# Antimicrobial *C*-Glucoside from Aerial Parts of *Diospyros nigra*

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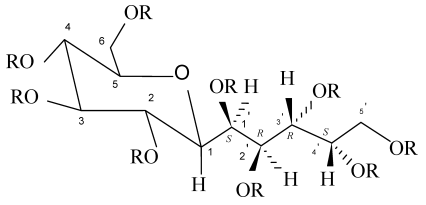
**A new *C*-alkylglucoside, diospyrodin [β-1*C*-(1′*S**,2′*R**,3′*R**,4′*S**-1′,2′,3′,4′,5′-pentahydroxypentyl)-glucopyranoside] (1) has been isolated as its nonaacetate from the leaves and stems of *Diospyros nigra*. Its structure was elucidated on the basis of chemical and spectral properties and a single crystal X-ray analysis. It showed antimicrobial activity against Gram-positive and Gram-negative bacteria.**

**Key words** *Diospyros nigra*; Ebenaceae; *C*-alkylglucoside; diospyrodin

As a part of our programme on the isolation of bioactive natural products from Indian medicinal plants, we chemically investigated for the first time the different parts of *Diospyros nigra* (J. F. GMEL) PIERRE of family Ebenaceae.[1)] The leaves and stems are used in the traditional medicine as laxative, styptic and antileucorrhoeic.[2)] Both the ethyl acetate and methanolic extracts of leaves and stems were active against Gram-positive and Gram-negative bacteria. We have previously reported a number of compounds, namely, lupeol, betulin, gallic acid and 1-*O*-bisgalloylhydroxyacetone from the stems; and cylindrin and 9,11-epoxycylindrin from the leaves.[3)] Our continued search for other chemical constituents, has resulted in the isolation of diospyrodin **1** as its nonaacetate **1a** from both ethyl acetate and methanolic extracts of the leaves and stems. Herein we report the isolation, structure elucidation and antimicrobial activity of this compound.

## Results and Discussion

Both the ethyl acetate and methanolic extracts of the leaves and stems were separately suspended in water and successively extracted with benzene, chloroform and *n*-butanol. The *n*-butanol extract was subjected to column chromatography to afford an oily mass of diospyrodin **1** containing another compound of close *Rf* value as minor impurity (approx. 5—10% from spot intensity). Repeated attempts to isolate the compound **1** in pure state by column chromatography were in vain. Hence, the major part of this oily mass was acetylated with acetic anhydride and pyridine at room temperature and the acetylated mixture on column chromatography afforded colorless needles of diospyrodin nonaacetate **1a**. It may be noted that no acetoxyl group had been detected by NMR spectral analysis of the oily mass prior to acetylation.



**1** R = H
**1a** R = Ac

Diospyrodin nonaacetate **1a**, $[\alpha]_D^{24}$ −15.2° ($c$=0.22, $CHCl_3$), showed mass ion at $m/z$ 715.2061 $[M+Na]^+$ (relative abundance, 100%) in the positive ion HR-FAB-MS indicating a molecular formula of $C_{29}H_{40}O_{19}$ (Calcd for $C_{29}H_{40}O_{19}Na$ 715.2062). This molecular formula was also verified from its elemental analysis. The IR spectrum in KBr showed absorption band for acetoxy carbonyl at 1750 $cm^{-1}$. The $^1$H-NMR spectrum ($CDCl_3$) of **1a** (Table 1) showed anomeric proton signal at highfield ($\delta$ 3.64) compared to normal value at $\delta$ 4.80—5.00 suggesting that it could be a *C*-1-alkylglucoside. The $^{13}$C-NMR spectra with DEPT experiments ($CDCl_3$) of **1a** revealed the presence of nine methyl, two methylene, nine methine and nine quaternary carbons (Table 1). The nine methyl carbons in the region of $\delta$ 20.48 to 20.83 and nine quaternary carbons in the region of $\delta$ 169.14 to 170.46 indicated the presence of nine acetoxy groups. Both these $^1$H- and $^{13}$C-NMR spectra of **1a** could be explained by considering the attachment of a pentaacetoxypentyl unit to a tetraacetylglucosyl unit at the anomeric carbon. In the HMBC correlation spectrum of **1a** (Table 1), anomeric proton ($\delta$ 3.64) exhibited long-range interactions with C-2 ($\delta$ 67.34), C-5 ($\delta$ 76.27), C-1′ ($\delta$ 67.91) and C-2′ ($\delta$ 66.17) suggesting the attachment of alkyl unit to the anomeric carbon (C-1). The selective proton decoupling experiment at $\delta$ 4.95 in the $^1$H-NMR spectrum has resulted the appearance of C-1 proton at $\delta$ 3.64 as a sharp doublet ($J$=9.5 Hz) indicating the attachment of C-1′ carbon of alkyl moiety to C-1 carbon of the glucose unit. Therefore, the structure of **1a** was considered as β-*C*-1-(pentaacetoxypentyl)-tetraacetylglucopyranoside. The skeletal structure and relative stereochemistry of compound **1a** was confirmed by single-crystal X-ray analysis. The ORTEP drawing[4)] of compound **1a** (Fig. 1) clarified the said structure having relative 1′*S**,2′*R**,3′*R**,4′*S** configuration with $^4C_1$ puckered chair conformation for pyranose ring.

