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BIOSYNTHESIS OF PISATIN: EXPERIMENTS WITH ENANTIOMERIC PRECURSORS

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Key Word Index—*Pisum sativum*; Leguminosae; phytoalexin; pterocarpan; isoflavonoid; pisatin; biosynthesis.

Abstract—Feeding experiments in cupric chloride-treated *Pisum sativum* pods and seedlings have demonstrated the preferential incorporation of (+)-(6a*S*,11a*S*)-[^{3}H]maackiain over (−)-(6a*R*, 11a*R*)-[^{14}C]maackiain into (+)-(6a*R*, 11a*R*)-pisatin, establishing that the 6a-hydroxylation of pterocarpans proceeds with retention of configuration. (+)-(6a*R*,11a*R*)-6a-hydroxymaackiain was similarly incorporated much better than (−)-(6a*S*,11a*S*)-6a-hydroxymaackiain. Where (−)-isomers were incorporated, optical activity measurements on the pisatin produced indicated significant synthesis of (−)-pisatin as well as the normal (+)-pisatin. 7,2′-Dihydroxy-4′,5′-methylenedioxyisoflav-3-ene and both enantiomers of 7,2′-dihydroxy-4′,5′-methylenedioxyisoflavan were poor precursors of pisatin.

INTRODUCTION

Feeding experiments in cupric chloride-treated pea (*Pisum sativum*) tissues [1] have demonstrated the excellent incorporations of (±)-(6a*S*, 11a*S* + 6a*R*, 11a*R*)-[^{14}C]maackiain (**3** and **7**) and (+)-(6a*R*, 11a*R*)-[^{14}C]6a-hydroxymaackiain (**4**) into the 6a-hydroxypterocarpan phytoalexin (+)-(6a*R*, 11a*R*)-pisatin (**5**), establishing that pisatin is produced by 6a-hydroxylation of maackiain followed by methylation. Although racemic maackiain was employed in these studies, it is likely that the 6a-hydroxylation step occurs with retention of configuration at C-6a, since an inversion process would necessitate the additional inversion at C-11a, pterocarpans having a *cis*-fused ring system [2, 3]. In this case, only (+)-(6a*S*, 11a*S*)-maackiain (**3**) would have served as the precursor of (+)-pisatin (**5**). However, the significant (1–9%) incorporation of (−)-(6a*R*, 11a*R*)-[^{14}C]maackiain (**7**) into pisatin [4] demonstrated that pea tissue was also able to utilize this enantiomer, but analysis of the phytoalexin showed this was converted into (−)-(6a*S*, 11a*S*)-pisatin (**9**) rather than the normal (+)-isomer [4]. With these observations, and the knowledge that (−)-(6a*R*, 11a*R*)-maackiain (**7**) functions as a minor phytoalexin of *P. sativum* [4, 5] we decided to study further the biosynthesis of pisatin from enantiomeric maackiain precursors labelled with ^{3}H or ^{14}C. The results demonstrate that the biosynthetic precursor of (+)-pisatin is almost certainly (+)-maackiain, and that the 6a-hydroxylation occurs with retention of configuration at C-6a.

RESULTS AND DISCUSSION

Feeding experiments

Feeding experiments with radioactive labelled precursors were performed in 7-day-old *Pisum sativum* seedlings or young partially-expanded pods from garden-grown plants. As previously [1], phytoalexin synthesis was induced by treating the roots of seedlings with dilute aqueous cupric chloride, or by injecting a similar solution into the pods. After 12 hr, this induction solution was replaced by a solution of the precursor, and the plant tissue was worked-up after a 36 hr metabolism period. Pisatin was isolated and purified as previously described [1].

