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ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 3672–3677
Bioorganic & Medicinal Chemistry

Synthesis and antiproliferative activity of some novel derivatives of diospyrin, a plant-derived naphthoquinonoid

Madhushree Das Sarma,[a] Rina Ghosh,[b] Amarendra Patra[c] and Banasri Hazra[a,*]

[a]*Department of Pharmaceutical Technology, Jadavpur University, Calcutta 700 032, India*
[b]*Department of Chemistry, Jadavpur University, Calcutta 700 032, India*
[c]*Department of Chemistry, University College of Science, Calcutta 700 009, India*

Received 14 February 2007; revised 14 March 2007; accepted 15 March 2007
Available online 18 March 2007

Abstract—Derivatisation of diospyrin, a bisnaphthoquinonoid isolated from *Diospyros montana* Roxb., led to the modification of its inhibitory activity, in vitro, towards a murine tumour model, Ehrlich ascites carcinoma (EAC), and two human cancer cell lines, viz., malignant skin melanoma (A375) and epidermoid laryngeal carcinoma (Hep2). Among the novel derivatives, an epoxide exhibited the maximum antiproliferative activity (IC_{50} values in the range of 0.03–0.21 μM) and a comparatively lower toxicity ($IC_{50} \sim 98$ μM) in normal human peripheral blood mononuclear cells (PBMC). This compound might provide a novel 'lead' for the development of clinically effective antiproliferative agents against cancer.

1. Introduction

Natural products with diverse biological activities have contributed to the development of nearly 75% of the anticancer drugs in modern pharmacopoeia.[1] Several plant-derived molecules, for example, vincristine, podophyllotoxin, camptothecin, paclitaxel, etc., provided highly effective 'leads' for chemotherapeutic treatment of many forms of cancer.[2] Again, a large number of clinical anticancer agents, such as mitomycin C, doxorubicin and its analogous anthracyclines, etc., have been derived from microorganisms,[3] and, incidentally, belong to the quinonoid family, which are ubiquitous secondary metabolites of living systems where they play essential roles in the biochemistry of energy production.[4] These compounds, possessing cytostatic and antiproliferative activities, serve as vital links in the energy transport by virtue of their propensity to generate free radicals through facile redox cycling mechanism.[5] Recently, several plant-derived naphthoquinonoids, such as plumbagin, shikonin, β-lapachone, etc., have been studied for elucidation of their antiproliferative activity against cancer cell lines.[6–8] It has been postulated that reactive oxygen species were involved in the induction of apoptosis by these quinonoids. Similar observations were obtained in our laboratory for diospyrin, a bisnaphthoquinonoid isolated from an indigenous plant, *Diospyros montana* Roxb., which is under development to generate potential anticancer agents.[9–11] In this context, several derivatives of diospyrin were synthesized, some of which were found to be active against murine and human cancer cells.[10–13] Hence, in an endeavour to use diospyrin as a 'lead' compound to obtain more effective cytotoxic agents, some newer derivatives have now been synthesized, based on two dialkyl ethers which had exhibited significant enhancement of the tumour-inhibitory activity in comparison to their natural precursor. In fact, most of the analogues of diospyrin have been prepared by using the dimethyl ether as a synthon. Here, we describe the synthesis and evaluation of antiproliferative activity of the compounds vis-à-vis their precursors in vitro against both murine and human tumour cells, viz., EAC (Ehrlich ascites carcinoma), A375 (malignant skin melanoma) and Hep2 (epidermoid laryngeal carcinoma). Further, cytotoxicity of these novel quinonoids was also assessed against human peripheral blood mononuclear cells (PBMC). Taken together, the results indicated significant enhancement of the tumour-inhibitory potential of the semi-synthetic derivatives as compared to diospyrin itself.

