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Cyathane diterpenes from *Sarcodon cyrneus* and evaluation of their activities of neuritegenesis and nerve growth factor production

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Abstract—Two new cyathane diterpenes, cyrneine C (**4**) and D (**5**), were isolated from the mushroom *Sarcodon cyrneus*, along with previously isolated cyrneine A, B and glaucopine C. The structures of the novel diterpenoids were determined by the analysis of spectroscopic data. Effects of the cyrneines and glaucopine C on the NGF gene expression in 1321N1 cells and on neurite outgrowth on PC12 cells were evaluated.

1. Introduction

Sarcodon mushrooms have been shown to be a great source of cyathane diterpenes that show interesting biological activities such as the anti-inflammatory activity.[1–4] Furthermore, recently cyathanes have attracted attention for their action as stimulators of neurotrophic factors in vitro,[5–7] among these scabronine G-methylester resulted particularly active.[6,7]

The interesting biological activities of *Sarcodon scabrosus* prompted us to examine *Sarcodon cyrneus* Maas Gest (Basidiomycetes).

Phytochemical investigations on the constituents of this mushroom led to the isolation of five cyathane diterpenes (**1**–**5**) four of which are novel, while compound **3** was already isolated from the hexane extract of *Sarcodon glaucopus*.[4]

We have already reported the isolation and the structural determination of cyrneines A and B (**1** and **2**) from the methanolic extract of *S. cyrneus*.[8] These compounds were tested for their capability to induce neurite outgrowth in rat pheochromocytoma cells (PC12), used as a model system of neuronal differentiation. Cyrneines A and B (50 or 100 μM) significantly promoted neurite outgrowth in concentration-dependent manners, and the effects of both compounds were comparable to nerve growth factor (NGF) as a positive control.[8,9]

This paper describes the separation and the structural elucidation of the new compounds (**4** and **5**) and the evaluation of their activity on PC12 differentiation. Besides we studied the capability of all our compounds (**1**–**5**) to induce NGF gene expression in 1321N1 cells compared to scabronine G-methylester.

2. Results and discussion

All the compounds were isolated from the methanolic extract by sequential chromatographic techniques and their structures were identified by analytical and spectroscopical data (1D and 2D ^{1}H and ^{13}C NMR).

Keywords: *Sarcodon cyrneus*; Cyrneine C; Cyrneine D; Glaucopine C; Cyathane diterpenes; NGF expression; PC12; Neurite outgrowth.
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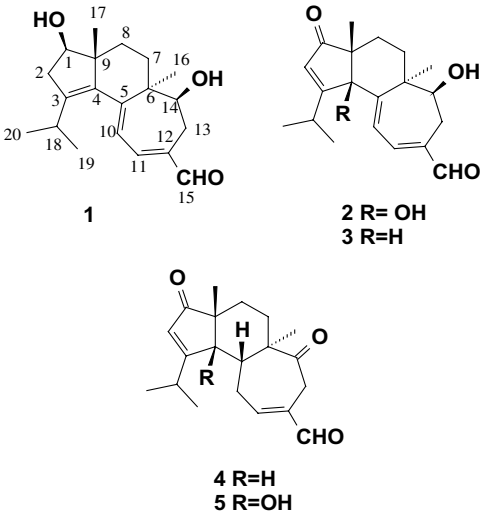

Compound **3** was identified by comparison of physical and spectral data (^{1}H and ^{13}C NMR, IR, [α]) with those of an authentic sample.[4]

The molecular formula of compound **4**, $C_{20}H_{26}O_3$, was deduced from MS, ^{1}H, and DEPT experiments.

The ^{1}H and ^{13}C NMR spectra are similar to those of glaucopine C (**3**) except for the absence of an hydroxy group at C-14 (δ 77.6) replaced by a keto group (δ 211.8) and the absence of the C-5/C-10 double bond (δ 156.5 and 125.0) replaced by a methine and a methylene (δ 38.0 and 33.7) in the ^{13}C NMR spectrum.

