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N,β-D-Glucopyranosyl vincosamide, a light regulated indole alkaloid from the shoots of *Psychotria leiocarpa*

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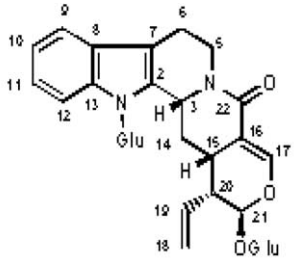
Abstract

From leaves of *Psychotria leiocarpa*, an indole alkaloid was isolated to which the structure *N*,β-D-glucopyranosyl vincosamide (**1**) was assigned. This represents the first report of an *N*-glycosylated monoterpenoid indole alkaloid. In field-grown plants highest amounts of **1** were found in the leaves (2.5% of dry wt) and fruit pulp (1.5% dry wt). Lower amounts were found in the stems (0.2% dry wt) and the seeds (0.1% of dry wt), whereas the alkaloid was not detected in the roots. The accumulation of **1** in aseptic seedlings was also restricted to the shoots and increased with plant age and light exposure, independent of the supply of sucrose in the culture medium.

Keywords: *Psychotria leiocarpa*; Rubiaceae; Light regulation; Organ distribution; Indole alkaloid; *N*,β-D-glucopyranosyl vincosamide

1. Introduction

The genus *Psychotria* (Rubiaceae) has been shown to produce alkaloids, mainly of the polyindolinic type (Adjibade et al., 1992; Hart et al., 1974; Libot et al., 1987). The analysis of several southern Brazilian species has revealed peculiar chemical features since all of the identified alkaloids are indole monoterpenic glucosides (Kerber et al., 2001; Santos et al., 2001). Therefore, alkaloids could be a useful chemotaxonomic character to distinguish groupings within a complex genus such as Psychotria, as previously proposed (Solís et al., 1995). A number of studies have reported different pharmacological effects of *Psychotria* alkaloids, such as inhibition of the aggregation of human platelets (Beretz et al., 1985), cytotoxicity (Roth et al., 1986), and analgesic activity (Leal and Elisabetsky, 1996). *Psychotria leiocarpa* Cham. et Schlecht. grows as a shrub, reaching up to 2 m in height, and is native to Argentina, Paraguay, and Brazil (Smith and Downs, 1956). It is widely distributed in the southern State of Rio Grande do Sul (Dillenburg and Porto, 1985). The crude ethanolic extract of *P. leiocarpa* leaves yielded a non-specific analgesic activity in the tail flick test (Elisabetsky et al., 1997). In the present study, an unusual *N*-glycosylated indole alkaloid, *N*,β-D-glucopyranosyl vincosamide (**1**) (Fig. 1), was identified as the major component from an ethanolic leaf extract of *P. leiocarpa*. The organ distribution in field-grown plants and the effects of age and light on the accumulation of **1** in aseptic seedlings were examined, indicating that amounts of this alkaloid are strongly regulated.



1 N, β-D-glucopyranosyl vincosamide
Glu = glucose

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2. Results and discussion

The major alkaloid in *P. leiocarpa* leaves, identified as *N*,β-D-glucopyranosyl vincosamide (**1**), was isolated as an amorphous pale yellow powder and the UV spectrum showed a maximum absorption at 225 nm and a broad shoulder in the region of 275 nm (typical heteroyohimbine chromophore). The ^{1}H NMR spectrum, measured in CD_3OD, revealed a number of characteristics of the alkaloid (Table 1). The presence of an unsubstituted indole ring system was deduced from the signals at δ 7.63 *d* (8.2), 7.44 *d* (7.8), 7.12 *dd* (8.2, 7.1) and 7.06 *dd* (7.8, 7.1) (Erdelmeier et al., 1991). A vinylidene substituent was indicated by the signals at δ 5.48 *ddd* (17.2, 10.1, 9.8), 5.26 *dd* (17.2, 1.9), and 5.14 *dd* (10.3, 2.1) . Moreover, the presence of an *O*-β-D-glucose substituent was suggested by the anomeric proton signal at δ 4.71 *d* (7.8) and the signals at δ 3.90 *dd* (12.1, 2.1) and 3.68 *dd* (12.1, 5.4) for the protons at C-6 (Erdelmeier et al., 1991).