Keywords: Diospyrin derivatives; Naphthoquinonoids; Tumour-inhibitory activity; Malignant skin melanoma (A375); Epidermoid laryngeal carcinoma (Hep2).
* Corresponding author. Tel.: +91 33 2337 0843; fax: +91 33 2337 0752; e-mail addresses: hazra1@vsnl.com; banasrihazra@yahoo.co.in

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doi:10.1016/j.bmc.2007.03.050

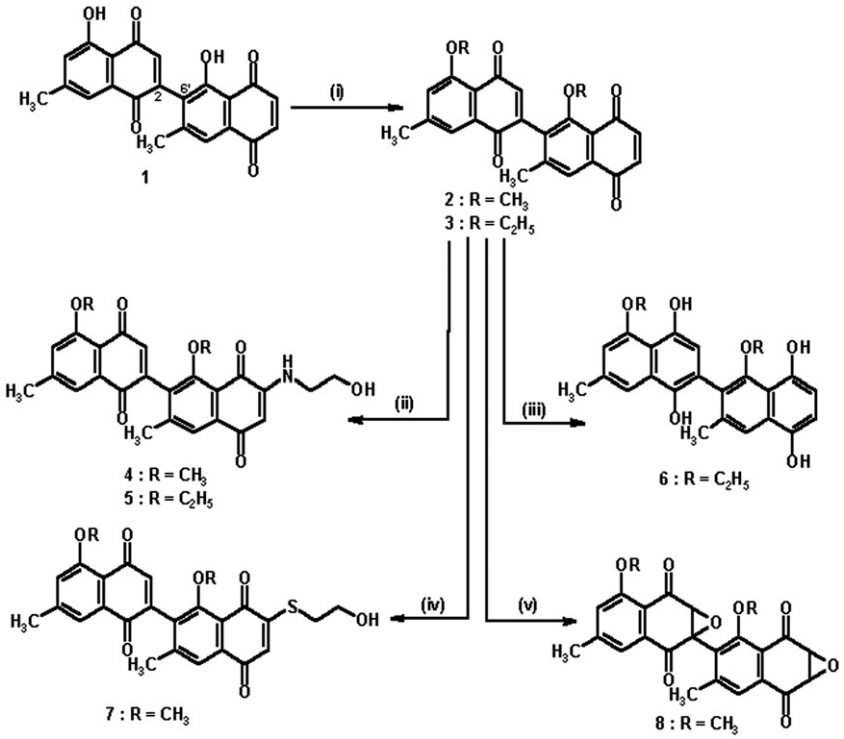
2. Results and discussion

2.1. Synthesis

Diospyrin (**1**) was isolated from the stem bark of *Diospyros montana* Roxb. (family: Ebenaceae) according to the procedure developed by modification of earlier reports,[9,14–16] and was used as the starting material for all the compounds presented in Scheme 1. Recently, the total synthesis[17] and crystallographic analysis[18] have unequivocally confirmed the structure of **1** to be 2,6′-bis(5-hydroxy-7-methyl-1,4-naphthoquinone). Incidentally, while carrying out the structural modification of **1**, this hydroxyquinonoid molecule resisted most of the conventional chemical transformations, presumably due to the presence of strong chelation of the peri-hydroxy groups (at C-5 and -5′) with the respective carbonyls at C-4 and -4′. However, this problem could be successfully overcome by the use of an alkyl iodide, in presence of excess of silver oxide, to produce the dialkyl ether derivatives of **1**, viz., **2** and **3**, in high yields,[11] which were then used as the synthons for producing the novel analogues of diospyrin (Scheme 1).

While preparing the ethanolamines (**4** and **5**), **5** could be produced more readily from diospyrin diethyl ether, and also in better yield than its dimethyl analogue (**4**), using the same reaction conditions. The epoxide, **8**, was obtained in excellent yield (95%) by using alkaline hydrogen peroxide. Earlier reports on the application of the same reagent for epoxidation of analogous plant-derived quinonoids, viz. plumbagin and juglone, show that the plumbagin derivative was produced in good yield (68%),[19] while only 22% of the epoxide was obtained from juglone.[20] However, Rashid and Read could improve the yield of juglone epoxide (73%) by using sodium perborate, although this reagent was not as successful for epoxidation of either naphthazarin (9%) or other quinonoids (5–15%).[20]

The position of the side-chain substitutions with ethanolamino moiety in compounds **4** and **5**, and mercaptoethanol group in **7**, was conclusively established from one-dimensional NMR spectral [^{1}H, ^{13}C (NDC and DEPT-135)] studies, and two-dimensional ^{13}C–^{1}H NMR correlations optimized for $^{1}J_{C-H} \approx 160$ Hz and $^{3}J_{C-H} \approx 7$ Hz. Taking **4** as a representative structure (Fig. 1), the most downfield proton resonance observed at δ 7.88 (s) was assignable to H-8′, and in the long-range HETCORR spectrum this proton showed correlation to the carbonyl carbon (C-1′) resonating at δ 182.3. Incidentally NH proton resonating at δ 6.36 had correlation with a different carbonyl carbon resonance (displayed at δ 179.1 in **4**) and assignable to C-4′. The heteroatomic substituents in **4** could be either at C-2′ or C-3′. The location at C-3′ was unequivocally settled from the long-range correlation observed between the C-4′ signal and H-2′ signal (δ 5.78) in consonance with the structure as 3′-substituted analogues of diospyrin dialkyl ether.



