isomer, 7-hydroxy-2′-methoxy-3′,4′-methylenedioxyisoflavan (**7**).

RESULTS AND DISCUSSION

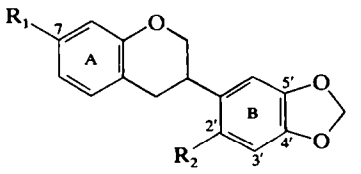
EtOAc extracts of diffusates [10, 11] from *H. carbonum*-inoculated leaflets were chromatographed (Si gel TLC, $CHCl_3$–MeOH, 50:1) to afford astraciceran (R_f 0.53) and mucronulatol (R_f 0.34) as well-separated bands. The isoflavans were eluted (EtOH) and further purified by TLC in *n*-pentane–Et_2O–HOAc, 75:25:3 (**5**, R_f 0.23; **6**, R_f 0.64). Diffusates from leaflets treated with de-ionized H_2O did not contain detectable amounts of either compound. Mucronulatol was firmly identified by UV, MS and TLC comparison with authentic material; **5** was readily distinguished from isomucronulatol (7,2′-dihydroxy-3′,4′-dimethoxyisoflavan, **8**) by co-TLC in C_6H_6–MeOH (9:1) as reported elsewhere [12]. Although **8** acts as a phytoalexin in *Glycyrrhiza glabra* (Galegeae) [12] and several *Astragalus* species (e.g. *A. glycyphyllos* and *A. penduliflorus*) (Ingham, unpublished), there was no evidence to suggest that it co-occurred with **5** and **6** in *A. cicer.*

The new phytoalexin, astraciceran (**6**), formed a monoMe ether **9** (M^+ 314) and a monoacetate (M^+ 342). MS analysis gave the molecular ion at *m/e* 300 together with prominent ions at *m/e* 178 (base), 165 and 135. These fragments can be rationalized if astraciceran is an isoflavan with substituted A- (OH) and B- (OMe; O—CH_2—O) rings. Whilst the OH and OMe groups could be respectively assigned to C-7 and 2′ by analogy with known isoflavans (e.g. **5**), location of the methylenedioxy substituent was difficult since two oxygenation patterns—3′,4′ and 4′,5′—have been associated with isoflavonoid compounds; the former system was slightly favoured because of the co-occurrence of astraciceran with **5**, a known 3′,4′-oxygenated isoflavan. In the absence of ^{1}H NMR data, synthesis of both structural alternatives (**6** and **7**) was undertaken in order to resolve this problem.

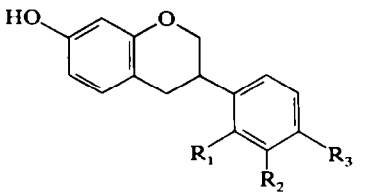
2-Methoxy-3,4-methylenedioxybenzaldehyde (croweacin aldehyde) was obtained by methylenation of pyrogallol 1-monomethyl ether and subsequent



	R_1	R_2	R_3
1	H	OMe	H
2	O—CH_2—O		H
11	OMe	OMe	H
12	H	OMe	OH
13	H	OH	OMe
14	H	OMe	OMe



	R_1	R_2
4	OH	OH
6	OH	OMe
9	OMe	OMe
10	OMe	OH



	R_1	R_2	R_3
3	OH	H	OMe
5	OMe	OH	OMe
7	OMe	O—CH_2—O	
8	OH	OMe	OMe

formylation of the resulting 1-methoxy-2,3-methylenedioxybenzene. Base condensation of this aldehyde with 4-*O*-benzylresacetophenone afforded 4′-benzyloxy-2′-hydroxy-2-methoxy-3,4-methylenedioxychalcone which was acetylated and then converted to the corresponding benzyloxyisoflavone via $Tl(NO_3)_3$ oxidation and treatment of the intermediate acetal with conc HCl. Catalytic hydrogenation of the isoflavone (1 hr) gave **7** in high yield; when treated with H_2 for a shorter period (15 min), the benzyloxyisoflavone afforded large amounts of 7-hydroxy-2′-methoxy-3′,4′-methylenedioxyisoflavone and its isoflavanone analogue but only traces of **7**. The synthesis of **6** has already been reported [13]; in the present study this compound was obtained by catalytic hydrogenation of 7-benzyloxy-2′-methoxy-4′,5′-methylenedioxyisoflavone, itself resulting from selective 7-*O*-benzylation and subsequent 2′-methylation of 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavone.