The combined use of H–H COSY and HMQC evidenced the presence of three spin systems (see **a**–**c** in Fig. 1). These substructures were connected in the HMBC spectrum by CH correlations between the protons H-4 (δ 2.71) and H-18 (δ 2.68) and C-3 (δ 188.6), H-17 (δ 1.09) and C-4 (δ 54.1), C-1 (δ 212.7) and C-9 (δ 49.7), H-8 (δ 1.86) and C-9, H-7 (δ 1.86) and H-16 (δ 0.96) and C-6 (δ 51.1) and H-16 and H-13 (δ 3.53) and C-14 (δ 211.8), H-13 and C-15 (δ 192.4).

The β stereochemistry of H-4 and H-5 was established by NOESY experiment (Fig. 2). Compound **4** was thus identified as 1,14-dioxo-cyatha-2,11-dien-12-carbaldehyde.

The molecular formula of compound **5** was determined as $C_{20}H_{26}O_4$ on the base of DEPT spectrum multiplicities.

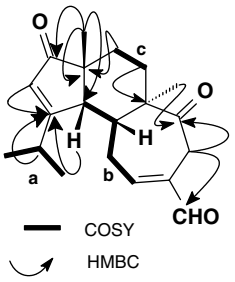

Figure 1. Key COSY and HMBC correlations in compound **4**.

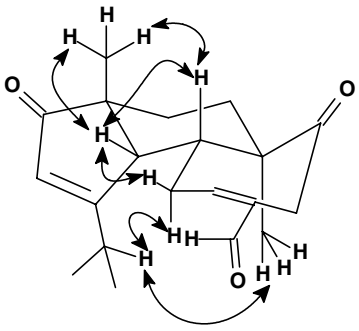

Figure 2. Key NOESY correlations in compound **4**.

Compound **5** differed from cyrneine C (**4**) for the replacement of the methine at C-4 (δ 54.1) with an oxygenated tetrasubstituted carbon (δ 82.6) and showed the same basic pattern of HMBC (Fig. 3) and NOESY (Fig. 4) correlations as in **4**.

The major differences between the two compounds resided in the chemical shifts of C17 and C5. Due to the γ-effect exerted by the hydroxy group in C4, C17 in compound **5** resonated at higher fields (14.7 ppm vs 22.2 ppm), while C5 (β-effect) is downshielded (44.8 ppm vs 38.0 ppm). As already observed in glaucopine C (**3**)[4] versus cyrneine B (**2**),[8] the presence of a β-hydroxy group in C4, δ to C16, determines a shielding of the methyl group in C6 with respect to compound **4** (15.3 ppm vs 18.9 ppm).[10] All these data allowed us to identify compound **5** as 4β-hydroxy-cyrneine C.

In view of the biological activity of cyrneines A and B[8] we examined the activity of compounds **3**, **4**, and **5** to stimulate neurite outgrowth in PC12 cells (see Section 3).[8]

In this assay, cyrneine A and NGF were used as positive control. The cells were stimulated with cyrneines C (**4**),

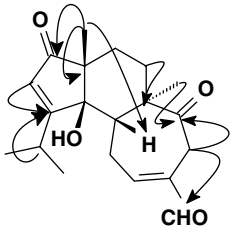

Figure 3. Key HMBC correlations in compound **5**.

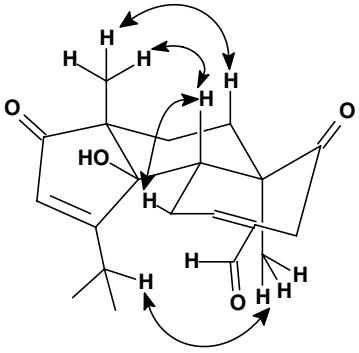

Figure 4. Key NOESY correlations in compound **5**.

D (**5**) and glaucopine C (**3**) (200 μM) or cyrneine A (200 μM) and NGF (100 ng/mL). As shown in Figure 5 cyrneines C and D, and glaucopine C did not promote neurite outgrowth significantly in PC12 cells.