Several *O*-glycosides of monoterpene indole alkaloids are known, e.g. strictosidine, vincoside, strictosamide, and vincosamide. These alkaloids are frequently found in Rubiaceae, particularly in neotropical species of the genera *Psychotria* and *Palicourea*, an exclusively neotropical genus, (Achenbach et al., 1995, Kerber et al. 2001) and *Nauclea* or *Neonauclea* (Itoh et al., 2003). All of these share the characteristics mentioned before. The data reported for vincosamide (Erdelmeier et al., 1991) most resembled the ^{1}H and ^{13}C NMR values of **1**. In fact, all of the signals reported for vincosamide were found in the spectra, but some signals showed discrete differences in the chemical shift values . In the ^{1}H NMR spectrum the signal of H-12 appeared at 7.63 ppm instead of 7.30 ppm, one of the H-14 protons at 2.34 ppm instead of 2.47 ppm, the H-3 signal at 5.12 ppm instead of 4.94 ppm, and some smaller differences in other signals (Table 1). In the ^{13}C NMR spectrum, the signals of C-2, C-7, C-8, C-12, and C-14 showed chemical shift differences of up to 2.7 ppm (Table 2). Most importantly, the alkaloid showed an additional set of β-D-glucose signals, with the anomeric proton at δ 5.12 *d* (8.9), the C-6 protons at δ 3.97 *dd* (12.1, 2.0) and 3.79 *dd* (12.1, 5.9), and the signal of the anomeric carbon at δ 85.9. This characteristic chemical shift of the anomeric carbon, and the fact that the protons and carbons near

Table 1
^{1}H NMR data, ^{1}H–^{1}H COSY correlations (600 MHz), and NOE interactions in the NOESY spectrum (600 MHz) of *N*-β-D-glucopyranosyl vincosamide (**1**) in methanol-d_4 (protons in brackets indicate weak correlations)