Scheme 1. Synthesis of derivatives of diospyrin. Reagents and conditions: (i) CH_3I, Ag_2O, $CHCl_3$, stir, rt, 6–8 h; (ii) $H_2NCH_2CH_2OH$, $CHCl_3$, C_2H_5OH, stir, 0–10 °C, 30 min–3 h; (iii) $Na_2S_2O_4$, ether, stir, rt, 20 min; (iv) $HSCH_2CH_2OH$, $CHCl_3$, C_2H_5OH, stir, 0 °C, 15 min; (v) H_2O_2, Na_2CO_3, CH_2Cl_2, stir, CH_3OH, 1 h.

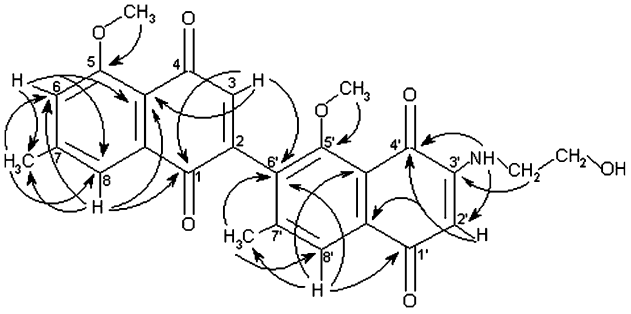


Figure 1. Long range ^{13}C–^{1}H correlations of **4**.

2.2. Antiproliferative activity

The inhibitory activity of diospyrin (**1**) against EAC was substantially enhanced in the synthetic alkyl ethers, **2** and **3**.[11,13] Presently, the cytotoxicity of **1** and its derivatives has been evaluated by MTT assay in EAC, A375 and Hep2 tumour cells, as well as PBMC.[21] Doxorubicin, a clinically used quinonoid anticancer agent, was taken as the positive control. The cytotoxicity induced by each of the compounds, cultured for 24 h with the respective cells, was observed in terms of IC_{50} and are summarised in Table 1. Although the cytotoxicity of the quinonoids, **2** and **3**, against the tumour cells was substantially enhanced for most of their derivatives, their IC_{50} values in human PBMC remained more or less unchanged, indicating the therapeutic advantage achieved through the synthetic modifications carried out on the natural product. The alkylation of **1** had led to an increase in its cytotoxicity against all the tumour cells, the dimethyl derivative, **2**, being comparatively more active than **3**. However, it was interesting to find a dramatic improvement in the activity of **3** by preparing its ethanolamine derivative (**5**). On the contrary, conversion of **2** to its ethanolamine analogue (**4**) was not so effective, although its mercapto derivative, **7**, proved to be substantially better than its nitrogenous analogue, **4**, in EAC and A375 cells. Presumably, the enhanced cytotoxicity exhibited by **5** and **7**, in comparison to **4**, could be attributed to the relative difference in lipophilicity and membrane permeability of the compounds in the tumour cells.[22] A moderate improvement was obtained by reducing the quinonoid moiety of **3** to its hydroquinone, **6**. However, a dramatic change was achieved through synthesis of the epoxide derivative, **8**, which was found to show a remarkable antiproliferative activity against all the tumour cells, with IC_{50} values between 0.03 and 0.21 μM, while the same for doxorubicin was found to be 0.01–0.42 μM. Furthermore, compared to its precursors (**1** and **2**), **8** was markedly less toxic in normal PBMC, the respective IC_{50} values being in the order **2** < **1** < **8**. In fact, considering the IC_{50} values in PBMC, a distinctly favourable therapeutic prospect of the epoxide (**8**: ~98 μM) was indicated with respect to the natural precursor (**1**: 78.3 μM) and doxorubicin (15.5 μM). Here, it may be noted that diosquinone, a similar plant-derived naphthoquinone epoxide, has also exhibited significant cytotoxicity in human cancer cell lines.[23] Undoubtedly, introduction of the oxygen heteroatoms through epoxidation of the quinonic double bonds in **2** could effectively influence the electronic distribution around the active pharmacophore, thereby leading to the enhanced tumour-inhibitory activity of **8**, concomitant with a reduction in toxicity towards normal human lymphocytes. Taken together, the results would justify the importance of rational modification of quinonoid natural products for designing anticancer drugs with improved pharmacological profile.