Since scabronines, whose structures are similar to cyrneines, induce NGF expression in 1321N1 human astrocytoma cells,[6,7] NGF-synthesis stimulating activity of cyrneines and glaucopine C was directly examined in 1321N1 cells as described in Section 3.

In this assay, scabronine G-methylester was used as a positive control.[7] The cells were stimulated with cyrneines A (**1**), B (**2**), C (**4**), and D (**5**), and glaucopine C (**3**) (50–200 μM) or scabronine G-methylester as positive control

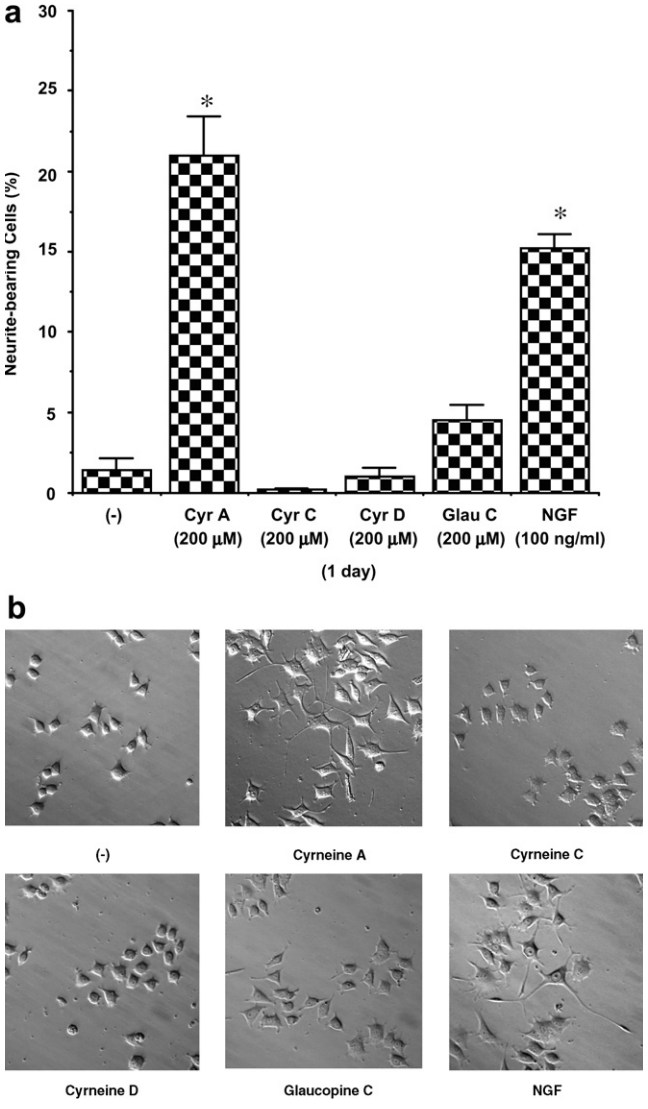

Figure 5. Effects of cyathane diterpenes on neurite outgrowth in PC12 cells. (a) PC12 cells were cultured in the presence of NGF (100 ng/mL), cyrneine A (200 μM), or cyrneine C (200 μM), cyrneine D (200 μM), and glaucopine C (200 μM) for 24 h, then the morphological changes of the cells were observed. (b) PC12 cells were cultured in the presence of cyrneine A (200 μM), cyrneine C, cyrneine D, and glaucopine C (200 μM) or NGF (100 ng/mL) for 24 h. Percentage of neurite-positive cells was calculated as described in Section 3. Values are means ± SEM of three wells. $^{*}P < 0.05$ versus control (without drug).

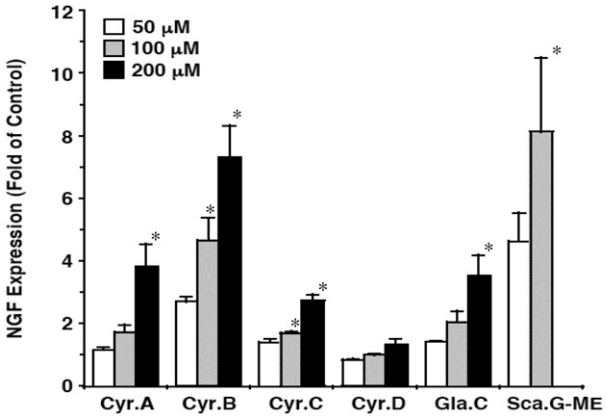

Figure 6. Effects of the cyrneines and glaucopine C on the NGF gene expression in 1321N1 cells. Data are expressed as fold of control (no drug treatment) and values are means ± standard error of mean of three independent experiments. $^{*}P < 0.05$ versus control (without drug).