In a series of experiments (Table 1), (+)-[^{3}H]maackiain was compared with (−)-[^{14}C]maackiain as a precursor of pisatin, either in single-labelled comparative feedings, or together as the racemate in double-labelled experiments. In pods, (+)-maackiain was a better precursor than (−)-maackiain, and when fed together, the ^{3}H : ^{14}C ratio increased significantly (4.7 → 30). The incorporations of tritiated precursors must be regarded as minimum values, since a similar feeding of tritiated (−)-maackiain (experiment ii) showed a loss of 50% of the ^{3}H label due to biological exchange (see later). If this were general, then a doubling of the ^{3}H figures would be appropriate, stressing further the preferred role of (+)-maackiain as a precursor of (+)-pisatin. Checks of the optical activities of the pisatin produced indicated the synthesis of significant amounts of (−)-pisatin in those feedings involving (−)-maackiain.

With seedling tissue, the results were less clear-cut, and although results almost identical to the pod studies were obtained in some of the experiments (v and vi), the ability of the plant to metabolize (−)-maackiain was quite marked. Thus, in single- and double-labelling experiments (iv), (−)-maackiain appeared a better precursor than (+)-maackiain, although after correcting for loss of ^{3}H, incorporation levels were very similar. The presence of (−)-maackiain in the feeding solution again resulted in the production of some (−)-pisatin, as indicated by the lower specific rotations recorded. Overall though, there can be little doubt that (+)-maackiain is the precursor of (+)-pisatin, and that the 6a-hydroxylation must, therefore, proceed with retention of configuration.

In the earlier feedings [1], (+)-6a-hydroxymaackiain (**4**) had proved to be an extremely efficient biosynthetic

Table 1. Incorporation of (+)- and (−)-maackiain into pisatin in cupric chloride-treated *Pisum sativum**

Experiment†	Maackiain fed	Pisatin produced (μg/g)	$[\alpha]_D$ (EtOH)‡	% incorporation (Dilution) ^3H	^{14}C	Change in ^3H:^{14}C ratio
(i) P	(+)-[^{3}H]	162	+278°	4.7 (320)	—	—
	(−)-[^{14}C]	97	+207°	—	1.3 (830)	—
	(+)-[^{3}H]+(−)-[^{14}C]	118	+263°	8.0 (76)	1.0 (480)	4.7 → 30
(ii) P	(+)-[^{3}H]+(−)-[^{14}C]	180	+276°	5.4 (320)	0.84(2170)	4.7 → 30
	(−)-[^{3}H]+(−)-[^{14}C]	167	+265°	0.84(210)	1.67(1060)	5.4 → 2.7
(iii) P	(+)-[^{3}H]	99	—	3.6 (390)	—	—
	(−)-[^{14}C]	47	—	—	6.7 (140)	—
(iv) S	(+)-[^{3}H]	70	+289°	2.2 (540)	—	—
	(−)-[^{14}C]	68	+250°	—	6.2 (200)	—
	(+)-[^{3}H]+(−)-[^{14}C]	61	+221°	4.7 (220)	7.5 (130)	5.2 → 3.2
(v) S	(+)-[^{3}H]+(−)-[^{14}C]	92	—	2.0 (260)	0.27(1850)	5.4 → 42
	(−)-[^{3}H]+(−)-[^{14}C]	81	+255°	0.40(2500)	0.50(1740)	5.1 → 4.3
(vi) S	(+)-[^{3}H]+(−)-[^{14}C]	70	+208°	3.7 (140)	0.46(2940)	4.7 → 39
	(−)-[^{3}H]+(−)-[^{14}C]	57	+217°	0.14(2430)	0.24(1430)	6.7 → 3.9

*Induction period 12 hr, feeding period 36 hr.
†P, Pod; S, seedling.
‡$[\alpha]_D$ (+)-pisatin, +288°.

precursor of pisatin with incorporations of 18–27%. The utilization of (−)-maackiain in pisatin biosynthesis made it desirable to test also the precursor efficiency of (−)-6a-hydroxymaackiain (**8**). Thus, (+)- and (−)-[^{14}C]6a-hydroxymaackiain were separately fed in comparative experiments with pod tissue, and the results are presented in Table 2. Although the (−)-isomer was significantly incorporated, the extent (1–3%) was very low indeed compared with that of the (+)-isomer (25–34%). In these cases also, the optical rotation of the pisatin formed was lower than usual, and the incorporations must thus represent incorporations into (−)-pisatin.

