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Production of (+)-5-deoxystrigol by *Lotus japonicus* root culture

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Abstract

Lotus japonicus roots, cultured in a modified B5 medium, produced and secreted germination stimulants that induced *Striga hermonthica* seed germination. The germination-inducing activity was detected both in the roots and the culture filtrate. Following bioassay-guided purification procedures, an active compound was isolated from hexane extracts of the roots and the culture filtrate. Based on chromatographic behaviour on HPLC, and ^{1}H NMR, UV, MS and CD spectroscopic analyses, the germination stimulant was identified as (+)-5-deoxystrigol.

Keywords: *Lotus japonicus*; Leguminosae; *Striga hermonthica*; Orobanchaceae; Root culture; Germination stimulant; (+)-5-Deoxystrigol

1. Introduction

Striga and *Orobanche* species (Orobanchaceae) are obligate root parasitic weeds, which survive only when attached to the roots of appropriate host plants. They deprive water and nutrients of the host plants and cause devastating effects on their hosts. *Orobanche* is a holoparasite, which lacks chlorophyll, while *Striga* is a hemiparasite and can fix some but not all of its own carbon. Seed germination requirements of these parasitic weeds include an after-ripening period of several months, followed by preconditioning in a warm and moist environment for one to two weeks, and finally exposure to specific chemical signals, including strigolactone compounds (Butler, 1995). Classically the strigolactones have been described as sesquiterpene lactones; however, little was known about their biogenesis until Matusova et al. (2005) reported detailed experiments using *vp*14 maize mutants. They proposed that strigolactones are a product of carotenoid cleavage and (+)-5-deoxystrigol (**1**) is a key intermediate for the biosynthesis of a series of strigolactones. Isolation of (+)-5-deoxystrigol (**1**) from hydroponic culture of *Lotus japonicus* as a branching factor for arbuscular mycorrhizal fungi (Akiyama et al., 2005), lent credit to the proposed biogenesis and revealed an ecological significance of strigolactones (Hamphrey et al., 2006).

Plant tissue culture with high productivity of a target molecule is useful for investigating the biosynthetic pathway of the metabolite because the culture is kept aseptic and cultural parameters are easily manipulated. We have previously reported production of (+)-strigol (**2**) by *Menispermum dauricum* root culture (Yasuda et al., 2003). Isolation of the strigolactone from the aseptic root culture provided the first unambiguous demonstration that strigolactones are genuine products of the plant roots. The isolation also suggested that strigolactones occur even more widely in the plant kingdom. In the present study, we report the isolation of (+)-5-deoxystrigol (**1**) from *L. japonicus* root culture, the second example of *in vitro* production of a strigolactone.

2. Results and discussion

Root tips were collected from aseptic *L. japonicus* seedlings and cultured on a rotary shaker in the dark in liquid B5 media (Gamborg et al., 1968) containing various

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concentrations of indole-3-butyric acid (IBA), indole-3-acetic acid or 1-naphthaleneacetic acid. Preliminary experiments showed that the media containing 5 μM IBA gave the highest root growth and *Striga hermonthica* seed germination-inducing activity. The culture media, in which no roots were grown, did not stimulate *Striga* seed germination. Temporal changes of root growth, activity in the culture filtrate, and hexane and EtOAc extracts sequentially prepared from the culture filtrate were examined (Fig. 1). Roots grew well, with about a 60-fold increase in 20 days. Formation of the active compounds accelerated as roots entered late-log phase, typical to the formation of secondary metabolites (Sugimoto et al., 2007). Finding the activity in the hexane extracts suggested that the roots produce and secrete less polar stimulant(s) than (+)-strigol (**2**), which was not extracted by an apolar solvent (Yasuda et al., 2003). In the light of a previous finding that production of strigolactones by red clover roots was stimulated under low phosphate conditions (Yoneyama et al., 2001), *L. japonicus* roots were cultured in media with different concentrations of phosphate for 20 days and germination-inducing activity in each of the culture filtrates was evaluated (Fig. 2). At 10 and 100 μM, the activity was much higher than that at 1000 μM, the original phosphate concentration in B5 medium (Gamborg et al., 1968). No significant difference in the activity was observed between 10 and 100 μM, but since the latter gave a better root growth than the former, the medium with 100 μM phosphate was employed for further work. Phosphate concentrations did not affect pH values in the media after 20 days of culture.