(50 or 100 μM) for 4 h. As shown in Figure 6, NGF gene expression was enhanced by cyrneines and glaucopine C.

Among them, cyrneine B caused the strongest NGF expression (7.3-fold at 200 μM). Cyrneines A, C, D and glaucopine C induced NGF expression level by 3.8-, 2.7-, 1.3- and 3.5-fold, respectively.

These results indicate that among our compounds, cyrneines A and B[8] and, to a lesser extent, glaucopine C induce neurite outgrowth in PC12 cells and stimulate NGF gene expression in 1321N1 cells.

While cyrneines A and B can promote both neurite outgrowth in PC12 cells and NGF production in 1321N1 cells, scabronines failed to induce neurite outgrowth in PC12 cells.[6,7] It has been reported that other similar diterpenes, such as erinacines, enhanced NGF biosynthesis in rat astroglial cells.[11] Looking at the structures of cyrneines, erinacines, and scabronines it is very difficult to outline a structural feature that can justify the different biological activities toward neurite outgrowth or NGF gene expression.

Judging from our observations concerning with cyrneines, it seems that the presence of the hydroxy cycloheptadienyl carbaldehyde system could be important both for the activity on 1321N1 and PC12 cells. As a matter of fact erinacines incorporate this kind of structural feature and in our molecule those that have this system show a higher activity. The hydroxy group in C4 could be important for the direct action on neurite outgrowth in PC12 cells. For the activity on NGF expression it looks that the carboxymethyl group in C9 is determinant[7] and that all the substitutions on the 5-membered ring (e.g., alcoholic or keto functions) other than C3–C4 double bond can decrease the activity toward the expression of NGF.

In other words, minor differences in functional groups on cyathane structure can influence the cellular responses, and further studies are essential to investigate the structure–activity relationship in more detail.

3. Experimental

3.1. General

IR spectra were obtained in neat on a Jasco FT/IR 410 infrared spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$, except otherwise specified, on a Bruker Avance-DRX 400 spectrometer at 400 and 100.62 MHz, respectively, using TMS as the internal standard. Optical rotations were measured with a JASCO DIP-1000 digital polarimeter. GC-MS analysis was performed on a HP-6890 instrument equipped with a mass selective detector HP-5973. Elemental analyses were carried out on a Carlo Erba Model 1106 Elemental Analyzer. Column chromatography was performed using Merck silica gel 60 (70–120 mesh). Thin layer chromatography was performed on Merck silica gel 60 F_{254}.

3.2. Plant material

Sarcodon cyrneus was collected in October 2004 near Perugia (Italy) and identified by Prof. Rita Pagiotti. A voucher specimen (RP #62) is deposited at the Dipartimento di Biologia Vegetale e Biotecnologie Agroambientali, Università degli Studi di Perugia.

3.3. Extraction and isolation

Forty-eight grams of the fruiting bodies of lyophilized *S. cyrneus* was extracted with hexane (1 L) at rt for 24 h. After vacuum filtration, the residue was extracted with MeOH (1 L) at rt for 24 h. The extract was evaporated giving 11.3 g of residue. A solution of this crude extract in EtOAc (100 mL) was washed with water (5× 20 mL). The organic phase was dried over Na_2SO_4 and evaporated leading to 2.27 g of a brown syrup that was fractionated by silica gel column chromatography (4 × 45 cm) using CH_2Cl_2/EtOAc (9:1, 200 mL; 5:1, 150 mL; 4:1, 100 mL; 7:3, 300 mL; 3:2, 100 mL) and MeOH (100 mL). The eluents were combined to 18 fractions (1–18) on the basis of TLC analysis. Fraction 6 (54 mg) was further purified by SiO_2 column chromatography, eluent CH_2Cl_2, giving 10 mg of cyrneine C (**4**, 0.02% yield) as a white powder after crystallization from Et_2O. Fraction 11 (168 mg) by TLC on crystallization using Et_2O yielded **5** as a white powder (4 mg, 0.008% yield). Purification of the mother liquor on SiO_2, eluent CH_2Cl_2–EtOAc 9:1, gave 71 mg of glaucopine C (**3**, 0.15% yield).