H	δ (ppm)	m	*J* (Hz) [coupled H]	COSY correlations	NOESY correlations
3	5.12	*d br*	11.3 [14a]	H-14a, (H-14b)	(H-5a), (H-5b), (H-14a), H-14b, (H-15)
5a	2.84	*ddd*	12.4 [5b], 11.8 [6a], 3.3 [6b]	H-5b, H-6a, H-6b	(H-3), H-5b, H-6a
5b	5.05	*ddd*	12.4 [5a], 4.5 [6a], 1.5 [6b]	H-5a, H-6a, H-6b	(H-3), H-5a, H-6a
6a	2.65	*ddd*	16.0 [6b], 11.8 [5a], 4.5 [5b]	H-5a, H5-b, H-6b	H-5a, H-5b, H-6b
6b	2.78	*ddd*	16.0 [6a], 3.3 [5a], 1.5 [5b]	H-5a, H-5b, H-6a	H-6a, (H-9)
9	7.44	*d*	7.8 [10]	H-10, (H-11), (H-12)	(H-6b)
10	7.06	*ddd*	7.8 [9], 7.1 [11], 1.1 [12]	H-9, H-11, (H-12)	–
11	7.12	*ddd*	8.2 [12], 7.1 [10], 1.1 [9]	(H-9), H-10,H-12	–
12	7.63	*d*	8.2 [11]	(H9), (H-10), H-11	H2″, (H-3″), (H-5″)
14a	1.38	*dddd*	13.5 [14b], 13.5 [15], 11.3 [3], 2.5 [20]	H-3, H-14b, H-15	(H-3), H-14b, H-15, H-19
14b	2.30	*d br*	13.5 [14a]	(H-3), H-14b, H-15	H-3, H-14a, (H-15), (H-20)
15	3.40	*m*		H-14a, H-14b, (H-17), H-20	(H-3), H-14a, (H-14b), (H-17), H-20
17	7.48	*d*	2.7 [15]	(H-15)	(H-15), (H-21)
18a	5.14	*dd*	10.2 [19], 2.0 [18b]	H-18b, H-19	(H-18b), H-19
18b	5.26	*dd*	17.2 [19], 2.0 [18]	H-18a, H-19	(H-18a), H-19, H-20
19	5.48	*ddd*	17.2 [18b], 10.2 [18a], 9.9 [20]	H-18a, H-18b, H-20	H-14a, H-18a, H-18b, H-20, H-21
20	2.75	*m*		H-15, H-19	(H-14b), H-15, H-18b, H-19, (H-2′)
21	5.51	*d*	1.9 [20]	–	(H-17), H-19, H-1′
1′	4.71	*d*	8.1 [2′]	H-2′	H-21, H-2′, H-3′, H-5′
2′	3.24	*dd*	9.2 [3′], 8.1 [1′]	H-1′, H-3′	(H-20), H-1′, H-4′, H-5′
3′	3.37	*dd*	9.2 [2′], 8.8 [4′]	H-2′, (H-4′), H-5′	H-1′, H-4′, H-5′, H-6′a
4′	3.54	*m*		(H-3′)	H-2′, H-3′, H-5′, H-6′a
5′	3.50	*m*		H-3′, H-6′a, H-6′b	H-1′, (H-2′), H-3′, H-4′, H-6′a, H-6′b
6′a	3.68	*dd*	12.1 [6′b], 5.4 [5′]	(H-4′), H-5′, H-6′b	H-3′, H-4′, H-5′, H-6′b
6′b	3.90	*dd*	12.1 [6′a], 2.1 [5′]	H-5′, H-6′a	H-5′, H-6′a
1″	5.12	*d br*	8.9 [2″]	H-2″	(H-2″), H-3″, H-5″
2″	4.13	*dd*	8.9 [1″], 9.0 [3″]	H-1″, H-3″	H-12, (H1″), H-3″
3″	3.54	*m*		H-2″	(H-12), H-1″, H-2″
4″	3.63	*m*		–	–
5″	3.54	*m*		H-6″a, H-6″b	(H-12), H1″, H-6″a, H-6″b
6″a	3.79	*dd*	12.1 [6″b], 5.9 [5″]	H-5″, H-6″b	H-5″, H-6″b
6″b	3.97	*dd*	12.1 [6″a], 2.0 [5″]	H-5″, H-6″a	H-5″, H-6″a

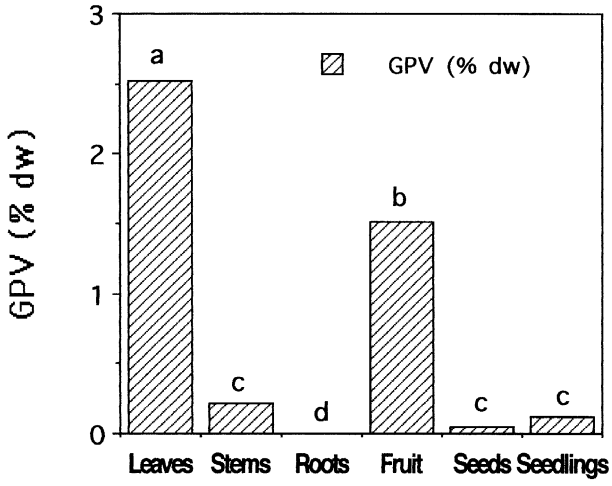

Fig. 1. Distribution of *N*-β-D-glucopyranosyl vincosamide (**1**) (GPV, % dry wt) in various parts of field-grown adult plants and in whole seedlings of *Psychotria leiocarpa*. Bars not sharing a letter are significantly different by a Duncan test ($P \leqslant 0.05$).

the indole nitrogen showed chemical shift differences, indicated that the additional glucose group should be attached to this indole nitrogen.