L. japonicus roots were cultured for 20 days, and then roots and culture filtrate were collected separately. Based on preliminary experiments in which hexane extracts obtained from the roots and the culture filtrate gave the active compound with the same chromatographic behaviour at a final step of purification by HPLC, both of the hexane extracts were combined and subjected to purification procedures. The combined extracts were separated

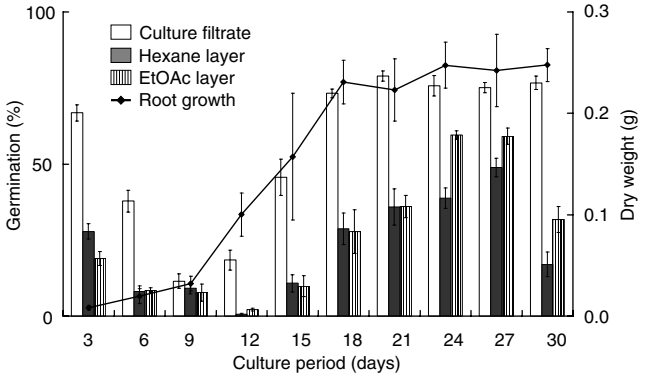


Fig. 1. Time course of *L. japonicus* root growth and germination-inducing activity in the culture filtrate. Excised roots (ca. 3.5 mg dry wt.) were cultured in 200-ml flasks containing 50 ml of medium. Culture filtrate was treated first with the same volume of hexane, and then with EtOAc. Data are the means ± SE ($n = 3$).

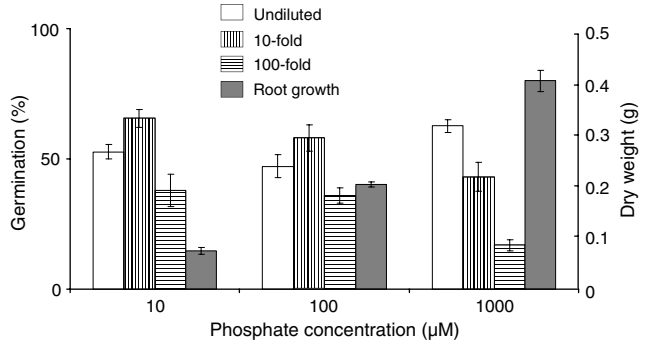


Fig. 2. Effects of phosphate concentration in culture media on *L. japonicus* root growth and germination-inducing activity in the culture filtrate. Data are the means ± SE ($n = 5$).

by Sephadex LH-20 column chromatography using a mixed solvent of MeOH–$CHCl_3$ (4:1) (Fig. 3a). Residues obtained from active fractions 14–22 were subjected to further Sephadex LH-20 column chromatography using MeOH–H_2O (1:1) (Fig. 3b). The active fractions 21–42 in the second chromatography were combined, MeOH was removed, and then active components were extracted with EtOAc from the aqueous phase. After removing the EtOAc, the residue was further purified by semi-preparative HPLC, resulting in the separation of a highly active

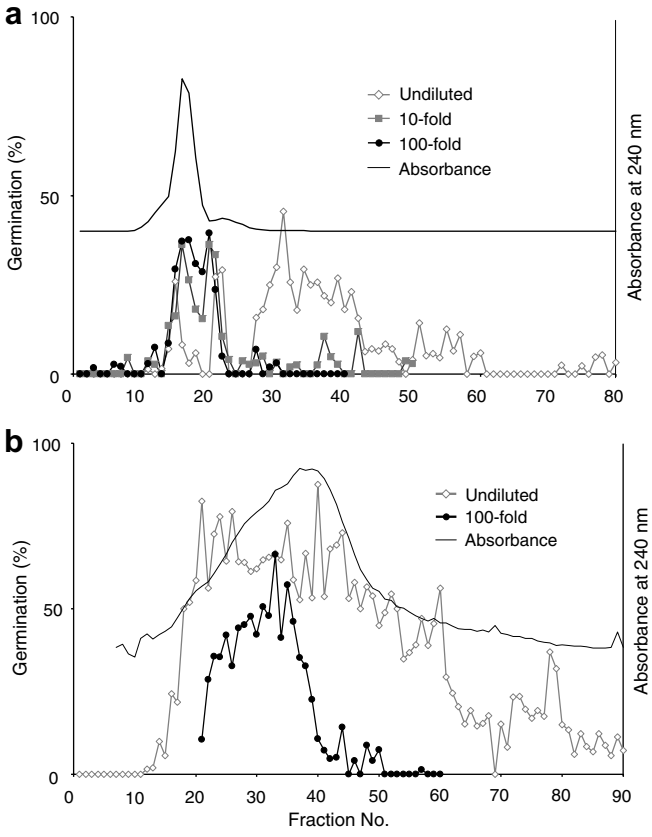


Fig. 3. Separation of the germination stimulant extracted from *L. japonicus* roots and culture filtrate by LH-20 chromatography. MeOH–$CHCl_3$ (4:1) (a) and MeOH–H_2O (1:1) (b) were used as a mobile phase.