Nonaacetate **1a** on deacetylation with 0.5 M aqueous methanolic HCl afforded a viscous oil, which was identical on co-TLC with the major component (compound **1**) of the oily mass used for acetylation. The viscous oil recorded mass ion at $m/z$ 313.1132 $[M-H]^-$ in the negative ion HR-FAB-MS corroborating a molecular formula of $C_{11}H_{22}O_{10}$ (Calcd for $C_{11}H_{21}O_{10}$ 313.1135). The $^1$H- and $^{13}$C-NMR spectra ($D_2O$) of **1** (Table 1) corroborated its β-1-*C*-(1′,2′,3′,4′,5′-pentahydroxypentyl)-glucopyranoside structure. The HMBC correlations (Table 1) also supported the said structure. Con-

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Table 1. $^{1}H$- and $^{13}C$-NMR and HMBC-Correlation Data of **1a** ($CDCl_3$) and **1** ($D_2O$)

| Carbon No. | **1a** | | | **1** | | |
|---|---|---|---|---|---|---|
| | $\delta_H$ | $\delta_C$ | HMBC(H→C) | $\delta_H$ | $\delta_C$ | HMBC(H→C) |
| 1 | 3.64 (dd, 4.5, 9.5) | 77.58 (d) | C-2, C-5, C-1′, C-2′ | 3.35 (dd, 4.5, 9.5) | 84.98 (d) | C-2, C-5 |
| 2 | 5.27 (t, 9.5) | 67.34 (d) | C-1, C-3, C-4 | 3.68 (t, 9.5) | 74.23 (d) | C-1, C-3 |
| 3 | 5.14 (t, 9.5) | 74.17 (d) | C-2, C-4 | 3.48 (t, 9.5) | 84.90 (d) | C-2, C-4 |
| 4 | 4.97 (t, 9.5) | 67.81 (d) | C-3, C-5, C-6 | 3.37 (t, 9.5) | 74.83 (d) | C-2, C-5 |
| 5 | 3.49 (ddd, 2.5, 4.5, 9.5) | 76.27 (d) | C-4 | 3.36 (ddd, 2.0, 4.5, 9.5) | 82.67 (d) | C-4, C-6 |
| 6A | 3.97 (dd, 2.5, 12.0) | 61.80 (t) | C-4, C-5 | 3.66 (dd, 2.0, 12.0) | 66.24 (t) | C-4, C-5 |
| B | 4.33 (dd, 4.5, 12.0) | | C-5 | 3.91 (dd, 4.5, 12.0) | | C-5 |
| 1′ | 4.95 (dd, 1.5, 4.5) | 67.91 (d) | C-1, C-2′ | 3.51 (dd, 1.5, 4.5) | 76.81 (d) | C-1, C-2′ |
| 2′ | 5.62 (dd, 1.5, 4.5) | 66.17 (d) | C-1′, C-3′ | 4.13 (dd, 1.5, 4.5) | 74.96 (d) | C-1, C-1′ |
| 3′ | 5.24 (dd, 1.5, 9.5) | 67.75 (d) | C-1′, C-2′ | 3.89 (dd, 1.5, 9.5) | 73.82 (d) | C-1′, C-2′ |
| 4′ | 5.27 (m) | 69.16 (d) | C-2′, C-3′, C-5′ | 3.93 (ddd, 1.5, 4.5, 9.5) | 75.34 (d) | C-2′, C-3′, C-5′ |
| 5′A | 3.82 (dd, 7.5, 11.5) | 62.16 (t) | C-3′, C-4′ | 3.61 (dd, 6.5, 11.0) | 68.45 (t) | C-3′, C-4′ |
| B | 4.24 (dd, 4.5, 11.5) | | C-3′ | 3.64 (dd, 4.5, 11.0) | | C-3′ |
| Ac (×9) | 1.99, 2.00, 2.01, 2.04, 2.05, 2.06, 2.08, 2.11, 2.14, (each s) | 20.48, 20.52 (×2), 20.58, 20.60, 20.67, 20.69, 20.80, 20.83 (each q) 169.14, 169.21, 169.58, 169.62, 170.22, 170.31, 170.37, 170.40, 170.46 (each s) | | | | |