Comparison of the synthetic and natural isoflavans revealed that astraciceran was identical (UV, MS, TLC) with 7-hydroxy-2′-methoxy-4′,5′-methylenedioxyisoflavan (**6**). The *Astragalus* phytoalexin (R_f 0.54) could be completely separated from synthetic **7** (R_f 0.60) by TLC in *n*-pentane–Et_2O–HOAc (75:25:3). Apart from this chromatographic difference, **6** and **7** also proved to be spectroscopically distinct. Thus, **6**, its methyl ether (**9**) and acetoxy derivative all exhibited clear UV (EtOH) maxima at *ca* 300 nm in accord with other correspondingly oxygenated isoflavonoids such as **4**, pterocarpinisoflavan (7-methoxy-2′-hydroxy-4′,5′-methylenedioxyisoflavan, **10**) and 3-hydroxy-8,9-dimethoxypterocarpan (**11**) [14, 15]. The UV maximum was, however, much less pronounced than that associated with 8,9-methylenedioxypterocarpans such as maackiain (**2**). In contrast, **7** and the hydrogenation product of pseudobaptigenin (7-hydroxy-3′,4′-methylenedioxyisoflavone) exhibited no significant absorption at 300 nm (cf. the related isoflavans **5** and **8**, and the 9,10-substituted pterocarpans, vesticarpan (**12**), nissolin (**13**) and 9-*O*-methylnissolin (**14**)) [12, 16], a fact which may be useful in distinguishing between isoflavans/pterocarpans with 3′,4′/9,10 and 4′,5′/8,9 oxygenation.

Astraciceran is the first simple methylenedioxyisoflavan to be isolated from a higher plant, the previously reported maackiainisoflavan (**4**) being a fungal metabolite of maackiain (**2**) [17]. Three complex constitutive isoflavans (leiocin, nitidulan and leiocinol) with the same B-ring oxygenation as **6** were recently obtained from bark of *Dalbergia nitidula* (Dalbergieae) [13]; however, no simple isoflavans were associated with this legume. Apart from *A. cicer*, the only known source of **6** is *A. pyrenaicus* which also produces **5** as a leaf phytoalexin (Ingham, unpublished). Neither *A. cicer* nor *A. pyrenaicus* has been found to accumulate **2** or any other compounds with 4′,5′/8,9-substitution.

In a typical experiment, fungus-induced diffusates contained **6** at a concentration (based on log $\varepsilon = 3.82$ at 291 nm for **4** [14]) of *ca* 50 μg/ml; the corresponding value for **5** (log $\varepsilon = 3.62$ at 282 nm [18]) was *ca* 15 μg/ml. When tested (TLC bioassay) against spore germination of *Cladosporium herbarum* Fr. [19], **5** and **6** had comparable antifungal activity giving inhibition zones of *ca* 20 and 40 mm^2 at applied levels of 10 and 20 μg, respectively. Both isoflavans could be easily located on TLC plates by direct bioassay of diffusate

(*H. carbonum*-induced) extracts; no other fungitoxic material was detected.

EXPERIMENTAL

MS and UV spectra were determined as previously described [20]. All TLC/PLC separations were undertaken using pre-coated, glass-backed plates (Merck Si gel 60, F 254, layer thickness 0.25/0.5 mm.

Induction of compounds **5** *and* **6**. Freshly collected leaflets of *Astragalus cicer* L. (obtained from the Royal Botanic Gardens, Kew, U.K.) were treated with de-ionized H_2O or conidial suspensions of *Helminthosporium carbonum* [11, 19]; after 48 hr incubation, the diffusates were extracted (EtOAc) and components of the organic phase separated and purified as outlined under Results and Discussion.