3.4. 14-Hydroxy-1-oxo-cyatha-2,5(10),11-trien-12-carbaldehyde (3)

For analytical and spectroscopical data, see Ref. 4.

3.5. 1,14-Dioxo-cyatha-2,11-dien-12-carbaldehyde (4)

White powder; mp: 228–230 °C; $[\alpha]_D^{26}$: −147.2 (*c* 0.45, CH_2Cl_2); R_f: 0.65 (CH_2Cl_2–EtOAc, 7:3); IR (neat) ν_{max} 2929, 1685 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 0.96 (3H, s, H-16), 1.06–1.11 (1H, m, H-7b), 1.09 (3H, s, H-17), 1.16 (3H, d, J = 6.9 Hz, H-20), 1.20 (3H, d, J = 6.9 Hz, H-19), 1.38 (1H, ddd, J = 3.3 10.7, 17.2 Hz, H-8b), 1.74 (1H, ddd, J = 3.3, 7.3, 14.1 Hz, H-8a), 1.86 (1H, ddd, J = 3, 7.2, 14.2 Hz, H-7a), 2.68 (1H, sept, J = 6.6 Hz, H-18), 2.71 (1H, dd, J = 2.1, 5.1 Hz, H-4), 2.79 (1H, dd, J = 5.8, 19.5 Hz, H-10b), 2.91 (1H, dd, J = 5.0, 12.8 Hz, H-5), 3.07 (1H, m, H-10a), 3.53 (2H, m, H-13), 5.98 (1H, br s, H-2), 6.70 (1H, m, H-11), 9.32 (1H, s, H-15); ^{13}C NMR: see Table 1; MS: *m/z* (%) = 314 [M$^+$] (100), 299 (35); Anal. Calcd for $C_{20}H_{26}O_3$: C, 76.40; H, 8.33. Found C, 76.35; H, 8.40.

3.6. 1,14-Dioxo-4-hydroxy-cyatha-2,11-dien-12-carbaldehyde (5)

White powder; mp: 238–239 °C (dec); $[\alpha]_D^{28.7}$ −66.5 (*c* 1.95, CH_2Cl_2); R_f: 0.46 (CH_2Cl_2–EtOAc, 7:3); IR (neat) ν_{max} 3364, 2944, 1697 cm^{-1}; ^{1}H NMR (CD_3OD): δ 0.99 (3H, s, H-16), 1.08 (3H, s, H-17), 1.10–1.14 (1H, m, H-7b), 1.19 (3H, d, J = 6.6 Hz, H-20), 1.25 (3H, d, J = 6.6 Hz, H-19), 1.49 (1H, m, H-8b), 1.65 (1H, td, J = 3.8, 14.4 Hz, H-8a), 1.90 (1H, m, H-7a), 2.70–2.75 (1H, m, H-10b), 2.76 (1H, sept, J = 6.6 Hz, H-18), 2.91 (1H, d, J = 12.5 Hz, H-5), 3.35 (1H, d, J = 14.0 Hz, H-13b), 3.43 (1H, dd, J = 5.7, 18.6 Hz, H-10a), 3.77 (1H, br d, J = 13.9 Hz, H-13a), 6.06 (1H, s, H-2), 6.91 (1H, m, H-11), 9.30 (1H, s, H-15); ^{13}C NMR: see Table 1; MS: *m/z* (%) = 330 [M$^+$] (70), 312 (10), 153 (100); Anal. Calcd for $C_{20}H_{26}O_4$: C, 72.70; H, 7.39. Found C, 72.75; H, 7.34.