3. Conclusion

In summary, derivatives of diospyrin were successfully synthesized in high yields, and evaluated against EAC, A375 and Hep2 cells for the antiproliferative property in vitro. Significant enhancement of the tumour inhibition was exhibited by most of the products. When compared to doxorubicin, the reference drug, the novel quinonoids exhibited a lower toxicity against normal human lymphocytes. The best result was obtained with an epoxide derivative, which might lead to more effective utilisation of the diospyrin template for anticancer drug design. Presently this has been precluded by our absolute dependence on the scarce natural product as the

Table 1. Evaluation of cytotoxicity towards tumour cells and PBMC by diospyrin and its derivatives

Compound	IC_{50} (μM) ± SE[a,b]			
	A375	Hep2	EAC	PBMC
1	0.82 ± 0.03	3.58 ± 0.56	0.84 ± 0.01	78.32 ± 3.41
2	0.20 ± 0.02***	0.39 ± 0.03**	0.65 ± 0.02**	53.26 ± 3.80
3	1.07 ± 0.04	2.39 ± 0.84	0.58 ± 0.01**	55.49 ± 0.32
4	3.18 ± 0.09	1.41 ± 0.16*	1.07 ± 0.09	21.82 ± 1.40
5	0.07 ± 0.01***	0.85 ± 0.13**	0.03 ± 0.01***	65.42 ± 2.54
6	0.96 ± 0.08	1.67 ± 0.23*	0.39 ± 0.07**	59.62 ± 1.53
7	0.19 ± 0.02***	1.73 ± 0.32*	0.26 ± 0.08**	95.51 ± 2.18
8	0.03 ± 0.01***	0.21 ± 0.05**	0.09 ± 0.01***	98.25 ± 1.61
Doxorubicin[c]	0.01 ± 0.01	0.42 ± 0.04	>10.0	15.51 ± 1.72

*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, determined by Student's *t*-test, indicate significant enhancement of tumour-inhibitory activity with respect to compound **1**.

[a] Inhibitory concentration to reduce 50% cell growth evaluated by MTT assay.

[b] Data represent mean values (±SE) for three independent determinations.

[c] Anticancer drug as 'standard clinical agent'.

starting material. However, modification of the synthetic procedure to obtain diospyrin in adequate quantity would open up such possibility in future.

4. Experimental

4.1. Chemistry

Sodium dithionite was purchased from Loba Chemie, India, ethanolamine from BDH Chemicals Ltd, Poole, England, and β-mercaptoethanol from Sigma Chemical Company, USA. All other reagents and solvents used were obtained from Sisco Research Laboratory, India. Column chromatography was performed on silica gel (60–120 mesh) from Merck, India. Petroleum ether was used in the boiling range of 60–80 °C. All organic solvents were distilled prior to use. Melting points were determined on Toshniwal melting point apparatus (Cat no: CL-0301) and are uncorrected. UV–vis absorption spectra were recorded with Shimadzu UV 1601 spectrophotometer. IR spectra were obtained on a Perkin-Elmer RXI FT-IR spectrometer system in KBr pellets. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM 300L Supercon NMR spectrometer operating at 300.13 and 75.47 MHz, respectively. Chemical shifts were expressed in parts per million (δ) downfield relative to internal reference Me_4Si and J values were reported in Hertz (Hz). The splitting pattern abbreviations in ^{1}H spectra are as follows: s, singlet, d, doublet, t, triplet, q, quartet, br s, broad singlet. EI-MS were recorded on an AEI MS902 spectrometer equipped with an MSS data acquisition system, version 10 (Mass Spectrometer Services, Manchester, UK), and ESI MS was run on WATERS Micromass Q-Tof microinstrument. Elemental analyses were carried out on a Perkin-Elmer instrument 2400 Series II CHN analyzer. Results obtained were within ±0.3% of the theoretical value.