7-*Hydroxy*-2'-*methoxy*-4',5'-*methylenedioxyisoflavan* **6** (*astraciceran*). Diazotized *p*-nitroaniline, yellow; Gibbs reagent, no reaction. λ_{max}^{EtOH} nm: 210 (100%), 230 sh (46%), 286 sh (28%), 292 (31%), 300 (28%); $\lambda_{max}^{EtOH+NaOH}$ nm: 210, 242 sh, 300. MS *m/e* (rel. int.): 301 (5), 300 (M^+; 39), 179 (10), 178 (100), 166 (13), 165 (36), 163 (11), 151 (6), 149 (7), 135 (11), 133 (29), 105 (7). *MonoMe ether* **9** (CH_2N_2) (R_f 0.81, $CHCl_3$–CCl_4, 1:1) λ_{max}^{EtOH} nm: 210 (100%), 230 sh (45%), 285 (24%), 290 (28%), 300 (26%). MS *m/e* (rel. int.): 315 (4), 314 (M^+; 13), 179 (9), 178 (100), 167 (6), 166 (11), 165 (25), 164 (7), 163 (11), 150 (5), 149 (52), 135 (7), 133 (31), 121 (6), 105 (9). *Monoacetate* (Py–Ac_2O) (R_f 0.75, $CHCl_3$) λ_{max}^{EtOH} nm: 210 (100%), 230 sh (43%), 280 sh (20%), 286 (24%), 301 (26%). MS *m/e* (rel. int.): 343 (4), 342 (M^+; 21), 300 (9), 179 (12), 178 (100), 166 (16), 165 (46), 164 (5), 163 (9), 151 (6), 149 (21), 147 (7), 135 (10), 133 (23), 105 (9).

Synthesis of **7**. 1-*Methoxy*-2,3-*methylenedioxybenzene*. Pyrogallol 1-monomethyl ether (17.5 g; Aldrich) and CH_2Br_2 (20 ml) in dry Me_2CO (200 ml) were stirred under reflux with dry K_2CO_3 (30 g) for 48 hr. The hot mixture was suction-filtered, the residue washed with boiling Me_2CO (150 ml) and the combined Me_2CO filtrates reduced (*in vacuo*, 40°) to *ca* 25 ml. EtOAc (200 ml) was then added and the soln shaken with aq. NaOH (1 N; 3×200 ml) followed by H_2O (3×200 ml). Removal of EtOAc gave an oil which crystallized on standing (16 hr) at room temp., mp 40–42° (lit. 41–42° [21]). Yield 10 g. MS *m/e* (rel. int.): 153 (7), 152 (M^+; 100), 151 (54), 137 (22), 121 (4), 109 (7), 108 (8), 107 (73), 95 (10).

2-*Methoxy*-3,4-*methylenedioxybenzaldehyde* (*croweacin aldehyde*). A mixture of $POCl_3$ (23 g) and dry *N*-methylformanilide (20 g) was allowed to stand (room temp.) for 30 min at which point finely powdered 1-methoxy-2,3-methylenedioxybenzene (9 g) was added. The mixture was swirled, and then heated (oil bath) at 100° (±3°) for 2 hr in a flask fitted with a reflux condenser/$CaCl_2$ guard tube; swirling was repeated at 10 min intervals throughout the heating period. After cooling to room temp., the soln was poured into ice–H_2O (200 ml) and the impure aldehyde recovered by suction filtration. Repeated crystallization (×4) from EtOH–H_2O gave croweacin aldehyde, mp 103–105° (lit. 103° [22] and 107–108° [23]; cf. 4-methoxy-2,3-methylenedioxybenzaldehyde, mp 84–86° [23]). Yield 4.5 g. MS *m/e* (rel. int.): 181 (9), 180 (M^+; 100), 179 (52), 165 (8), 164 (34), 162 (20), 152 (14), 151 (26), 149 (22), 134 (30), 133 (14).

4'-*Benzyloxy*-2'-*hydroxy*-2-*methoxy*-3,4-*methylenedioxychalcone*. Croweacin aldehyde (4.5 g) and 4-*O*-benzylresacetophenone (4.5 g) were dissolved in EtOH (150 ml; 60°). KOH (45 g) in H_2O (45 ml) was then added and the soln stirred (room temp.) for 7 hr. The pptd chalcone was removed by filtration, washed with H_2O (500 ml; 45°) and then dried *in vacuo* (50°) prior to crystallization from MeOH, mp 155–158°. Yield 5 g. MS *m/e* (rel. int.): 405 (2), 404 (M^+; 7), 373 (13), 178 (11), 165 (11), 133 (8), 92 (8), 91 (100).