3.7. Cell culture and evaluation of neurite outgrowth

PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated 10% fetal calf serum (FCS) (Cell Culture Laboratory, Cleveland, OH) and 5% horse serum (HS) (Invitrogen,

Table 1. ^{1}H and ^{13}C NMR data

Position	4		5[a]	
	H	C	H	C
1		212.7		212.5
2	5.98	128.5	6.06	127.0
3		188.6		187.0
4	2.71	54.1		82.6
5	2.91	38.0	2.91	44.8
6		51.1		52.2
7a	1.86	32.7	1.90	31.6
7b	1.06–1.11		1.10–1.14	
8a	1.74	30.7	1.65	31.7
8b	1.38		1.49	
9		49.7		53.7
10a	3.07	33.7	3.43	28.1
10b	2.79		2.70–2.75	
11	6.70	152.1	6.91	155.1
12		136.1		136.2
13a	3.53	36.7	3.77	34.6
13b			3.35	
14		211.8		211.7
15	9.32	192.4	9.30	193.2
16	0.96	18.9	0.99	15.3
17	1.09	22.2	1.08	14.7
18	2.68	32.2	2.76	30.5
19	1.20[b]	21.3[b]	1.25[b]	24.5[b]
20	1.16[b]	23.9[b]	1.19[b]	21.3[b]

[a] Spectra registered in CD_3OD.
[b] Values within the column may be interchanged.

Grand Island, NY), penicillin (50 U/mL), and streptomycin (50 μg/mL) in a 5% CO_2 incubator. 1321N1 human astrocytoma cells were grown in DMEM supplemented with heat-inactivated 5% FCS and above antibiotics. Neurite outgrowth assay was performed as described previously.[12] In brief, PC12 cells were seeded onto 48-well plates (2.5×10^4 cells/well) and cultured for 24 h. Media were replaced with DMEM containing 1% FCS, 0.5% HS, antibiotics, and drugs (cyrneines or NGF (Sigma, St. Louis, MO)), then the cells were cultured for additional 24 h. The cell morphology was assessed under microscope. Neurite extension from PC12 cells was regarded as an index of neuronal differentiation, processes with a length equivalent to one or more diameters of the cell body were regarded as neuritis. The differentiation of PC12 cells was evaluated by examining the proportion of neurite-positive cells to total cells in randomly selected fields (a field/well). The mean differentiation score was obtained for more than a 100 cells in each well. Data were expressed as means ± standard error of mean (SEM) of three different wells, and the significant differences were analyzed with Tukey's multi-comparison test.

3.8. Reverse transcription-real time polymerase chain reaction (RT-real time PCR)

NGF expression in 1321N1 cells was determined by RT-real time PCR. Briefly, 1321N1 cells were seeded onto 12-well plates (1×10^5 cells/well). After serum-starvation for a day, the cells were stimulated with drugs for 4 h. Total RNA was extracted by using a total RNA extraction kit (Nippon Gene, Toyama, Japan). RT reaction and real-time PCR were carried out by using a RT-real time PCR kit (Takara, Otsu, Japan) with a thermal cycler (Opticon real-time PCR system, Japan Bio-Rad Laboratories, Tokyo, Japan). For analysis of human NGF mRNA, the sense primer (5′-CCA AGG GAG CAG CTT TCT ATC CTG G-3′) and the antisense primer (5′-GGC AGT GTC AAG GGA ATG CTG AAG T-3′) were used. The human NGF cDNA fragment (189 bp) was amplified (94 °C for 5 s, 61 °C for 20 s, 72 °C for 15 s) and the fluorescence of SYBR green was measured every cycle. β-Actin transcripts amplified with sense primer (5′-AGG GAA ATC GTG CGT GAC AT-3′) and antisense primer (5′-TCC TGC TTG CTG ATC CAC AT-3′) were used as an internal control for normalization. Data are expressed as means ± standard error of mean of three independent experiments. The significant differences were analyzed with Dunnett's test.

Acknowledgments

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