4.2. Synthesis of derivatives of diospyrin

4.2.1. General procedure for the preparation of ethanolamine derivatives, 4 and 5, from 2 and 3, respectively. Ethanolamine (25 μL, 0.5 mmol) in ethanol (1 mL) was added to an ice-cold solution of the appropriate diospyrin dialkyl ether (0.2 mmol) dissolved in chloroform (3 mL). The mixture was stirred vigorously at 0–10 °C until the starting material was completely consumed (TLC). The mixture was worked up with dichloromethane (3× 10 mL), washed with water (3× 10 mL), and the organic part was evaporated after drying over anhydrous sodium sulfate. The residue was purified by column chromatography using chloroform–ethyl acetate as the eluent to produce, in each case, the respective ethanolamine derivatives (**4** and **5**) in good yield.

4.2.1.1. 3′-(2-Hydroxyethylamino)diospyrin dimethyl ether (4). Stirring **2** with ethanolamine for 3 h. Elution with chloroform/ethyl acetate = 3:2, (v/v), yield 64 mg, 70%, orange powder, mp 246 °C (dichloromethane–petroleum ether). TLC R_f 0.39 (chloroform/ethyl acetate = 1:4, v/v). UV–vis ($CHCl_3$): λ_{max} ($\log\varepsilon$) 235 nm (3.91), 268 nm (3.88), 305 nm (3.47), 410 nm (3.30). IR (KBr): ν_{max} (cm^{-1}) 3308, 2931, 1662, 1598, 1510, 1454, 1360, 1253, 1066. ^{1}H NMR ($CDCl_3$): δ 2.07 (1H, br s, –NHCH$_2$CH$_2$O*H*), 2.27 (3H, s, 7′-CH$_3$), 2.49 (3H, s, 7-CH$_3$), 3.37 (2H, q, J = 5.4 Hz, –NHC*H*$_2$CH$_2$OH), 3.63 (3H, s, 5′-OCH$_3$), 3.87 (2H, t, J = 5.0 Hz, –NHCH$_2$C*H*$_2$OH), 4.03 (3H, s, 5-OCH$_3$), 5.78 (1H, s, H-2′), 6.36 (1H, t, J = 5.2 Hz, N*H*CH$_2$CH$_2$OH), 6.78 (1H, s, H-3), 7.15 (1H, s, H-6), 7.61 (1H, s, H-8), 7.88 (1H, s, H-8′). ^{13}C NMR ($CDCl_3$): δ 20.3 (7′-CH$_3$), 21.7 (7-CH$_3$), 44.5 (NH*C*H$_2$CH$_2$OH), 55.9 (5-OCH$_3$), 58.9 (NHCH$_2$*C*H$_2$OH), 61.8 (5′-OCH$_3$), 98.8 (C-2′), 117.3 (C-4a), 118.5 (C-6), 120.0 (C-4′a), 120.4 (C-8), 123.8 (C-8′), 133.3 (C-1′a), 133.7 (C-6′), 135.4 (C-3′), 139.7 (C-3), 143.5 (C-1a), 145.6 (C-7′), 146.9 (C-7), 149.4 (C-2), 158.2 (C-5′), 159.8 (C-5), 179.1 (C-4′), 182.3 (C-1′), 183.3 (C-4), 183.9 (C-1). MS (EI, relative intensity, %) m/z 459.3 (100) [M-2], 429 (70), 402 (12), 472.1 (2), 256 (1), 199.5 (6), 149 (8), 90 (11), 31 (4). Anal. Calcd for $C_{26}H_{23}O_7N$: C, 67.67; H, 5.02; N, 3.04. Found: C, 67.63; H, 4.98; N, 3.10.

