

Induced and Constitutive Isoflavonoids in *Phaseolus mungo* L. Leguminosae

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Sixteen isoflavonoids have been isolated from the black gram, *Phaseolus mungo* L. (syn. *Vigna mungo*) following treatment with aqueous CuCl₂. These include 12 known isoflavonoids: genistein, 2'-hydroxygenistein, 2'-hydroxydaidzein, kievitone, dalbergioidin, cyclokievitone, 5-deoxykievitone, 2'-hydroxydihydrodaidzein, isoferreirin, aureol, glycinol and demethylvestitol. In addition, kievitone hydrate, which was previously known only as a fungal metabolite of kievitone, was isolated together with 3 novel natural isoflavanones which were characterized as 4'-O-methylkievitone, cyclokievitone hydrate and 5-deoxykievitone hydrate.

Introduction

The black gram, *Phaseolus mungo* L. (syn *Vigna mungo*), has been described as perhaps the most important pulse cultivated in India where the beans are eaten whole or split, boiled or roasted, ground into flour and used to make cakes, breads and porridge [1]. In view of the agricultural value of the crop it is surprising that virtually nothing is known of the phytoalexin content of the plant. To date in the literature, the only report of a study of antifungal substances produced by the black gram, failed to demonstrate any detectable fungal inhibitors in *P. mungo* pod cavities following infection with *Colletotrichum lindemuthianum* [2]. Considering that other *Phaseolus* and *Vigna* species are known to produce potent fungitoxic isoflavonoids as phytoalexins [3], we have investigated the inducible and constitutive isoflavonoids of *P. mungo*. The present communication describes the isolation and characterization of 16 isoflavonoids from *P. mungo* seedlings following treatment with CuCl₂.

Results and Discussion

TLC-bioassay [4] against *Cladosporium cucumerinum* of aliquots of the EtOH extracts from CuCl₂-treated *P. mungo* seedlings indicated 3 inhibitory zones at R_fs 0.25–0.30, 0.40–0.50 and 0.60–0.75. Subsequent purification of the com-

ponents of the zones afforded 16 isoflavonoids which were identified by their UV, MS and PMR characteristics. Five of these compounds: genistein (**1**), 2'-hydroxygenistein (**2**), kievitone (**3**), dalbergioidin (**4**) and demethylvestitol (**5**) are of widespread occurrence as phytoalexins in the Leguminosae [3] and have been reported previously to occur in the genus *Phaseolus*. Five of the remaining compounds were identified as the isoflavone 2'-hydroxydaidzein (**6**), the isoflavanones cyclokievitone (**7**), 5-deoxykievitone (**8**), 2'-hydroxydihydrodaidzein (**9**) and the coumestan aureol (**10**). These substances are so far known to occur only in the genus *Phaseolus* [3, 5]. Two further *P. mungo* isoflavonoids which have been characterised as the isoflavanone isoferreirin (**11**) and the pterocarpin glycinol (**12**) have not yet been reported in either the *Phaseolus* or the *Vigna* genera. The four remaining isoflavonoids isolated from *P. mungo* were characterised as isoflavanones. One of these, **13**, has so far been encountered only as a fungal metabolite of kievitone. The other 3 are new natural products.

The first of the new isoflavonoids possessed a UV spectrum which closely resembled that of kievitone, having principal maxima at 292 nm and 345 nm. The compound was revealed to be a prenylated isoflavanone closely related to kievitone by its PMR spectrum. This included a complex multiplet at δ 4.47 which integrated for 3 protons and arises from the C-2a, C-2b and C-3 protons in the isoflavanone nucleus and signals for 4 aromatic protons, three of which (δ 6.43, δ 6.49 and δ 7.07) formed an ABX system and the fourth (δ 6.04) showed no coupling.

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The presence of a dimethylallyl side chain was indicated by signals for 2-CH₃ groups at δ 1.64 and δ 1.74, 2 methylene protons forming a doublet at δ 3.24 and an allylic proton which gave rise to a broad triplet at δ 5.20. The only significant difference in this spectrum compared to that of kievitone was the presence of a signal typical of methoxyl protons in the spectrum of the new compound. Thus this compound appears to be a mono O-methylated derivative of kievitone. Considering that the singlet for the C-6 proton in the new compound occurs at precisely the same shift (δ 6.04) as that for the same proton in kievitone, whereas the signals for the ABX protons at C-6', C-5' and C-3' in the new compound occurred at δ 7.07, δ 6.43 and δ 6.49 respectively compared with values of δ 6.96, δ 6.34 and δ 6.45 for corresponding protons in kievitone, it seemed likely that the new compound was methylated at either the 2' or 4' hydroxyl of kievitone.