The presence of the second sugar residue was also revealed by the mass spectra. High-resolution electrospray mass spectrometry yielded a mass of 659.323 for the ion $[M-H]^-$, compatible with the molecular formula $C_{32}H_{40}O_{13}N_2$. The molecular mass difference between **1** and vincosamide revealed the presence of the second sugar residue.

The stereochemistry of the vincosamide part was established by the exact reproduction of most chemical shifts in the aliphatic part of the molecule, and exact coincidence of the coupling constants with literature data published for vincosamide (Erdelmeier et al., 1991). The NOESY spectrum yielded additional proof for the stereochemistry (Table 1) and the position of the *N*-glucosyl group. The signal at δ 7.62 for H-12 showed a strong cross peak with that for H-2″ and a weak cross peak with the signal at δ 3.54 for H-3″ and H-5″. The HMBC spectrum gave $^3J_{CH}$ long-range correlations connected through the nitrogen atom between the anomeric proton at δ 5.12 and C-2 (δ 136.1) and C-13 (δ 137.7) (Atta-Ur-Rahman et al., 1987; Ouyang et al., 1994). These data served to establish the attachment of the sugar unit to N-1 of the indole moiety. Intraresidual NOE effects (NOESY) were used to verify the anomeric configuration of the *N*-pyranoside. An intense cross peak between H-1″ (δ 5.12) and the signals for H-3″ and H-5″ (δ 3.54) indicated the β-anomeric configuration (Agrawal et al., 1992). The alkaloid was then assigned as *N*, β-D-glucopyranosyl vincosamide.

The first naturally occurring *N*-D-glucopyranosyl indole derivatives were the blue pigments trichotomine G1 and a mixture of anomers of *N,N*-di-(D-glucopyranosyl) trichotomine isolated from the fruits of *Clerodendron trichotomum* Thunb (Verbenaceae) (Iwadare et al., 1974). Dihydroindole alkaloids, bearing a glycosidic linkage with sugar in an oxidized state on N_a, were isolated from leaves of *Rhazya stricta* Decaisne (Apocynaceae) (Atta-ur-Rahman, 1987; Habbib-ur-Rahman, 1996). In a survey of the literature, only two additional indole alkaloids, containing this unique chromophore, were found. The simple tryptophan derivatives bruceolline F and PFP-1 were obtained from the roots of *Brucea mollis* (Simaroubaceae) (Ouyang et al., 1994) and the flowers of *Pueraria lobata* (Fabaceae) (Kinjo et al., 1988), respectively.

During the extraction and concentration procedure, small hydrolysis of **1** can yield vincosamide (data not shown), but the HPLC C_{18} column step employed in the current study separated with baseline resolution the hydrolysis products from the main alkaloid; The fact that **1** is a genuine alkaloid of *P. leicarpa* was confirmed by the analysis of rapidly prepared methanolic extracts of fresh leaves, in which no hydrolysis takes

Table 2
^{13}C NMR data (150.9 MHz) and ^1H–^{13}C HMBC correlations of *N*-β-D-glucopyranosyl vincosamide (**1**) in methanol-d_4

C no.	δ (ppm)	HMBC correlations
2	136.10	H-3, H-1″
3	54.48	H-5b, H-14a
5	41.63	H-6b
6	22.29	H-5a
7	111.54	H-5b, H-6b, H-9
8	129.55	H-10, H-12
9	119.32	H-11
10	121.27	H-12
11	122.87	H-9
12	114.76	H-10
13	137.73	H-9, H-11, H1″
14	35.58	–
15	27.91	H-14a, H-17, H-20, H-21
16	109.13	H-17, H-20
17	149.25	H-21
18	120.70	H-20
19	133.41	H-18b, H-20
20	44.13	H-18a, H-18b, H-21
21	97.48	H-17, H-20, H-1
22	166.33	H-17
1′	99.63	H-19, H-2′
2′	74.87	H-3′, H-5′
3′	77.99	H-2′, H-4′
4′	71.56[a]	H-3′
5′	78.35	H-6′a
6′	62.70	H-5′
1″	87.56	–
2″	71.99	–
3″	79.15	H-2″, H-4″, H-5″
4″	71.61[a]	H-3″, H-5″
5″	81.24	H6″a
6″	62.96	H-5″

[a] Assignment might be interchanged.

place. This fast extraction procedure was used throughout the alkaloid distribution studies and the seedling experiments.