4.2.1.2. 3′-(2-Hydroxyethylamino)diospyrin diethyl ether (5). Stirring **3** with ethanolamine for 30 min. Elution with chloroform/ethyl acetate = 1:4, (v/v), yield 88 mg, 90%, orange powder, mp 212 °C (dichloromethane–petroleum ether). TLC R_f 0.48 (chloroform/ethyl acetate = 1:4, v/v). UV ($CHCl_3$): λ_{max} ($\log\varepsilon$) 242 nm (3.38), 267 nm (3.34), 308 nm (2.94), 387 nm (2.71). IR (KBr): ν_{max} (cm^{-1}) 3382, 2924, 1659, 1601, 1580, 1493, 1367, 1256, 1157. ^{1}H NMR (DMSO-d_6): δ 1.10 (3H, t, J = 6.9 Hz, 5′-OCH$_2$C*H*$_3$), 1.35 (3H, t, J = 6.9 Hz, 5-OCH$_2$C*H*$_3$), 2.19 (3H, s, 7′-CH$_3$), 2.40 (3 H, s, 7-CH$_3$), 3.17 (2H, m, –NHC*H*$_2$CH$_2$OH), 3.54 (2H, m, –NHCH$_2$C*H*$_2$OH), 3.70 (2H, q, J = 6.9 Hz, 5′-OC*H*$_2$CH$_3$), 4.15 (2H, q, J = 6.8 Hz, 5-OC*H*$_2$CH$_3$), 4.88 (1H, t, J = 5.4 Hz, –NHCH$_2$CH$_2$O*H*), 5.63 (1H, s, H-2′), 6.84 (1H, s, H-3), 7.30 (1H, t, J = 5.7 Hz, –N*H*CH$_2$CH$_2$OH), 7.37 (1H, s, H-6), 7.41 (1H, s, H-8), 7.66 (1H, s, H-8′). ^{13}C NMR (DMSO-d_6): δ 14.7 (5′-OCH$_2$*C*H$_3$), 15.5 (5-OCH$_2$*C*H$_3$), 20.6 (7′-CH$_3$), 21.8 (7-CH$_3$), 44.9 (NH*C*H$_2$CH$_2$OH), 58.6 (NHCH$_2$ *C*H$_2$OH), 64.4 (5′-O*C*H$_2$CH$_3$), 70.1 (5-O*C*H$_2$CH$_3$), 98.5 (C-2′), 117.1 (C-4a), 119.7 (C-6), 120.1 (C-4′a), 120.5 (C-8), 123.1 (C-8′), 133.6 (C-1′a), 133.7 (C-6′), 135.2 (C-3′), 140.0 (C-3), 143.1 (C-1a), 145.6 (C-7′), 146.8 (C-7), 147.8 (C-2), 156.8 (C-5′), 158.9 (C-5), 179.7 (C-4′), 180.6 (C-1′), 182.7 (C-4), 184.0 (C-1). ESI-MS: 512 (M+Na). Anal. Calcd for $C_{28}H_{27}O_7N$: C, 68.70; H, 5.56; N, 2.86. Found: C, 68.61; H, 5.48; N, 2.80.

4.2.2. 1,4,1′,4′-Tetrahydroxydiospyrin diethyl ether (6). Freshly prepared saturated solution of sodium dithionite (1 g in 5 mL distilled water) was added dropwise to the solution of **3** (75 mg, 0.1 mmol) in diethyl ether (4 mL), and the mixture was stirred vigorously in nitrogen atmosphere. After the ethereal layer gradually became completely colourless (20 min), the reaction mixture was washed with water (3× 15 mL), dried over anhydrous sodium sulfate and evaporated to dryness to furnish a crude residue. It was crystallised from a mixture of ether, petroleum ether and dichloromethane to get the desired hydroquinone derivative (**6**) as a light brown powder (70 mg, 92%), mp 192 °C. TLC R_f 0.56

(petroleum ether/ethyl acetate = 2:1, v/v). UV ($CHCl_3$): λ_{max} (log ε) 255 nm (3.71), 354 nm (2.26). IR (KBr): ν_{max} (cm^{-1}) 3400, 2984, 1636, 1460, 1435, 1396, 1371, 1331, 1285, 1242, 1190, 1155, 1076. ^1H NMR ($CDCl_3$): δ 1.06 (3H, t, J = 7.0 Hz, 5′-OCH_2CH_3), 1.25 (3H, s, 5-OCH_2CH_3), 2.27 (3H, s, 7′-CH_3), 2.51 (3H, s, 7-CH_3), 3.81 (2H, m, 5′-OCH_2CH_3), 4.33 (2H, q, J = 6.9 Hz, 5-OCH_2CH_3), 5.02 and 5.20 (1H each, s, 1-OH and 1′-OH), 6.64 (1H, s, H-3), 6.70 (1H, d, J = 8.2 Hz, H-2′ or H-3′), 6.73 (1H, s, H-6), 6.78 (1H, d, J = 8.2 Hz, H-3′ or H-2′), 7.71 (1H, s, H-8), 7.91 (1H, s, H-8′), 9.18 and 9.32 (1H each, s, 4-OH and 4′-OH). ESI-MS: 457 (M+Na). Anal. Calcd for $C_{26}H_{26}O_6$: C, 71.87; H, 6.03. Found: C, 71.75; H, 5.98.