The existence of a methoxyl group on the B-ring of the new compound was confirmed by the MS which contained an intense M⁺ at m/e 370 (67%) and prominent signals at m/e 221 (67%) and m/e 150 (78%) which are attributed to the A-ring and B-ring fragments respectively of the isoflavanone. Also since it is known [6] that in general 2'-hydroxylated isoflavanones have MS with prominent A- and B-ring fragments whereas the MS of 2'-methoxylated isoflavanones show only intense B-ring fragments, the new isoflavanone is characterized as 4'-O-methylkievitone (**13**). The location of the methoxyl at C-4' rather than at C-2' was supported by a positive reaction with Gibbs reagent.

The three remaining isoflavonoids have been found to be hydrated isoflavanones. One of these possessed a UV spectrum which was identical to that of kievitone although its behaviour on TLC suggested that it was much more polar than kievitone. MS revealed an M⁺ at m/e 374 (23%), 18 mass units higher than kievitone and a fragmentation pattern identical to that previously observed [7] for kievitone hydrate, a metabolite isolated following incubation of kievitone with *Fusarium solani*. PMR confirmed the identity of the *P. mungo* compound as kievitone hydrate (2',4',5,7-tetrahydroxy-8-(3''-hydroxy-3''-methylbutyl) isoflavanone (**14**).

The second of the hydrated isoflavanones to be isolated from *P. mungo* was also fairly polar on TLC and possessed a typical isoflavonoid UV spectrum

with a principal maximum at 284 nm. Apart from an intense peak at m/e 358 which appeared to be a plausible M⁺, the remaining major peaks in the MS at m/e 340, 205, 203, 176, 149 and 136 closely resembled the fragmentation pattern of 5-deoxykievitone (M.Wt. 340). Thus the new compound was provisionally identified as a 5-deoxykievitone hydrate. The PMR spectrum of this compound possessed multiplet signals at δ 4.40, δ 4.56 and δ 4.10 which are typical of the C-2a, C-2b and C-3 protons of an isoflavanone system [8]. Signals for 5 aromatic protons, 3 of which at δ 6.47, δ 6.19 and δ 6.30 formed an ABX system and are assigned to the protons at C-6', C-5' and C-3' respectively. The remaining 2 aromatic protons at δ 6.85 and δ 6.48, were both ortho coupled and are located at C-5 and C-6. The non-equivalent methyl groups of 5-deoxykievitone are replaced in the spectrum of 5-deoxykievitone hydrate by a 6H singlet at δ 1.19 which indicates that the C-3'' is not protonated and implies that the OH of the hydrating H₂O molecule is located at C-3'' as is the case in kievitone hydrate [7]. Signals for the methine and methylene protons at C-1'' and C-2'' in the spectrum of 5-deoxykievitone are replaced in the spectrum of the hydrate by two multiplets which show the AA'XX' structure expected for protons in adjacent methylene units. The chemical shifts of these protons are typical for methylene in an aliphatic chain (δ 1.49) and methylene substituted by an aromatic ring (δ 2.65). Thus the new hydrate is characterized as 2',4',7-trihydroxy-8-(3''-hydroxy-3''-methyl-butyl) isoflavanone (**15**).

The final new compound isolated from *P. mungo* has been identified as cyclokievitone hydrate (**16**). Its isoflavonoid nature was initially suggested by its UV spectrum which contained a principal maximum at 294 nm. MS indicated a probable M⁺ at m/e 372 (50%). Other significant peaks at m/e 354, 339, 337, 321, 219, 203 and 136 resembled the fragmentation pattern of cyclokievitone. Thus it seemed possible that the new compound would be a hydrated derivative of cyclokievitone. The principal mass breakdown in cyclokievitone involves loss of Me from the M⁺ at m/e 354 to produce an ion at m/e 339 which is the base peak in the spectrum. This ion undergoes an RDA fragmentation in the typical manner of isoflavanones [9] to produce a ring A fragment ion at m/e 203 and a ring B fragment ion at m/e 136. The peak at m/e 321 in this

spectrum presumably results from loss of H₂O from the ion at *m/e* 339. The peak at *m/e* 219 could be due to the ring A fragment resulting from RDA fragmentation of the M⁺.