A series of studies in *Medicago sativa* and *Trifolium pratense* [6] had demonstrated the interconversions of pterocarpans and 2′-hydroxyisoflavans. Whilst no isoflavan derivatives have been reported in *P. sativum*, and it was considered unlikely that isoflavans had any role in the biosynthesis of 6a-hydroxypterocarpans, the two enantiomers of [^{3}H]-7,2′-dihydroxy-4′,5′-methylenedioxyisoflavan (**10** and **11**) were tested as precursors (Table 3). Even after allowing for loss of ^{3}H label by analogy with maackiain precursors, the incorporations

Table 2. Incorporation of (+)- and (−)-[^{14}C]6a-hydroxymaackiain into pisatin in cupric chloride-treated *P. sativum* pods*

Isomer fed	Pisatin produced (μg/g)	$[\alpha]_D$ (EtOH)†	Dilution	Incorporation (%)
(+)	124	+295°	120	25
(+)	92	+281°	61	34
(−)	100	+235°	1350	1.4
(−)	84	+170°	390	2.8

*Induction period 12 hr, feeding period 36 hr.
†$[\alpha]_D$ (+)-pisatin, +288°.

Table 3. Incorporation of labelled isoflavonoids into pisatin in cupric chloride-treated *P. sativum**

Compound	Tissue†	Pisatin produced (μg/g)	Dilution	Incorporation (%)
(3*S*)-[^{3}H]-7,2′-Dihydroxy-4′,5′-methylenedioxyisoflavan	S	47	19500	0.33
(3*R*)-[^{3}H]-7,2′-Dihydroxy-4′,5′-methylenedioxyisoflavan	S	54	22900	0.31
[4-^{14}C]-7,2′-Dihydroxy-4′,5′-methylenedioxyisoflav-3-ene	P	155	22900	0.22

*Induction period 12 hr, feeding period 36 hr.
†S, Seedling; P, pod.

cannot be regarded as particularly significant, but probably occur via the corresponding maackiain intermediates. The isoflavene, [4-^{14}C]-7,2'-dihydroxy-4',5'-methylenedioxyisoflav-3-ene (**12**) was similarly a poor precursor of pisatin. Isoflav-3-enes appear to be important intermediates in the biosynthetic pathways to coumestans [7] but seem to play no role in the production of pterocarpans [7] and similarly 6a-hydroxypterocarpans.

Synthesis of labelled compounds

(−)-[^{14}C]Maackiain was obtained by feeding L-[U-^{14}C]phenylalanine to cupric chloride-induced seedlings of red clover (*Trifolium pratense*), during which process approximately equal amounts of (−)-maackiain and (−)-medicarpin are produced as phytoalexins [8]. After partial purification of the pterocarpans by TLC, they were separated by gel filtration using Sephadex LH-20.

(+)-[^{3}H]Maackiain was synthesized by base-catalysed exchange of unlabelled (+)-maackiain with tritiated water [9]. (+)-Maackiain is a component of several tropical heartwoods [10] and was isolated for these studies from *Dalbergia oliveri* [11]. The position of labelling was established by a similar experiment with (±)-maackiain and D_2O, and ^{1}H NMR analysis indicated exchange occurred *ortho* to the hydroxyl. The signal for H-4 (δ 6.36, *d*, J = 2.4 Hz) was reduced in intensity to *ca* 9% of its normal value, indicating *ca* 91% deuteriation, and the signal for H-2 (δ 6.55, *dd*, J = 8.5, 2.4 Hz) collapsed to a doublet (J = 8.5 Hz). A reduction in intensity for this signal indicated some deuteriation at C-2, confirmed by a weak singlet at the centre of the H-1 doublet (δ 7.30, J = 8.5 Hz), possessing an intensity of *ca* 10% of the H-1 signal. Thus, there is preferential exchange at H-4 and only much smaller exchange at H-2 under the conditions used. Unfortunately, the label introduced was not completely stable in the feeding experiments since (−)-[^{3}H]maackiain prepared similarly and fed with an equal amount of (−)-[^{14}C]maackiain showed a loss of up to 50% ^{3}H relative to ^{14}C. This, however, did not seriously affect the interpretation of results.