Figures in parentheses are multiplicities and coupling constants, *J* (Hz).

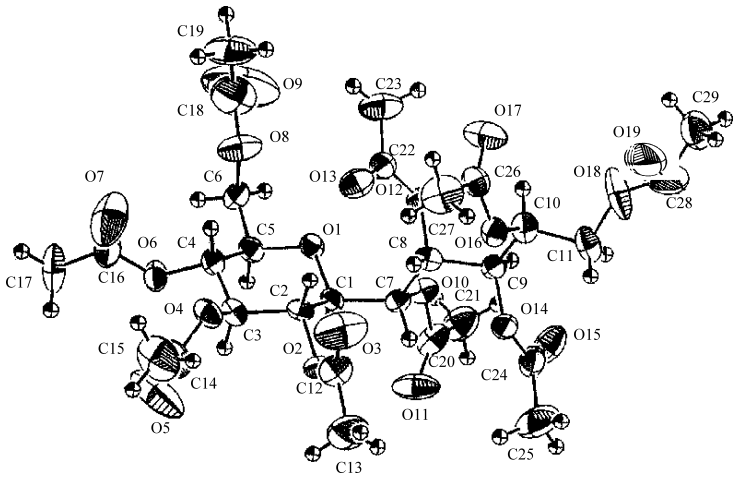


Fig. 1. ORTEP Drawing of **1a** from X-Ray Crystallographic Data with an Arbitrary Atomic Numbering

Table 2. Antimicrobial Assay Results of **1** and **1a**

| Compound | Antimicrobial activity (Inhibition zone, mm) | | | | |
|---|---|---|---|---|---|
| | Dose (μg/disc) | *B. subtilis* | *S. aureus* | *E. coli* | *P. aeruginosa* |
| **1** | 250 | 12 | 9 | 8 | 5 |
| | 125 | 6 | 5 | 3 | 2 |
| | 70 | 4 | 2 | 0 | 0 |
| **1a** | 250 | 6 | 4 | 4 | 3 |
| | 125 | 3 | 2 | 1 | 0 |
| | 70 | 0 | 0 | 0 | 0 |
| Amikacin | 30 | 18 | — | 25 | 18 |
| Penicillin | 10 units | — | 25 | — | — |

sidering all the foregoing facts, the structure of **1** was elucidated as β-1*C*-(1′*S**,2′*R**,3′*R**,4′*S**,1′,2′,3′,4′,5′-pentahydroxypentyl)-glucopyranoside.

The antibacterial activity of diospyrodin **1** and its nonaacetate **1a** was assayed by cup-plate method[5,6] against four microorganisms (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*) and the results are summarized in Table 2. The results indicated that both

disopyrodin **1** and its nonaacetate **1a** are mild active against the four tested microorganisms and **1** is relatively more active than its synthetic product **1a**. The compound **1** showed higher activity against Gram-positive bacteria compared to Gram-negative ones possibly because of unilayer cellwall of Gram-positive bacterium. Antibacterial drugs–amikacin and penicillin were screened under identical conditions for comparative antibacterial study of the compounds against each bacterium.