An important difference between the Mass spectra of the new compound and of cyclokievitone is the existence of the base peak at *m/e* 237, in the MS of the new compound, which is totally absent from the spectrum of cyclokievitone. This peak could be due to the ring A ion resulting from an RDA fragmentation of the molecular ion occurring before dehydration. Corresponding peaks do not appear in the MS of kievitone hydrate or 5-deoxykievitone hydrate in which the hydrated prenyl group is an open chain. Dreiding models revealed that an axial OH group at C-1'' or an equatorial group at C-2'' in cyclokievitone hydrate could possibly be stabilised by H-bonding respectively to the 7-oxygen or the 1-oxygen in the isoflavanone nucleus.

The PMR spectrum exhibited multiplets at δ 4.55, δ 4.40 and δ 4.23 which are typical of the C-2a, 2b and C-3 protons in an isoflavanone. Signals were also present for 4 aromatic protons, 3 of which formed an ABX system at almost identical shifts (δ 6.53, δ 6.37 and δ 6.45) to the signals assigned to the C-6', C-5' and C-3' protons respectively in cyclokievitone. The fourth aromatic proton resonates at δ 5.89, is not coupled and is located at C-6. The non-equivalent methyl groups of cyclokievitone are replaced by a 6H singlet at δ 1.28 in the spectrum of cyclokievitone hydrate. The signals for the C-1'' and C-2'' methine protons in the PMR spectrum of cyclokievitone are absent from the spectrum of the new compound and are replaced by multiplets at δ 4.76 and δ 3.05 which integrate respectively for 1H and 2H. Thus the new compound appears to possess a substituted 2,2-dimethylchroman ring. The location of the OH group at either the benzylic carbon C-1'' or at the vicinal position C-2'' is uncertain.

The biosynthetic interrelationship between the 5-deoxyisoflavonoids: 2'-hydroxydaidzein, 2'-hydroxydihydrodaidzein, 5-deoxykievitone and demethylvestitol in *P. vulgaris* and *P. aureus* has been proposed elsewhere [15, 10]. It seems likely that this pathway could be extended in *P. mungo* to account for the occurrence of 5-deoxykievitone hydrate and glycinol (Fig. 1). 5-deoxykievitone hydrate is formed presumably by hydration of 5-deoxykievitone and glycinol may be derived from 2'-hydroxydaidzein

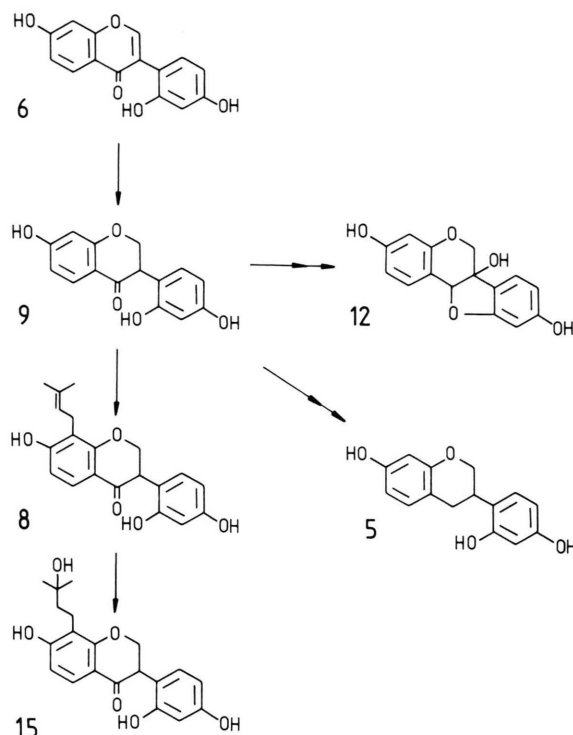


Fig. 1. Proposed biosynthetic interrelationships between 5-deoxy-isoflavonoids in *P. mungo*.

perhaps *via* 2'-hydroxydihydrodaidzein. 2'-hydroxydaidzein has been suggested as a biosynthetic precursor of glycinol in *Glycine max* [19].