(+)-[^{14}C]-6a-Hydroxymaackiain was obtained as earlier [1] by fungal demethylation of (+)-[^{14}C]pisatin using cultures of *Fusarium avenaceum* [12]. The enantiomer (−)-[^{14}C]-6a-hydroxymaackiain was prepared by fungal 6a-hydroxylation of (−)-[^{14}C]maackiain using an isolate of *Nectria haematococca* [13]. Optical activity measurements confirm that this hydroxylation occurs with retention of configuration.

(3*R*)- and (3*S*)-[^{3}H]-7,2'-Dihydroxy-4',5'-methylenedioxyisoflavan (**10** and **11**, respectively) were synthesized by catalytic hydrogenolysis of (−)- and (+)-[^{3}H]maackiain, respectively. The position of labelling would, thus, be mainly at H-8 with a little at H-6. [^{14}C]-7,2'-Dihydroxy-4',5'-methylenedioxyisoflav-3-ene was obtained by acid treatment [14] of (±)-[11a-^{14}C]maackiain synthesized for earlier experiments [1].

Biosynthesis of (+)- and (−)-pisatin

The results support the biosynthetic proposals published earlier [1, 4] (Scheme 1). Thus, (+)- and (−)-maackiain arise by reductive sequences from a common intermediate 7,2'-dihydroxy-4',5'-methylenedioxyisoflavone (**1**). An overall *Z* reduction occurs in the case of (+)-pisatin (and hence (+)-maackiain), whereas an overall *E* reduction is assumed to take place for (−)-maackiain by analogy with results for (−)-medicarpin [15]. Since *E* reduction of α, β-unsaturated ketones appears to be the biological norm, e.g. ref. [16], it is perhaps possible that the 'overall' *Z* reduction in the case of pisatin arose by epimerization of the isoflavanone (**6**) produced by *E* reduction. The enantiomeric maackiains are then 6a-hydroxylated with retention of configuration and finally methylated to give (+)- and (−)-pisatins.

The 6a-hydroxylation of pterocarpans thus parallels the sequence employed by several micro-organisms in their metabolic detoxification of pterocarpan phytoalexins. Where data is available, the fungal 6a-hydroxylation similarly occurs with retention of configuration [17].

EXPERIMENTAL

General. Pea pods and seedlings, feeding techniques and isolation of pisatin were as reported earlier [1]. Labelled compounds were fed in *ca* 0.2 mg amounts. In double-labelling expts, inactive (+)- or (−)-maackiain was added to the precursor mixture to produce ^{3}H:^{14}C ratios of *ca* 5 and 1:1 proportions of enantiomers (i.e. racemic mixtures) as appropriate. TLC was carried out using 0.5 mm layers of Si gel (Merck TLC-Kieselgel 60GF$_{254}$). Me_2CO was used for elution of TLC zones.