7-*Benzyloxy*-2'-*methoxy*-3',4'-*methylenedioxyisoflavone*. The above chalcone (1.7 g) was acetylated (dry Py (5 ml)–Ac_2O (10 ml), room temp. 16 hr) and the reaction mixture poured into H_2O (100 ml) and extracted with EtOAc (2× 100 ml). The extract was successively shaken with dil HCl (0.1 N, 2×200 ml) and H_2O (2×200 ml) before being reduced to dryness *in vacuo*. The acetate, without further purification, was dissolved in MeOH (200 ml)–$CHCl_3$ (25 ml) and stirred (room temp.) with $Tl(NO_3)_3 \cdot 3H_2O$ (2.5 g) for 16 hr. The vol. was then reduced (*in vacuo*, 40°) to *ca* 20 ml, conc H_2SO_4 (2 ml) and H_2O (100 ml) added, and the mixture extracted with EtOAc (2×100 ml). After removing EtOAc (*in vacuo*, 40°), the residue was dissolved in MeOH (250 ml)–$CHCl_3$ (30 ml) and refluxed with conc HCl (7 ml) for 90 min. The vol. was then reduced to *ca* 40 ml and the pptd benzyloxyisoflavone removed by filtration, washed with H_2O (100 ml) and crystallized from MeOH–H_2O, mp 201–203°. Yield 0.9 g. MS *m/e* (rel. int.): 403 (3), 402 (M^+; 12), 311 (12), 92 (8), 91 (100).

7-*Hydroxy*-2'-*methoxy*-3',4'-*methylenedioxyisoflavan* **7**. H_2 (generated by the action of conc HCl on granulated Zn) was bubbled through a soln of the above isoflavone (50 mg) in glacial HOAc (8 ml) containing Pd/C (10%; 80 mg) for 1 hr (room temp.). After removal of catalyst and solvent, the residue was purified (Si gel PLC in $CHCl_3$–MeOH, 25:1) to afford **7** (R_f 0.45; *ca* 20 mg) and its isoflavanone (R_f 0.29) and isoflavone (R_f 0.16) analogues. UV and MS data for **7** were as follows, λ_{max}^{EtOH} nm: 215 (100%), 230 sh (63%), 275 sh (18%), 281 sh (22%), 284 (24%), 290 sh (19%); $\lambda_{max}^{EtOH+NaOH}$ nm: 216, 246 sh, 287 sh, 297. MS *m/e* (rel. int.): 301 (9), 300 (M^+; 46), 179 (11), 178 (100), 166 (54), 165 (34), 163 (22), 150 (7), 149 (13), 147 (9), 135 (24), 133 (24), 107 (9), 105 (6). Diazotized *p*-nitroaniline, yellow; Gibbs reagent, no reaction. 7-*Hydroxy*-2'-*methoxy*-3',4'-*methylenedioxyisoflavanone*. λ_{max}^{MeOH} nm: 215 (100%), 234 sh (49%), 275 (45%), 312 (25%); $\lambda_{max}^{MeOH+NaOH}$ nm: 213, 245, 335; $\lambda_{max}^{MeOH+NaOAc}$ nm: 258 sh, 283, 332 (addition of boric acid regenerated the MeOH spectrum). MS *m/e* (rel. int.): 315 (4), 314 (M^+; 23), 179 (12), 178 (100), 177 (4), 163 (19), 150 (4), 149 (13), 135 (5), 133 (24), 105 (10). Diazotized *p*-nitroaniline, yellow/orange. 7-*Hydroxy*-2'-*methoxy*-3',4'-*methylenedioxyisoflavone*. λ_{max}^{MeOH} nm: 215 (100%). 240 (87%), 249 (84%), 298 (41%), 308 sh (38%); $\lambda_{max}^{MeOH+NaOH}$ nm: 215, 256, 337; $\lambda_{max}^{MeOH+NaOAc}$ nm: 255, 334 (addition of boric acid regenerated the MeOH spectrum). MS *m/e* (rel. int.): 313 (16), 312 (M^+; 100), 311 (8), 295 (14), 283 (18), 282 (20), 281 (79), 253 (12), 176 (21), 175 (38), 174 (8), 146 (10), 137 (55), 131 (14), 127 (11). Diazotized *p*-nitroaniline, orange/yellow. On TLC plates viewed under long wavelength UV light, the above isoflavone exhibited a pale blue fluorescence intensifying when fumed with NH_3.