4.2.3. 3′-(2-Hydroxyethylmercapto)diospyrin dimethyl ether (7). To an ice-cold solution of **2** (80 mg, 0.2 mmol) in dichloromethane (3 mL), a solution of β-mercaptoethanol (0.2 mL) in ice-cold ethanol (1 mL) was added under stirring condition. After 15 min, the reaction mixture was worked up with dichloromethane (3× 10 mL), washed with water (3× 10 mL), and the organic part was evaporated after drying over anhydrous sodium sulfate. The crude product thus obtained was purified by chromatography on a silica gel column using chloroform/ethyl acetate = 1:1, v/v, as the eluent, and crystallised from a mixture of dichloromethane and petroleum ether to get **7** as a brownish yellow powder (60 mg; 63%), mp 220 °C. TLC R_f 0.62 ($CHCl_3$/ethyl acetate = 2:3, v/v). UV–vis ($CHCl_3$): λ_{max} (log ε) 260 nm (4.56), 315 nm (3.99), 358 nm (3.83), 401 nm (3.86). IR (KBr): ν_{max} (cm^{-1}) 3405, 2932, 1654, 1583, 1458, 1342, 1252, 1154, 1064. ^1H NMR ($CDCl_3$): δ 2.29 (3H, s, 7′-CH_3), 2.50 (3H, s, 7-CH_3), 3.08 (2H, t, J = 5.4 Hz, –SCH_2CH_2OH), 3.69 (3H, s, 5′-OCH_3), 3.96 (2H, q, J = 5.0 Hz, –SCH_2CH_2OH), 4.04 (3H, s, 5-OCH_3), 6.66 (1H, s, H-2′), 6.78 (1H, s, H-3), 7.16 (1H, s, H-6), 7.60 (1H, s, H-8), 7.84 (1H, s, H-8′). ESI-MS: 501 (M+Na). Anal. Calcd for $C_{26}H_{22}O_7S$: C, 65.26, H, 4.63. Found: C, 65.19, H, 4.58.

4.2.4. Diospyrin dimethyl ether 2,3,2′,3′-diepoxide (8). An alkaline hydrogen peroxide solution [2 mL; 1:7 mixture of hydrogen peroxide (50 wt% in water) and aqueous sodium carbonate (5%, w/v)] was added to diospyrin dimethyl ether (80 mg, 0.2 mmol) in methanol (2 mL) and dichloromethane (3 mL). The mixture was stirred vigorously at room temperature for 1 h and subsequently extracted with dichloromethane (10 mL), washed with water (3× 10 mL). The pooled organic layer was dried over anhydrous sodium sulfate and evaporated to dryness to give a crude product (82 mg, 95%, mp 176–182 °C). It was crystallised (twice) from a mixture of ether, acetone and dichloromethane to obtain white shining powder (**8**), mp 248 °C. TLC R_f 0.51 (petroleum ether/ethyl acetate = 3:2, v/v). UV ($CHCl_3$): λ_{max} (log ε) 258 nm (4.23), 347 nm (3.83). IR (KBr): ν_{max} (cm^{-1}) 3431, 2944, 1694, 1595, 1458, 1337, 1266, 1172, 1085. ^1H NMR ($CDCl_3$): δ 2.45 (3H, s, 7′-CH_3), 2.47 (3H, s, 7-CH_3), 3.77 (3H, s, 5′-OCH_3), 3.98 (3H, s, 5-OCH_3), 4.00 (2H, d, H-2′ and H-3′), 4.01 (1H, s, H-3), 7.13 (1H, s, H-6), 7.44 (1H, s, H-8), 7.63 (1H, s, H-8′). ^{13}C NMR ($CDCl_3$): δ 20.1 (7′-CH_3), 22.3 (7-CH_3), 55.5 (C-3′), 55.6 (C-2′), 56.5 (5-OCH_3), 61.4 (C-2), 62.1 (C-3), 63.3 (5′-OCH_3), 117.9 (C-4a), 118.7 (C-6), 120.6 (C-8), 121.5 (C-4′a), 124.7 (C-8′), 132.2 (C-6′), 133.6 (C-1′a), 133.8 (C-1a), 146.9 (C-7′), 148.1 (C-7), 159.2 (C-5′), 159.4 (C-5), 189.7 (C-1′ and C-4′), 190.7 (C-1), 190.8 (C-4). ESI-MS: 435 (M+H), 457 (M+Na). Anal. Calcd for $C_{24}H_{18}O_8$: C, 66.36; H, 4.18. Found: C, 66.43; H, 4.30.