(+)-Maackiain. Fine shavings (30 g) of *Dalbergia oliveri* heartwood were extracted with boiling EtOH (6 × 300 ml). The combined extracts were evaporated to dryness and the residue purified by TLC (hexane–EtOAc, 3:2). The band corresponding to maackiain was eluted, and UV spectroscopy indicated both maackiain and medicarpin to be present. This material was rechromatographed (hexane–Me_2CO, 2:1), achieving some separation of the two compounds. The maackiain portion was further purified by gel filtration (Sephadex LH-20, column size 30 × 1.5 cm, eluting solvent EtOH, 12 ml/hr), medicarpin appearing in the 58–70 ml eluate and maackiain in the 68–81 ml eluate. Maackiain-containing fractions were bulked and rechromatographed until free from medicarpin. (+)-Maackiain was then recrystallized from aq. MeOH, yield 22 mg, mp 178–180°, lit. [18] 180–181°; $[\alpha]_D$ + 230° (EtOH; *c* 1.30).

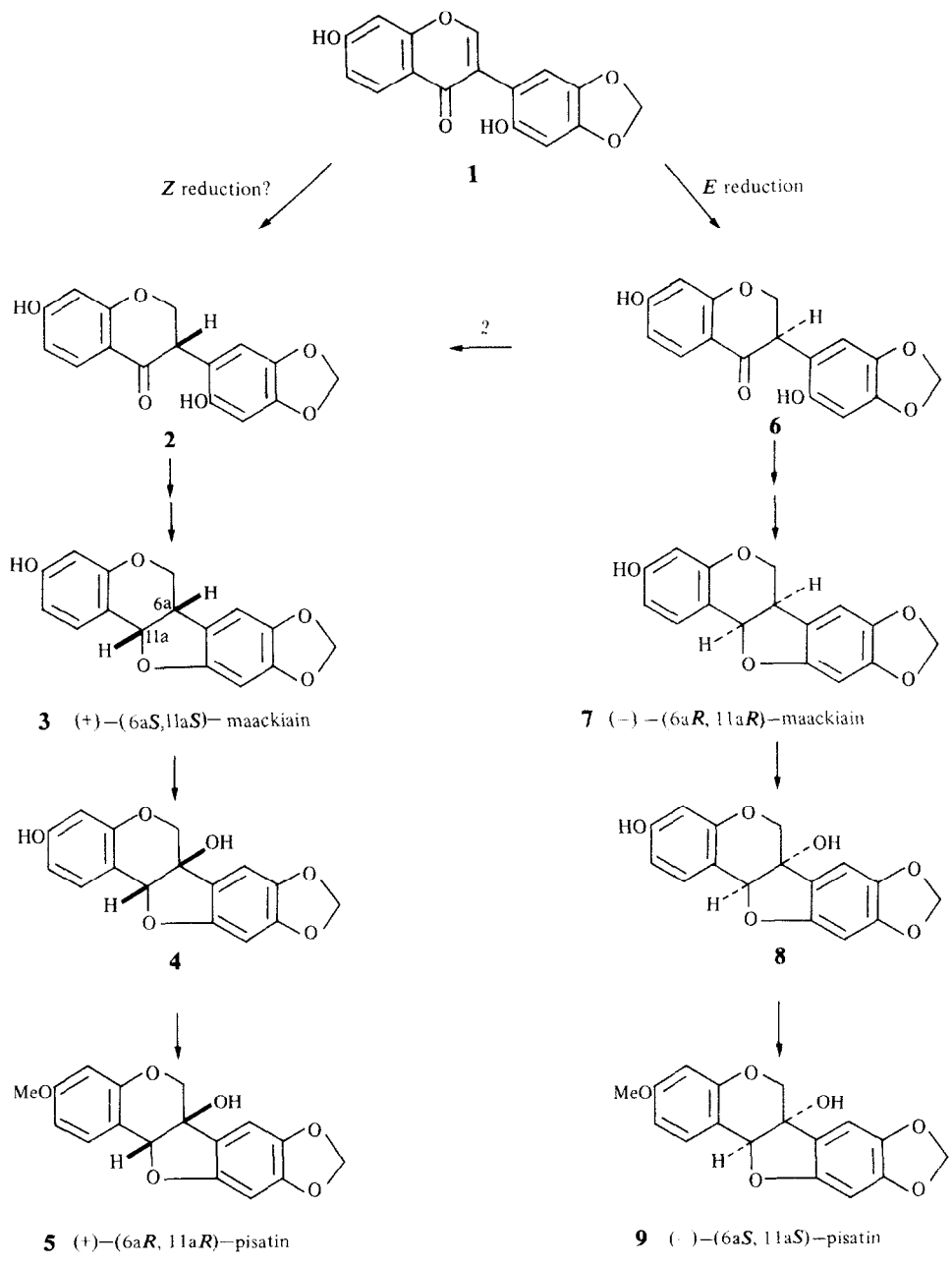
(±)-[^{2}H]Maackiain. A mixture of (±)-maackiain [8] (33 mg) in DMF (0.27 ml), D_2O (0.17 ml) and Et_3N (0.018 ml) in a Reactivial was flushed with N_2, then sealed and heated at 80° for 96 hr. The mixture was cooled, pipetted into MeOH–H_2O (9:1, 5 ml), evaporated to dryness and purified by TLC (hexane–EtOAc, 3:2; hexane–Me_2CO, 2:1) to yield (±)-[^{2}H]maackiain (20 mg). ^{1}H NMR, see Results and Discussion.