The concentration and distribution of **1** in various parts of the seedlings and adult plants of *P. leiocarpa* was examined. Germination was very slow for all treatments; mean germination times (in weeks) were as follows: dark-grown with sucrose = 7.7, dark-grown without sucrose = 10.9, light-grown with sucrose = 10.2, light-grown without sucrose = 9.8. Light-grown seedlings had from 0.05 to 0.23% of **1** on a dry weight basis (Figs. 1 and 2). Leaves of adult field-grown plants displayed approximately 2.5% of **1**, followed by the fruit pulp with approximately 1.5% of the alkaloid, and the stems with 0.24%. Seeds had the lowest amounts of **1** (0.05% dry wt.), whereas no alkaloid was detected in the roots, as was the case for the roots of seedlings (Fig. 1). The lack or low concentration of alkaloid in plant parts developed in darkness and the higher amounts present in light exposed organs suggests the involvement of light in controlling the accumulation of **1**.

The contents of **1** in the shoots and roots of light and dark-grown aseptic seedlings of two ages, both in the presence and absence of exogenous sugar were examined. *N*,β-D-glucopyranosyl vincosamide (**1**) was consistently not detected in the roots of seedlings from any of the treatments. The presence of this alkaloid in shoots of aseptically germinated seedlings shows that **1** is a genuine metabolite of *P. leiocarpa* and is not the product of microorganisms potentially present in field-grown plants. Shoots of dark-grown seedlings had significantly lower amounts of the alkaloid (between 7 and 15 times less) compared to the same parts of light-grown seedlings at 100 and 150 days after inoculation, independent of the presence of sucrose in the culture medium (Fig. 2). Dark-grown seedlings not supplemented with sugar survived for this long period on seed reserves, compatible with the slow germination and early growth displayed by the species. The residual amount of **1** in dark grown seedlings may be related to brief irradiation with light while checking for germination and explant contamination. Light has been shown to be essential for the accumulation of vindoline and its derivatives in seedlings of *Catharanthus roseus* due to light requirements for the expression of tabersonine 16-hydroxylase, as well as for the activation of the later enzymatic steps involving deacetoxyvindoline 4-hydroxylase (D4H) and deacetylvindoline 4-*O*-acetyltransferase (DAT) (Schröder et al., 1999; De Luca, 2000). The lower amount of **1** in dark-grown seedlings, both in the presence and absence of sucrose, compared to the light-grown ones suggests that alkaloid biosynthesis in the dark is not limited by carbon supply. Light may be necessary for the activation of enzymes or transcription of the biosynthetic genes involved in alkaloid assembly, in conjunction with chloroplast differentiation. Alternatively, photosynthetic carbon metabolism may be related to the higher accumulation of **1** in light-grown seedlings by providing specific biosynthetic intermediates such as terpene moieties. Secologanin, used in the biosynthesis of most monoterpene indole alkaloids, derives from the plastid deoxyxylulose phosphate pathway, at least in *C. roseus* (Memelink et al., 2001). The roles for light proposed above are further supported by the fact that sucrose supplementation in younger light-grown seedlings (100 days after sowing) resulted in lower alkaloid accumulation compared to non-supplemented seedlings (+Suc Light versus −Suc Light, open bars) (Fig. 2). Carbohydrates are known to repress the expression of various photosynthetic genes (Sheen et al., 1999). In older seedlings (150 days after sowing), this difference was no longer observed (+Suc Light versus −Suc Light, hatched bars) probably due to depletion of sucrose in the medium, leading to enhanced photoautotrophic metabolism.