fraction at 21 min with a much less active fraction at 28 min (Fig. 4). Extracts obtained from the active fraction at 21 min were subjected to analytical HPLC connected with a mass spectrometer in the ESI mode as well as a UV detector. An active substance was eluted at 21.5 min (Fig. 5), and when subjected to ESI-MS analysis gave rise to major signals at *m*/*z* 683, 661, 353 and 331, suggesting that the molecular mass of the active substance is 330. This was confirmed by EI-MS, in which the molecular and fragment ions observed were almost the same as those reported for the authentic (±)-5-deoxystrigol (**1**) (Frischmuth et al., 1991). The ^{1}H NMR spectrum of the active substance showed major peaks identical with those reported for the authentic (±)-5-deoxystrigol (Frischmuth et al., 1991). Chromatographic behaviour on the semi-preparative and analytical HPLC as well as the UV spectrum of the active substance were also identical with those of authentic (±)-5-deoxystrigol. A CD spectrum of the active substance showed positive and negative cotton effects at around 230 nm and 206 nm, respectively, which are consistent with those reported for (+)-5-deoxystrigol (**1**) (Akiyama et al., 2005). Based on the results mentioned above, the major germination stimulant in the hexane extracts obtained from *L. japonicus* root culture was unambiguously identified as (+)-5-deoxystrigol (**1**). Approximately 13 μg of the stimulant was isolated from 500 g of cultured *L. japonicus* roots and 100 l of the culture filtrate. Isolation of (+)-5-deoxystrigol (**1**) from the *Lotus* root culture suggests that the roots are responsible for strigolactone biosynthesis.

Dose response curves of the authentic (±)-5-deoxystrigol and GR24 (**3**, equimolar mixture of all four stereoisomers) for *S. hermonthica*, *O. crenata* and *O. minor* seeds are shown in Fig. 6a–c, respectively. In all cases, no germination was observed when seeds were treated with distilled water. For *S. hermonthica* and *O. crenata* seeds, (±)-5-deoxystrigol is as active as GR24 (**3**); however, the curves differed in the activity for *O. minor* seeds at 10^{-7} and 10^{-8} M. (±)-5-Deoxystrigol (**1**) is about 10 times more active than GR24 (**3**) in inducing *O. minor* seed germination.

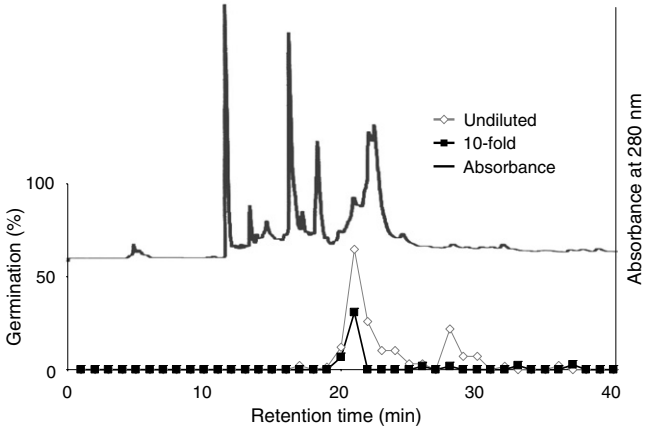


Fig. 4. Separation of germination stimulants by semi-preparative HPLC on Senshu-Pac Aquasil. Mixed solvents $CHCl_3$–hexane (3:7) and $CHCl_3$–EtOAc (1:1) were used (see Section 3).