The isolation of diospyrodin **1** is interesting from chemotaxonomic viewpoint as this type of *C*-glucoside is reported from plant species for the first time. Earlier glycerol-*O*-glucosides have been reported from several *Lilium* species.[7,8]

## Experimental

Melting point was determined on an open capillary tube and is uncorrected. Optical rotation was measured on a JASCO DIP-370 digital polarimeter. IR spectra were recorded on a Shimadzu FTIR 8100 spectrometer. $^1$H- and $^{13}$C-NMR spectra were taken on a Varian XL 400 spectrometer and chemical shifts were recorded in $\delta$ value. $^1$H–$^1$H COSY, $^1$H–$^{13}$C correlation spectroscopy (HMQC), and HMBC spectra were obtained with the usual pulse sequence. FAB-MS were recorded on a JEOL JMS AX505 HA and a JEOL JMS 700 Mstation spectrometers using *m*-nitrobenzyl alcohol and NaI or PEG as matrix and Xe as bombarding gas. Column chromatography (C.C.) was carried out with silica gel (60—120 mesh, Qualigens, India) and thin layer chromatography (TLC) on silica gel G (Merck, India), respectively.

**Plant Material** The fresh plant material (leaves, stems) was collected from college tilla area, Agartala (Tripura) in October, 1998. The plant was identified by Prof. N.K. Chakraborty, Taxonomist, Department of Botany, M.B.B. College, Agartala. A voucher specimen (# BD-3/05) has been deposited in the National Herbarium, Govt of India, Shibpur, Howrah.

**Extraction and Isolation** Air-dried powdered leaves (3 kg) of *D. nigra* were extracted in a Soxhlet extractor with ethyl acetate and methanol, respectively. Both the ethyl acetate and methanolic extracts were concentrated to resinous residues (75 g, 42 g, respectively). Each resinous residue was suspended in water and extracted with benzene, chloroform and *n*-butanol, respectively. The *n*-butanol fraction of ethyl acetate and methanolic extracts on C.C. afforded an oily mass (0.4 g, 1.2 g, respectively, % yield $5.3\times10^{-2}$) of diospyrodin **1** having minor impurity of another compound.

Air-dried coarse powdered stems (1 kg) of *D. nigra* were extracted in a Soxhlet extractor with ethyl acetate and methanol, respectively. Both the concentrated ethyl acetate and methanolic extracts (32 g, 18 g, respectively) were dissolved in water separately and fractionated into benzene, chloroform and *n*-butanol soluble fractions. The *n*-butanol fraction of both ethyl acetate and methanolic extracts on C.C. afforded diospyrodin **1** (0.05 g, 0.2 g, respectively, % yield $2.5\times10^{-2}$) having minor impurity of another compound.

**Acetylation of Diospyrodin** The oily mass (1.5 g) of diospyrodin was acetylated with $Ac_2O$ and pyridine at room temperature (28 °C) for 2 d. The acetylated mixture was poured in ice cold water and extracted with chloroform. The chloroform extract was dried, concentrated and subjected to C.C. Elution of the column with ethyl acetate–benzene (2 : 1) gave a solid, which was homogeneous on TLC. This solid on crystallization from ethyl acetate–benzene mixture afforded crystalline diospyrodin nonaacetate **1a** (800 mg). (*Rf*=0.66, silica gel TLC, benzene–ethyl acetate, 4 : 1 as solvent system).

Diospyrodin Nonaacetate **1a**: Colorless needles, mp 192 °C; $[\alpha]_D^{23}$ −15.2° (*c*=0.22, $CHCl_3$); IR (KBr) cm$^{-1}$: 3024, 2985, 2880, 1750, 1430, 1375, 1235, 1138, 1105, 1090, 1050, 956, 905. $^1$H-NMR ($CDCl_3$, 400 MHz) $\delta$: Table 1. $^{13}$C-NMR ($CDCl_3$, 100 MHz) $\delta$: Table 1. HMBC Correlations: Table 1. Positive ion FAB-MS *m/z* (rel. int., %): 715 $[M+Na]^+$ (48), 693 $[M+H]^+$ (11), 633 $[M+H-AcOH]^+$ (100), 591 $[633-42]^+$ (9), 291 (8), 249 (14), 207 (12), 189 (12), 169 (12), 137 (26), 43 (68). *Anal.* Calcd for $C_{29}H_{40}O_{19}$: C, 50.29; H, 5.82. Found: C, 50.21; H, 5.75.