Radiochemicals. L-[U-^{14}C]Phenylalanine (10 mCi/mM) and $^{3}H_2O$ (140 mCi/mM) were purchased (Amersham). The syntheses of (±)-[11a-^{14}C]maackiain (0.0426 mCi/mM) and (+)-[^{14}C]-6a-hydroxymaackiain (0.0219 mCi/mM) have been described earlier [1].

(+)-[^{3}H]Maackiain. A mixture of (+)-maackiain (9.4 mg) in DMF (0.15 ml), H_2O (25 μl), $^{3}H_2O$ (25 μl, 100 mCi) and Et_3N (4.9 μl) was heated as above for 45 hr. After work-up and purification by TLC (hexane EtOAc, 3:2; hexane–EtOAc–MeOH, 60:40:1; hexane–Me_2CO, 2:1), (+)-[^{3}H]maackiain (5.2 mg, sp. act. 21.2 mCi/mM) was obtained.

(−)-[^{3}H]Maackiain. A similar procedure using (−)-maackiain [4] (11 mg) gave (−)-[^{3}H]maackiain (6.3 mg, sp. act. 2.75 mCi/mM).

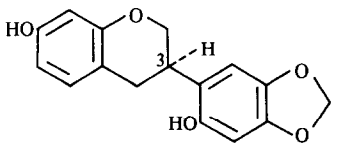
(−)-[^{14}C]Maackiain. Seeds (27 g) of *Trifolium pratense* cv Essex were germinated on moist filter paper in the dark at 25° for 5 days. The seedlings were transferred to a large Petri dish (15 cm) and sufficient aq. $CuCl_2$ (3 mM) was added to cover the roots. The seedings were grown on in the light for 8 hr, then the inducer soln



Scheme 1. Biosynthesis of (+)- and (−)-pisatin in *P. sativum*.

was removed, the roots washed with H_2O and a soln of L-[U-^{14}C]phenylalanine (50 μCi) in sufficient H_2O to just cover the roots was added. The seedlings were grown on in the light for 16 hr, then homogenized in a mortar with ground glass. The slurry was extracted with boiling EtOH (3 × 100 ml), the extracts combined, evaporated, treated with H_2O (50 ml) and extracted with Et_2O (100 ml, then 3 × 50 ml). The combined Et_2O extracts were evaporated and a mixture of (−)-maackiain and (−)-medicarpin was isolated and purified by TLC (hexane–EtOAc, 3:2; $CHCl_3$–MeOH, 25:1, hexane–Me_2CO, 2:1). (−)-[^{14}C]Maackiain was separated from [^{14}C]medicarpin by gel filtration (Sephadex LH-20, EtOH) as above. (−)-[^{14}C]Maackiain was further purified to constant sp. act. by TLC [hexane–Me_2CO, 2:1; C_6H_6–EtOAc–2-propanol, 90:10:1; C_6H_6–EtOAc–MeOH–petrol (60–80°), 6:4:1:3; C_6H_6–EtOAc–MeOH–petrol (60–80°), 6:4:1:6]. Yields *ca* 4 mg, sp. act. 0.005–0.015 mCi/mM.