Synthesis of **6** (*astraciceran*). 7-*Benzyloxy*-2'-*methoxy*-4',5'-*methylenedioxyisoflavone*. 7,2'-Dihydroxy-4',5'-methylenedioxyisoflavone (100 mg) [24] in dry Me_2CO (20 ml) was stirred under reflux with BzCl (46 mg), dry K_2CO_3 (2 g) and dry KI (0.2 g) for 2 hr. The mixture was filtered, and the filtrate evapd to dryness. The residue was purified by TLC (C_6H_6–EtOAc–MeOH–petrol (60–80°), 6:4:1:6) to yield 7-benzyloxy-2'-hydroxy-4',5'-methylenedioxyisoflavone (64 mg) [13] as the major product. This isoflavone (35 mg),

without further purification, was methylated (Me_2CO (20 ml), K_2CO_3 (2 g), MeI (0.5 ml)) and the product isolated in the usual manner. 7-Benzyloxy-2'-methoxy-4',5'-methylenedioxyisoflavone (15 mg) was obtained after TLC (C_6H_6–EtOAc–MeOH–petrol (60–80°), 6:4:1:6) and recrystallization from MeOH, mp 147–148° (lit. 150° [13]). MS *m/e* (rel. int.): 403 (3), 402 (M^+; 16), 371 (9), 311 (13), 280 (6), 92 (7), 91 (100).

7-Hydroxy-2'-methoxy-4',5'-methylenedioxyisoflavan. The above isoflavone (4 mg) was hydrogenated in glacial HOAc (5 ml) over Pd/C catalyst (10%; 10 mg) for 16 hr. The mixture was reduced to dryness *in vacuo* and the residue chromatographed (C_6H_6–EtOAc–MeOH–petrol (60–80°)) to afford the desired isoflavan (2 mg) as crystals from MeOH, mp 168–169° (lit. 168° [13]). Synthetic astraciceran was indistinguishable by UV, MS and TLC from the *Astragalus*-derived phytoalexin.

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REFERENCES

1. Ingham, J. L. (1978) *Biochem. Syst. Ecol.* **6,** 217.
2. Ingham, J. L. (1977) *Z. Naturforsch. Teil C* **32,** 449.
3. Ingham, J. L. (1979) *Z. Naturforsch. Teil C* **34,** 293.
4. Ingham, J. L. (1979) *Biochem. Syst. Ecol.* **7,** 29.
5. Cruickshank, I. A. M. and Perrin, D. R. (1965) *Aust. J. Biol. Sci.* **18,** 829.
6. Robeson, D. J. and Harborne, J. B. (1977) *Z. Naturforsch. Teil C* **32,** 289.
7. Hutchinson, J. (1964) *The Genera of Flowering Plants* Vol. 1. Clarendon Press, Oxford.
8. Bell, E. A., Lackey, J. A. and Polhill, R. M. (1978) *Biochem. Syst. Ecol.* **6,** 201.
9. Harborne, J. B. and Ingham, J. L. (1978) in *Biochemical Aspects of Plant and Animal Coevolution* (Harborne, J. B., ed.) p. 343. Academic Press, London and New York.
10. Higgins, V. J. and Millar, R. L. (1968) *Phytopathology* **58,** 1377.
11. Ingham, J. L. and Millar, R. L. (1973) *Nature* **242,** 125.
12. Ingham, J. L. (1977) *Phytochemistry* **16,** 1457.
13. Van Heerden, F. R., Brandt, E. V. and Roux, D. G. (1978) *J. Chem. Soc. Perkin Trans.* 1, 137.
14. Shibata, S. and Nishikawa, Y. (1963) *Chem. Pharm. Bull. Tokyo* **11,** 167.
15. Fukui, K. and Nakayama, M. (1969) *Bull. Chem. Soc. Jpn.* **42,** 1408.
16. Robeson, D. J. and Ingham, J. L. (1979) *Phytochemistry* **18,** 1715.
17. Higgins, V. J. (1975) *Physiol. Plant Pathol.* **6,** 5.
18. Donnelly, D. M. X., Keenan, P. J. and Prendergast, J. P. (1973) *Phytochemistry* **12,** 1157.
19. Ingham, J. L. (1976) *Phytopathol. Z.* **87,** 353.
20. Ingham, J. L. (1976) *Z. Naturforsch. Teil C* **31,** 504.
21. Baker, W. and Savage, R. I. (1938) *J. Chem. Soc.* 1602.
22. Brownell, W. B. and Weston, A. W. (1951) *J. Am. Chem. Soc.* **73,** 4971.
23. Bick, I. R. C. and Russell, R. A. (1969) *Aust. J. Chem.* **22,** 1536.
24. Farkas, L., Gottsegen, A., Nógrádi, M. and Antus, S. (1974) *J. Chem. Soc. Perkin Trans.* 1, 305.