**Single Crystal X-Ray Analysis of 1a** A colorless prismatic crystal of approximate dimensions of 0.20×0.40×0.20 mm was mounted on a glass fibre and transferred to a Rigaku AFC-5S diffractometer. All crystal data were collected from the diffractometer having graphite monochromated Cu$K\alpha$ radiation ($\lambda$=1.54178 Å) at 23 °C and a 12 kW rotating anode generator. The structure was solved by direct method.[9,10] The non-hydrogen atoms were refined anisotropically and the hydrogen atoms were refined isotropically. The final cycle of full-matrix least-squares refinement was based on 2466 observed reflections ($I>5.00\sigma(I)$) and 451 variable parameters and converged with unweighted (*R*) and weighted agreement factors ($R_w$) of 0.076 and 0.084, respectively.

All calculations were performed using the TEXSAN[11] Crystallographic Software Package of Molecular Structure Corporation.

Crystal Data of **1a**: $C_{29}H_{40}O_{19}$, *M*=692.62, orthorhombic, *a*=46.749(8) Å, *b*=8.190(9) Å, *c*=9.064(6) Å, *V*=3470(7) Å$^3$, space group $P2_12_12_1$, *Z*=4, $D_c$=1.326, $D_m$=1.325 (g/cm$^3$), $\mu$(Cu$K\alpha$)=9.26 cm$^{-1}$, scan mode=$\omega-2\theta,2\theta$, $2\theta_{max}$=126.9°, No. of reflections measured=3305.

**Supporting Information Available** X-Ray crystallographic data for the structure of **1a** have been deposited at the Cambridge Crystallographic Data Centre (CCDC 269253).

**Deacetylation of 1a** A solution of compound **1a** (150 mg) in aqueous methanolic 0.5 M HCl (10 ml) was stirred at *ca*. 40 °C for 2 h and monitored by TLC. TLC of the reaction mixture showed the disappearance of the starting material. The reaction mixture was cooled, concentrated and subjected to C.C. to get diospyrodin **1** as colorless viscous oil (50 mg), (*Rf*=0.25, silica gel TLC, solvent system EtOAc).

Diospyrodin **1**: IR (film) cm$^{-1}$: 3400, 2945, 1460, 1350, 1250, 1040. $^1$H-NMR ($D_2O$, 400 MHz ) $\delta$: Table 1. $^{13}$C-NMR ($D_2O$, 100 MHz) $\delta$: Table 1. HMBC Correlations: Table 1.

**Antimicrobial Assay** The agar cup diffusion and dilution methods were used for assay. Four bacteria namely, *Bacillus subtilis*, *Staphylococcus aureus* (Gram-positive), *Escherichia coli* and *Pseudomonas aeruginosa* (Gram-negative) were used as test organisms. All the bacteria were incubated at 37 °C for 24 h by inoculating into Nutrient Broth (Hi-media). Inoculums containing about $10^6$ bacterial cells/ml were spread by a micro glass spreader on each Nutrient agar plate (100 $\mu$l inoculums/plate). The plates were dried at room temperature for 4 h. The solution of the compound **1** was made in 50% aqueous ethanol and of compound **1a** in DMSO at a concentration of 10 mg/ml. A part of the stock solutions of the compounds were diluted to lower concentrations for the tests. On each dry bacterium seeded plate wells of 5 mm diameter were made by a sterilized micro glass tip and solutions (25 $\mu$l) of different concentrations of the compounds were added. On one well of the plate only solvent was used as a control. On each plate an appropriate reference antibiotic disc (amikacin for the bacterium, *B. subtilis*/*E.coli*/*P. aeruginosa*; and penicillin for bacterium, *S. aureus*; 5 mm diameter, BEACON) was placed by pressing slightly and the plates were then incubated at 37 °C for 24 h. After this period, the positive antibacterial activity was ascertained by measuring the growth inhibition zone (mm). The data reported in Table 2 are the average data of five experiments.

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