4.3. Biological assay

4.3.1. Cell culture. Two human cancer cell lines, viz. A375 (malignant skin melanoma) and Hep2 (epidermoid laryngeal carcinoma), were obtained from National Centre for Cell Science, Pune, India. The cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated foetal calf serum (FCS, Gibco-BRL, Gaithersburg, MD, USA) containing 5% mixture of penicillin (100 U/mL), streptomycin (100 μg/mL) and gentamicin (3 μg/mL) in the presence of 5% CO_2 in air at 37 °C and routinely subcultured using a 0.25% trypsin–0.02% EDTA solution.

Ehrlich ascites carcinoma (EAC) cells, obtained from Chittaranjan National Cancer Institute, Calcutta, were serially maintained in female Swiss A mice (6–8 weeks old; 18–20 g) by routine intraperitoneal (ip) transplantation. For this experiment, cells were collected on day 12–14 post-transplantation, suspended in phosphate-buffered saline (PBS; pH 7.4), centrifuged and washed with cold PBS. The pellet was resuspended in the RPMI 1640 medium without phenol red (Sigma Chemical Company, USA), supplemented with serum and antibiotics as above for incubation to be carried out for the desired experiment in a humidified atmosphere of 5% CO_2 in air at 37 °C.

Fresh heparinized whole blood was collected from normal human volunteer with informed consent. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque density gradient centrifugation.[24] The blood (5 mL) was layered carefully over the Hypaque (3 mL, Sigma Diagnostics, USA) and centrifuged at room temperature at 1000 rpm for 45 min. The buffy coat layer containing PBMC at the interface was carefully taken out, washed twice with PBS and centrifuged at 1500–2000 rpm for 10 min. The cells were suspended in RPMI 1640 with phenol red (Gibco-BRL, Gaithersburg, MD, USA), supplemented with 20% FCS and antibiotics (as above), and incubated in the presence of 5% CO_2 in air at 37 °C.

4.3.2. Assessment of cytotoxicity in vitro. In vitro growth inhibition effect of the test-compounds on A375, Hep2, EAC and normal human lymphocyte was assessed by colorimetric determination of the conversion of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemicals, USA) into 'formazan blue' by the living cells.[21] Briefly, cells (2×10^5/mL) were seeded in 96-well flat-bottomed microplates (Nunc, Roskilide, Denmark) and treated with different concentrations, in triplicate, of the test compounds

appropriately diluted with DMSO. After 24-h incubation at 37 °C in a 5% CO_2 atmosphere, the medium was replaced with MTT solution (100 μL, 1 mg/mL in sterile PBS) for further 24-h incubation (4 h in case of EAC cells). The supernatant was aspirated carefully, the precipitated crystals of 'formazan blue' were solubilized by adding DMSO (200 μL) to each well, and the optical density was measured with a microplate reader (Emax precision microplate reader, Molecular Devices, USA) at a wavelength of 570 nm. Doxorubicin (Sigma Chemicals, USA) was used as the positive control in this experiment. The result represents the mean of three independent experiments and is expressed as IC_{50}, the concentration at which the optical density of the treated cells was reduced by 50% with respect to the untreated control.

4.4. Statistical analysis

The IC_{50} values were calculated by using linear regression analysis (MINITAB Release 13.31, USA). Student's *t*-test was performed to analyse the statistical significance of the activity of the novel derivatives vis-à-vis diospyrin.

Acknowledgements

B.H. received financial support as a Research Scientist from U.G.C., and ICMR [Grant No. 58/4/2000/BMS], New Delhi. Authors acknowledge C.S.I.R., New Delhi, for a Senior Research Fellowship Grant [No. 9/96(377)/2002-EMR-I] to M.D.S., and a Research Project Grant [No. 01/1951/04/EMR-II] to R.G. Thanks are accorded to the instrumentation facility of the CAS on Natural Products, Department of Chemistry, Calcutta University, for spectroscopic measurements. The facility to maintain human cancer cell lines was kindly provided by Dr. Keya Chaudhuri, IICB, Calcutta. Authentic stem-bark of *D. montana* Roxb. was supplied by Dr. R.B. Mishra, Nagarjuna Education Society, Bolangir, Orissa.

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