Woodward [8, 15] has postulated that two biosynthetic pathways leading to kievitone operate in *P. vulgaris*. One pathway would proceed from genistein to 2'-hydroxygenistein to dalbergioidin to kievitone whereas the second pathway would diverge at 2'-hydroxygenistein by prenylation to produce 2,3-dehydrokievitone. We have earlier proposed that the same two pathways operate in *P. aureus* [10]. However in *P. mungo* no 2,3-dehydrokievitone could be detected and so presumably only the former route to kievitone is feasible. This scheme could be extended (Fig. 2) to account for the other 5-hydroxyisoflavonoids: cyclokievitone, cyclokievitone hydrate, kievitone hydrate, isoferreirin, 4'-O-methylkievitone and aureol in *P. mungo*.

Radioactive feeding experiments on the biosynthesis of the 4'-methoxyisoflavone formononetin [20] and pterocarpan with a 9-methoxy or 8,9-methylenedioxy group *e.g.* medicarpin (3, maackiain [20, 21]) and pisatin [22] suggest that the major

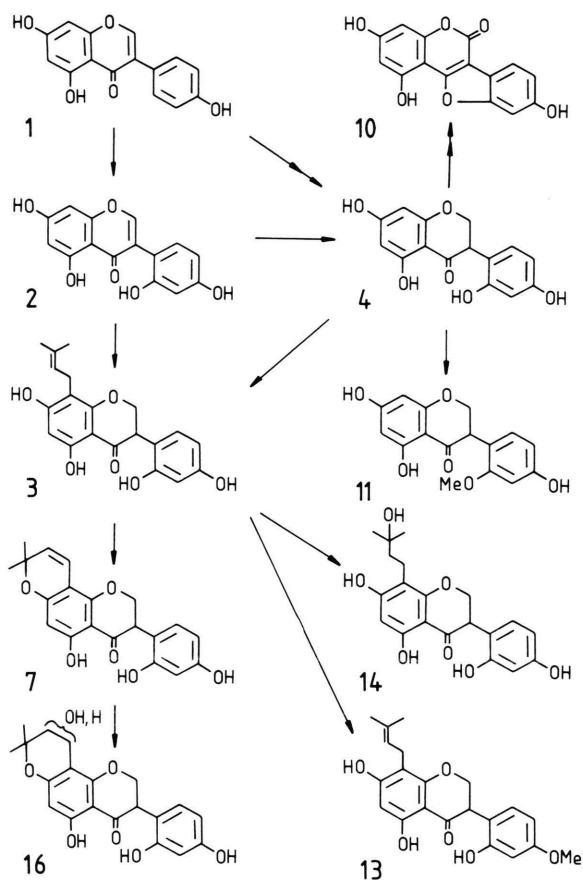


Fig. 2. Proposed biosynthetic interrelationships between 5-hydroxyisoflavonoids in *P. mungo*.

biosynthetic route to 4'-methoxyisoflavonoids involves methylation of the 4'-hydroxy group during the aryl migration step which generates the isoflavonoid from the chalcone or flavanone. However there is some support for the existence of a minor route to 4'-methoxyisoflavonoids through 4'-hydroxyisoflavonoids [23]. In *P. mungo*, no 4'-methoxyisoflavonoids other than 4'-O-methylkievitone could be detected. It seems likely therefore that in this plant the compound is derived from kievitone.

The hydration of isopentenyl side chains in kievitone and phaseollidin has so far been encountered only as a means of fungal detoxification of these compounds [7, 24]. In the present study, the hydrates of kievitone, cyclokievitone and 5-deoxykievitone were detected in *P. mungo* after treatment of the plant with the abiotic elicitor CuCl_2 and thus

appear to be produced as a result of activity by host enzymes. The ability of a plant to catabolise phytoalexins has been recognised and metabolites have been identified in experiments in which exogenous phytoalexins were added to plant tissue [25]. The sesquiterpenoid phytoalexin rishitin can be converted to hydrated derivatives by potato tissue [26], although the importance of this type of transformation in the detoxification of these compounds is unclear. Thus the detection of kievitone hydrate, cyclokievitone hydrate and 5-deoxykievitone hydrate in *P. mungo* may be the first indication of the existence of plant enzymes capable of catalysing the hydration of prenylated isoflavonones.

There is growing evidence in the literature that the black gram *P. mungo* L. is more appropriately assigned to the genus *Vigna* [27–29]. The present study has revealed differences in the isoflavonoids produced by the black gram and other *Phaseolus* species examined to date [3, 10, 18]. *P. mungo* produces no prenylated pterocarpans comparable to phaseollin or phaseollidin. Also the ability of the plant to produce methylated isoflavonoids is a feature in common with *Vigna* species [30, 31] whereas other members of the genus *Phaseolus* do not appear to synthesize these derivatives.