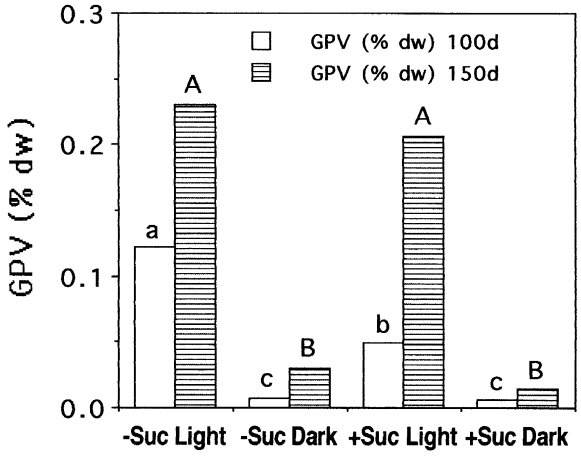

Fig. 2. Content of *N*-β-D-glucopyranosyl vincosamide (**1**) (GPV, % dry wt) in shoots of aseptic *Psychotria leiocarpa* seedlings grown under light or darkness in the presence or absence of sucrose in the culture medium. Seedlings were extracted 100 (open bars) and 150 (hatched bars) days after sowing. Bars within the same age group not sharing a letter are significantly different by a Duncan test ($P \leqslant 0.05$).

The yield of **1** in shoots of light-grown seedlings cultivated in medium devoid of sucrose increased with seedling age from approximately 0.12% dry wt at 100 days after sowing to 0.23% dry wt 50 days later (Fig. 2). In leaves of adult field-grown plants, the amount of **1** raises by an order of magnitude to approximately 2.5% dry wt. relative to the quantities observed in shoots of seedlings 150 days after sowing. Taken together, these data suggest that the accumulation of **1** is also developmentally regulated.

In conclusion, the major alkaloid of *Psychotria leiocarpa* leaves has been defined as *N*,β-D-glucopyranosyl vincosamide, representing the first *N*-glycosylated monoterpenoid indole alkaloid to be reported. The accumulation of the alkaloid was shown to be tempor-

ally and spatially regulated, displaying organ specific distribution (restricted to shoots, mainly leaves), developmental control (increasing with age of seedlings), and light activation (increasing in photomorphogenic conditions, independent of the presence of carbohydrates).

3. Experimental

3.1. General procedures

UV spectra were obtained in MeOH, using a Cintra 5 spectrophotometer. NMR spectra were determined in CD_3OD. Chemical shifts are given in δ relative to the solvent signal at δ 3.30 for protons, or δ 49.00 for carbon. The ^1H NMR spectrum was recorded at 600 MHz and ^{13}C NMR at 150.9 MHz. Mass spectra were recorded in a Finnigan MAT TQS-70 double quadrupole spectrometer with an electrospray ionization interface. Samples were introduced in column by-pass mode at 1 μg per injection. The eluent was MeOH-0.1 mM NaOAc at a flow rate of 1.2 ml min^{-1}. vaporizer, repeller and source temp. were at 70°, 50 V and 250°, respectively. The HPLC system consisted of a Waters 2690 separation module, a reversed phase column (C_{18} Nova Pak Waters 3.9×150 mm) and a photodiode-array detector (PDA 996 Waters).

3.2. Plant material

P. leiocarpa Cham. et Schlecht. was collected (May, 1995 and 2001) at Morro Santana in Porto Alegre, Brazil, and was identified by Marcos Sobral (Faculty of Pharmacy, UFRGS). A voucher specimen (ICN SOBRAL 7898) is deposited at the Herbarium of the Universidade Federal do Rio Grande do Sul (UFRGS, ICN).