(−)[^{14}C]-*6a-Hydroxymaackiain.* (−)-[^{14}C]Maackiain (4.1 mg, sp. act. 0.0108 mCi/mM) in 2-methoxyethanol (0.5 ml) was added to an actively growing culture of *Nectria haematococca* MPVI T-110 [13] in glucose–asparagine medium [19] (100 ml). The culture was incubated at 27° on a rotary shaker (120 rpm) for 86 hr, then extracted with Et_2O (5 × 100 ml) and the Et_2O extracts combined and evaporated. The residue was purified by TLC ($CHCl_3$–MeOH, 97:3; hexane–EtOAc–MeOH, 60:40:1; C_6H_6–EtOAc–2-propanol, 90:10:1) to yield (−)-[^{14}C]-6a-



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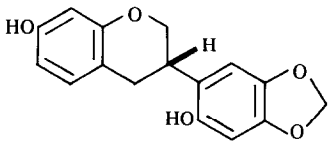

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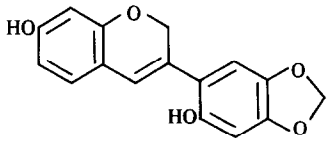

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hydroxymaackiain 1.8 mg, sp. act. 0.0108 mCi/mM, $[\alpha]_D - 328°$ (EtOH; *c* 1.80), identical to (+)-6a-hydroxymaackiain [1], except optical rotation.

(3R)-[^{3}H]-7,2′-*Dihydroxy*-4′,5′-*methylenedioxyisoflavan.* (−)-[^{3}H]Maackiain (1.3 mg, sp. act. 1.05 mCi/mM) was dissolved in EtOAc (10 ml) and hydrogenated over a Pd–C catalyst (10%, 20 mg) at room temp. for 2 hr. The soln was filtered, the filtrate evaporated and purified by TLC [hexane–Me_2CO, 2:1; C_6H_6–EtOAc–MeOH–petrol (60–80°), 6:4:1:6; hexane–EtOAc–MeOH, 60:40:1]. Yield 0.82 mg sp. act. 1.05 mCi/mM, mp 169–171°, UV λ_{max}^{EtOH} nm: 289 (log ε 3.84), 298.

(3S)-[^{3}H]-7,2′-*Dihydroxy*-4′, 5′-*methylenedioxyisoflavan.* (+)-[^{3}H]Maackiain (1.3 mg, sp. act. 1.15 mCi/mM) was hydrogenated as described above. Yield 0.80 mg sp. act. 1.15 mCi/mM, mp 172–174°.

[4-^{14}C]-7,2′-*Dihydroxy*-4′,5′-*methylenedioxyisoflav*-3-*ene.* (±)-[11a-^{14}C]Maackiain (1.0 mg sp. act. 0.0426 mCi/mM) in EtOH (3 ml) was heated under reflux for 30 min with conc. HCl (0.1 ml). The mixture was cooled, concd, treated with H_2O (20 ml) and extracted with EtOAc (3 × 20 ml). The combined extracts were washed with H_2O (3 × 50 ml), evaporated and purified by TLC [hexane–Me_2CO, 2:1; hexane–EtOAc–MeOH, 60:40:1; C_6H_6–EtOAc–MeOH–petrol (60–80°), 6:4:1:6]. Yield 0.12 mg (calculated assuming no change in sp. act.). UV λ_{max}^{EtOH} nm: 282 sh, 335.

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