Experimental

Plant material and extraction of isoflavonoids

Seeds of *P. mungo* L., obtained from Thomson and Morgan Ltd., were surface sterilized and allowed to germinate. Control and CuCl_2 -treated seedlings were produced and isoflavonoids were extracted into EtOH and then into EtOAc as previously described [10].

Purification and characterisation of isoflavonoids

Small aliquots of the EtOAc fractions from the control and treated seedlings were subjected to TLC bioassay against *Cladosporium cucumerinum* [10] which revealed the presence of several fungitoxic substances in the extract from the treated seedlings. Appreciable fungitoxicity could not be detected in the extract from the control seedlings. Purification of the fungitoxic substances in the treated seedling extract was achieved by column chromatography on polyamide (Polyclar, Gaf and Co. Ltd., London).

Table I. Chromatographic properties and yields of *P. mungo* isoflavonoids.

Compound	Column fraction	TLC system			Yield $\mu\text{g/g}$ fr. wt.
		1	2	3	
Genistein (1)	C	0.65	0.18	n.d.	3.6
2'-Hydroxygenistein (2)	H	0.59	0.50	n.d.	3.4
Kievitone (3)	F	0.65	0.72	n.d.	19.8
Dalbergioidin (4)	I	0.63	0.72	n.d.	5.6
Demethylvestitol (5)	H	0.62	n.d.	n.d.	0.4
2'-Hydroxydaidzein (6)	D	0.50	0.69	n.d.	0.7
Cyclokievitone (7)	A	0.50	0.56	n.d.	1.0
5-Deoxykievitone (8)	D	0.60	0.70	n.d.	0.9
2'-Hydroxydihydrodaidzein (9)	G	0.40	0.52	n.d.	0.1
Aureol (10)	C	0.70	0.36	n.d.	2.1
Isoferreirin (11)	B	0.50	0.72	0.35	2.0
Glycerinol (12)	E	0.62	0.60	n.d.	0.1
4'-Methylkievitone (13)	A	0.60	0.65	0.26	1.0
Kievitone hydrate (14)	J	0.31	0.45	n.d.	1.2
5-Deoxykievitone hydrate (15)	E	0.27	0.45	n.d.	0.1
Cyclokievitone hydrate (16)	B	0.27	0.35	n.d.	3.0

The initial eluting solvent was CHCl_3 -MeOH (9-1) and elution was continued with an increasing MeOH gradient until fractions no longer contained Fast Blue Salt B-positive substances. The column yielded ten fractions containing isoflavonoids and these were further purified by TLC on silica gel GF₂₅₄ in the following solvent systems: Hexane-EtOAc-MeOH: 6-4-1 (solvent 1), CHCl_3 -isopropanol: 9-1 (solvent 2), Hexane-acetone: 2-1 (solvent 3). The distribution of isoflavonoids in the column fractions and their R_f values on TLC are given in Table I.

Compounds **1–10**, **12**: UV, MS and PMR characteristics were identical to literature values [5, 6, 8, 10–17].

Isoferreirin (**11**): UV MeOH λ_{max} nm: 225 sh, 288, 330 sh; MS m/e (rel. intens.): 302(23) M⁺, 271(13), 270(71), 269(17), 268(11), 242(4), 154(11), 153(100), 152(29), 150(64), 137(15), 135(15), 125(20), 118(21), 107(37), PMR 250 MHz (CD_3)₂CO: δ 3.80 (3 H, s, 2'-OMe), δ 4.29 (1H, d, d, J = 11.0 Hz, 6.2 Hz, C-3), δ 4.44 (1H, d, d, J = 11.0 Hz, 6.3 Hz, C-2b), δ 4.55 (1H, t, J = 7.0 Hz, C-2a), δ 5.96 (1H, d, J = 2.0 Hz, C-6), δ 5.99 (1H, d, J = 2.0 Hz, C-8), δ 6.42 (1H, d, d, J = 8.0 Hz, 2.1 Hz, C-5'), δ 6.54 (1H, d, J = 2.2 Hz, C-3'), δ 7.0 (1H, d, J = 8.2 Hz, C-6'); nOe: irradiation at δ 3.80 caused enhancement of signal at δ 6.54.