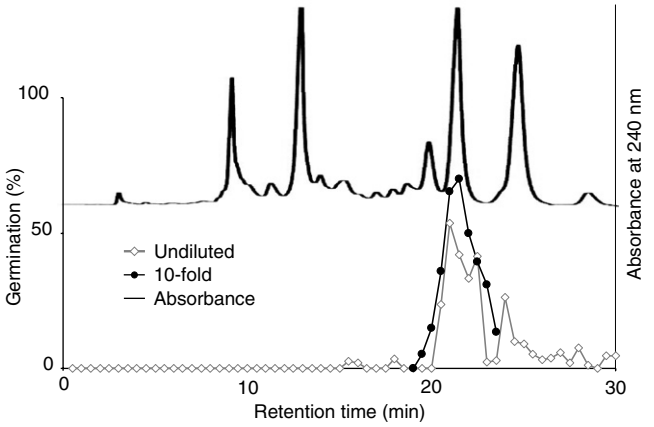


Fig. 5. Separation of germination stimulants by analytical HPLC on Develosil CNUG-5. MeOH–H_2O (1:1) was used as a mobile phase.

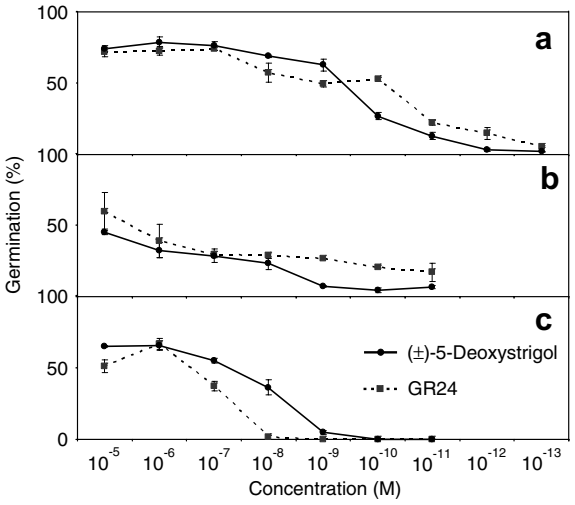
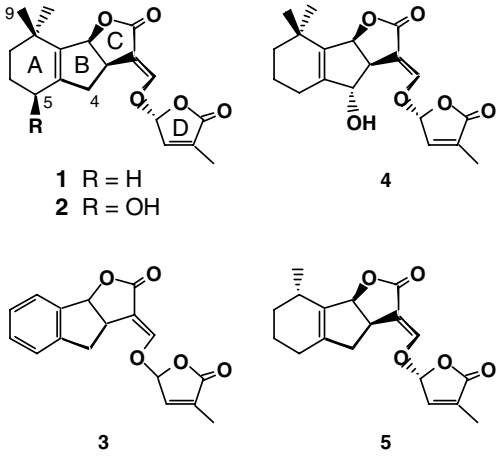


Fig. 6. Germination of *S. hermonthica* (a), *O. crenata* (b) and *O. minor* (c) seeds stimulated by GR24 and (±)-5-deoxystrigol. Data are the means ± SE ($n = 3$).



5-Deoxystrigol (**1**) was first synthesized in a racemic form as an analogue of strigol (Frischmuth et al., 1991) and its activity in inducing germination of *O. crenata* seeds was confirmed (Bergmann et al., 1993). (+)-5-Deoxystrigol (**1**) was isolated from the root exudate of *L. japonicus* by Akiyama et al. (2005) as a hyphal branching factor for arbuscular mycorrhizal fungi. Matusova et al. (2005) proposed the biosynthetic pathway of strigolactones from the carotenoid pathway, in an analogous manner to that of ABA. According to their proposal, cleavage by an enzyme of the 9-*cis*-epoxycarotenoid dioxygenase (NCED) family leads plastidic carotenoid to a strigolactone ABC-portion, which is exported to the cytosol prior to coupling with the D-ring precursor derived from the cytosolic terpene biosynthetic pathway. (+)-5-Deoxystrigol (**1**) is located in a branching point of strigolactone biosynthesis although (+)-5-deoxystrigol (**1**) itself had not been identified yet in nature when they made the proposal. Hydroxylation at C-4 and C-5 of (+)-5-deoxystrigol (**1**) converts the possible intermediate to orobanchol (**4**) and (+)-strigol (**2**), respectively, and demethylation at C-9 affords sorgolactone (**5**). However, no activity was detected in hexane extracts obtained from culture filtrate of high (+)-strigol-producing *M. dauricum* roots (Yasuda et al., 2003), although the deoxy-derivative appears to be more stable than strigol under our experimental conditions. Further work is necessary to elucidate the biosynthetic pathway of strigolactones.