3.3. Extraction and isolation

Dried and powdered leaves (100 g) were extracted with EtOH (3×0.5 l, 4 days each extraction) at room temp. After filtration, the removal of the solvent under red. pres. yielded a crude extract (7.5 g). The residue was suspended in H_2O (50 ml) and washed with petrol. The soluble portion was evapd to dryness at 55 °C and yielded a brownish syrup (4.0 g). An aliquot (3.0 g) was fractionated on a C-size column packed with Lichroprep C_{18} (Merck) and eluting from H_2O to MeOH. Fraction 18 (0.15 g) was obtained as a mixture in the proportion of H_2O to MeOH ca. 1:1. HPLC separation of this fraction using a radial pack column (PrepPack C_{18} 25×100 mm, Waters) eluting with a gradient of H_2O: MeOH (1:1 V/V) to MeOH gave alkaloid **1**, in a pure form (9 mg).

For experimental treatments, fresh seedlings and other plant parts were quick frozen in liquid nitrogen and ground in 100% MeOH, using mortar and pestle. After debris removal by centrifugation (5 min at 2500 *g*, room temp, in a bench-top clinical centrifuge), extracts were filtered (0.45 μm) and analyzed by HPLC. *N*,β-D-glucopyranosyl vincosamide (**1**) content was expressed as percentage of extracted dry wt.

3.4. Alkaloid analysis

HPLC analyses were carried out using a C_{18} reversed phase column and the mobile phase consisted of solvent A (MeOH–H_2O, 40:60) for the first 5 min, followed by a linear gradient ending with solvent B (MeOH) within 15 min; flow rate was 1 ml min^{-1}. For the quantitation of **1**, 10 μl samples of each extract were injected and an external standard curve was generated with pure **1** isolated from leaves. Eluting compounds were detected by UV spectra recorded with a photodiode-array detector.

3.5. *N*,β-D-Glucopyranosyl vincosamide (*1*)

Amorphous yellow powder; UV λ_{max}^{MeOH} nm: 225. ^1H and ^{13}C NMR data are shown in Tables 1 and 2, respectively. Positive FABMS *m/z* (rel. int.): 661 $[M+H]^+$ (50), 659 $[M-H]^+$(10), 499 $[M+H\text{-Glu}]^+$ (20), 185 (100). HR-ESI-MS *m/z*: 659.3235 (calc. for $C_{32}H_{39}O_{13}N_2$ $[M-H]^-$ 659.2452).

3.6. Seedling growth conditions

Seeds from the ripe fruit (purple colored) were collected, separated from the flesh, washed in distilled water and surface sterilized in 70% EtOH (v/v) for 1 min and 1.5% NaClO (v/v), with a few drops of neutral detergent, under agitation for 15 min. Following three washes in sterilized distilled water, seeds were inoculated in 0.1 ×salts of Murashige and Skoog (1962) and 0.75% (w/v) microbiological agar, with (15 g l^{-1}) or without sucrose supplementation. Germination was slow and mean germination times were calculated as a kinetic parameter (Labouriau and Osborne, 1984). Seedlings were grown in darkness or under white fluorescent light (approximately 40 μmol m^{-2} s^{-1} at explant level). At 100 and 150 days after sowing, seedlings were analyzed for the content of **1**. Criteria for harvesting of light-grown seedlings for phytochemical analysis was the presence of 2–4 leaves besides cotyledons; for dark-grown seedlings harvesting criteria was similar height at sampling time, since leaves do not expand in etiolated seedlings. In spite of the long period in darkness, seedlings were alive and viable at harvest time (as demonstrated by the capability of de-etiolation under light, data not shown) both with and without sucrose supplementation. Such long survival may be directly related to the very slow germination and early growth displayed by the species under the experimental conditions.

Statistical analyses were carried out by simple or factorial (sucrose×light) ANOVA, followed by Duncan test ($P \leqslant 0.05$). Extracts were obtained and analyzed at least in triplicates (of three individuals each) for seedlings and in quadruplicates for plant parts (four portions per plant part for each of four individuals).

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