4'-O-Methylkievitone (**13**): UVMethOH λ_{max} nm: 233 sh, 270 sh, 292, 345; MS m/e (rel. intens.): 370(67)M⁺, 340(33), 280(38), 221(67), 205(44),

192(39), 177(33), 167(44), 165(100), 150(78), 137(33), 136(7); PMR 250 MHz (CD_3)₂CO: δ 1.64 (3H, s, CH₃), δ 1.74 (3H, s, CH₃), δ 3.24 (2H, d, J = 7.0 Hz, C-1''), δ 3.74 (3H, s, OCH₃), δ 4.47 (1H, m, C-3), δ 4.53 (1H, m, C-2b), δ 4.63 (1H, m, C-2a), δ 5.20 (1H, br.t., C-2''), δ 6.04 (1H, s, C-6), δ 6.43 (1H, d, d, J = 8.4 Hz, 2.5 Hz, C-5'), δ 6.49 (1H, d, J = 2.5 Hz, C-3'), δ 7.07 (1H, d, J = 8.4 Hz, C-6'); Gibbs reagent: weak positive.

Kievitone hydrate (**14**): UV MeOH λ_{max} nm: 210, 293, 340 sh, MS m/e (rel. intens.): 374(23)M⁺, 356(21), 338(8), 300(44), 283(26), 221(49), 207(18), 192(18), 179(36), 177(16), 165(100), 149(46), 146(49), 145(26), 123(46), 107(16); PMR 80 MHz (CD_3)₂CO: δ 1.30 (6H, s, 2CH₃), δ 1.58 (2H, m, C-2''a, C-2''b), δ 2.85 (2H, m, C-1''a, C-1''b), δ 4.10 (1H, m, C-3), δ 4.25 (1H, m, C-2a), δ 4.65 (1H, m, C-2b), δ 6.03 (1H, s, C-6), δ 6.33 (1H, d, d, J = 8.3 Hz, 2.2 Hz, C-5'), δ 6.45 (1H, d, J = 2.2 Hz, C-3'), δ 6.95 (1H, d, J = 8.3 Hz, C-6').

5-deoxykievitone hydrate (**15**): UV MeOH λ_{max} nm: 230 sh, 284, 330 sh; MS m/e (rel. intens.): 358(22)M⁺, 340(27), 338(5), 325(10), 322(8), 296(5), 285(11), 270(8), 267(16), 266(17), 254(6), 253(5), 242(12), 223(7), 206(10), 205(100), 203(10), 189(5), 176(10), 167(20), 163(16), 161(10), 149(92), 137(15), 136(38), 135(22), 134(10), 123(16), 121(10), 107(16); PMR 400 MHz, (CD_3)₂CO: δ 1.19 (6H, s, 2CH₃), δ 1.49 (2H, m, C-2''a, C-2''b), δ 2.96 (2H, m, C-1''a, C-1''b), δ 4.10 (1H, d, J = 12.0 Hz, 4.8 Hz, C-3), δ 4.56 (1H, d, d, J = 12.0 Hz, 4.8 Hz, C-2b), δ 4.60

(1 H, m, C-2a), δ 6.19 (1 H, d, J = 8.4 Hz, 2.4 Hz, C-5'), δ 6.30 (1 H, d, J = 2.4 Hz, C-3'), δ 6.47 (1 H, d, J = 8.4 Hz, C-6'), δ 6.48 (1 H, d, J = 8.6 Hz, C-6), δ 6.85 (1 H, d, J = 8.6 Hz, C-5).

Cyclokievitone hydrate (**16**): UV MeOH λ_{\max} nm: 213 sh, 222 sh, 240 sh, 294, 335 sh; MS m/e (rel. intens.): 372(50)M⁺, 354(13), 353(3), 339(6), 337(5), 321(6), 254(63), 237(100), 219(13), 203(18), 137(63), 136(28); PMR 250 MHz (CD₃)₂CO: δ 1.28 (6 H, s, 2 CH₃), δ 3.05 (2 H, m, J \approx 10.3 Hz, C-1'' or C-2''), δ 4.23 (1 H, d, d, J = 11.3 Hz, 6.3 Hz, C-3), δ 4.55 (1 H, d, d, J = 11.0 Hz, 6.2 Hz, C-2b), δ 4.65 (1 H, m, C-2a), δ 4.76 (1 H, m, J \approx 10.2 Hz, C-2'' or C-1''),

δ 5.90 (1 H, s, C-6), δ 6.34 (1 H, d, d, J = 8.3 Hz, 2.4 Hz, C-5'), δ 6.45 (1 H, d, J = 2.3 Hz, C-3'), δ 6.94 (1 H, d, J = 8.3 Hz, C-6').

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