We also worked on EtOAc extracts with the same procedures employed for hexane extracts. However, while only one major fraction with germination-inducing activity was obtained from EtOAc extracts of *M. dauricum* root culture filtrate (Yasuda et al., 2003), more than seven fractions with the activity were found in EtOAc extracts of *L. japonicus* root culture filtrate after separation by analytical HPLC. Their purification is currently under way. EtOAc extracts of roots were also potent in inducing *Striga* seed germination; however, contaminants hampered further purification of the active ingredients. It should be noted that no germination inducing activity was detected in root culture filtrate of *L. corniculatus* although the plant also forms symbiotic relationship with arbuscular mycorrhizal fungi (Mendoza, 2001). Non-strigolactone compounds may also act as a branching factor for the fungi.

3. Experimental

3.1. General experimental procedures

^{1}H NMR spectra were recorded in $CDCl_3$ on a Bruker Avance 500 spectrometer. MS spectra were obtained on a JEOL JMS-700 mass spectrometer in the EI or ESI mode. UV and CD spectra were recorded in MeOH–H_2O (1:1) on a JASCO J-820 spectropolarimeter and a JASCO V-820 spectrometer, respectively.

3.2. Chemicals

GR24 (**3**) and authentic (±)-5-deoxystrigol (**1**) were kindly supplied by Prof. B. Zwanenburg, University of Nijmegen, The Netherlands, and Prof. K. Akiyama, Osaka Prefecture University, Japan, respectively.

3.3. Plant materials

Striga hermonthica (Del.) Benth. seeds were collected in 2003/04, from mature plants parasitizing sorghum, at the Gezira Research Station, Sudan, and were provided by Prof. A.G.T. Babiker, Agricultural Research Corporation, Sudan. *Orobanche crenata* seeds were provided by Dr. R. Vasey, Sheffield University, UK, from the stock collected in 1997. *Orobanche minor* seeds were provided by Prof. K. Yoneyama, Utsunomiya University, Japan, from the stock collected in 2005. *Lotus japonicus* (Miyakojima, MG20) seeds and *L. corniculatus* root culture were obtained from the Biological Resource Center in *Lotus* and *Glycine*, Miyazaki University, Japan.

3.4. Germination bioassay

Striga hermonthica seeds were surface sterilized by immersion in 0.5% (w/v) NaOCl, containing a few drops of Tween 20, and sonication for 3 min in an ultrasonic cleaner. After having been rinsed 3 times with distilled H_2O and surface-dried at 27 °C in a laminar hood, the seeds were pretreated (conditioned) for 10–12 days on 8 mm glass fiber filter paper disks (ca. 50 seeds each) placed on distilled H_2O-saturated filter paper. For each bioassay, distilled H_2O and aqueous solution of GR24 (**3**) were included as negative and positive controls, respectively. Dilution series were prepared for each sample. Aqueous solutions and extracts were assayed directly, by applying 20 μl aliquots of the respective test solution to conditioned *S. hermonthica* seeds on 8 mm disks. For solutions and extracts containing organic solvents, aliquots (20 μl each) of the test solution were applied to 8 mm disks of glass fiber filter paper, which were then allowed to dry for 1 h at room temp. A disk with conditioned *Striga* seeds was placed on top of each treated disk and moistened with distilled H_2O (40 μl). The treated seeds were incubated at 30 °C and microscopically evaluated for germination (radicle protrusion) 24 h later.

O. crenata and *O. minor* seeds were conditioned at 23 °C for 6 days, treated with test solutions, incubated for 5 days at 23 °C, and then examined for germination.

3.5. Root culture

L. japonicus seeds were surface sterilized and germinated aseptically on agar medium. Root tips were collected from the seedlings and cultured in a modified B5 medium containing 4% sucrose and 5 μM IBA. For germination-inducing stimulant production, a further modified medium, in which phosphate concentration was reduced to 100 μM,

was employed. The roots were placed in 200-ml flasks containing 50 ml of the medium, and then cultured in the dark at 27 °C on a rotary shaker at 70 rpm. Germination-inducing activity in the culture filtrate reached maximum activity after 18–27 days of culture. *L. corniculatus* roots were cultured in a modified MS medium containing 3% sucrose (Akashi et al., 2000).

3.6. Isolation of (+)-5-deoxystrigol (*1*) from the roots and culture filtrate

All separation steps were monitored by UV absorption at 240 or 280 nm and by bioassays, in which fractions were diluted 1:10 repeatedly and tested on conditioned *S. hermonthica* seeds as mentioned above. At the end of each LH-20 and HPLC procedure, the column was washed with MeOH in order to recover all applied materials.

The culture filtrate (1–2 l) was collected and treated with the same volume of hexane (×2) and then with EtOAc (×2). Activity was detected both in hexane and EtOAc solutions. This extraction procedure was repeated over a period of several months to treat 100 l of the culture filtrate in total. The roots (500 g fresh wt.) were soaked in hexane (5 l) for 8 days at a room temperature and the roots were removed by filtration. The hexane solutions obtained from the culture filtrate and the roots were combined and washed with 0.2 M K_2HPO_4 (pH 9), dried over Na_2SO_4 and concentrated under reduced pressure to give a neutral hexane fraction. The residue was dissolved in MeOH and subjected to a Sephadex LH-20 CC (450 × 25 mm) using MeOH–$CHCl_3$ (4:1) as a mobile phase. Active fractions (14–22) were combined and evaporated to dryness. The residue was dissolved in MeOH and subjected to further Sephadex LH-20 column chromatography (400 × 20 mm) using MeOH–H_2O (1:1) as a mobile phase. In both phases of the LH-20 chromatography, fractions were collected every 5 ml. In the second phase of the chromatography, activity was detected in fractions 21–42. These fractions were combined, diluted with distilled H_2O (1 l), and active ingredients were extracted from the aqueous phase with EtOAc (1 l). The EtOAc solution was washed with the same volume of distilled water, dried over Na_2SO_4 and concentrated under reduced pressure at room temp. The residue was dissolved in $CHCl_3$–hexane (3:7, half-satd. with water) and subjected to semi preparative HPLC on a Senshu-Pac Aquasil column (250 × 10 mm) using 40-min gradient from $CHCl_3$–hexane (3:7, half-satd. with water) to $CHCl_3$–EtOAc (1:1, half-satd. with water). A peak eluted at 21 min was collected and the solvent was removed. The residue was dissolved in MeOH and subjected to analytical HPLC on a Develosil CN-UG-5 column (250 × 4.6 mm), using MeOH–H_2O (1:1) as a mobile phase at a flow rate of 0.5 ml/min. The column effluents were divided into two, one portion subjected to a UV detector and another to a mass spectrometer in the ESI mode. A peak showing activity was detected in the chromatogram at 21.5 min. Extracts obtained from this active fraction were subjected to semi-preparative HPLC on the Aquasil column again, and the active fraction eluted at 21 min was collected. The fraction was concentrated to dryness (ca. 13 μg) and a part of the residue was used for EI-MS and NMR analysis. Another part was subjected to analytical HPLC on the CN-UG-5 column. The fraction at 21.5 min was collected and used for measurement of UV and CD spectra.

3.7. (+)-5-Deoxystrigol (*1*)

^{1}H NMR (500 MHz) δ: 1.10 (3 H, *s*, 8-Me), 1.12 (3H, *s*, 8-Me), 1.93–2.13 (2H, *m*, 5-CH_2), 2.03 (3H, *t*, $J = 1.5$ Hz, 4′-Me), 2.70 (1H, *dd*, $J = 16.5$, 9.5 Hz, 4-H), 3.59 (1H, *m*, 3a-H), 5.51 (1H, *d*, $J = 5.5$ Hz, 8b-H), 6.14 (1H, *d*, $J = 1.5$ Hz, 2′-H), 6.92 (1H, *t*, $J = 1.5$ Hz, 3′-H), 7.41 (1H, *d*, $J = 2.5$ Hz, 9-H). Signals at δ 1.5 (7-CH_2), δ 1.6 (6-CH_2) and δ 2.3 (4-H′) were hindered by those of contaminants. UV λ_{max} 238.1 nm. CD $\Delta\varepsilon_{206}$ –18.4, $\Delta\varepsilon_{230}$ + 24.9. ESI-MS *m/z* (rel. int.) 683 $[2M+Na]^+$ (100), 661 $[2M+H]^+$ (35.7), 353 $[M+Na]^+$ (39.7), 331 $[M+H]^+$ (76.3). EI-MS *m/z* (rel. int.) 330 $[M]^+$ (8.2), 215 (23.3), 187 (20.7), 97 (100).

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