Bioxanthracenes from the Insect Pathogenic Fungus *Cordyceps pseudomilitaris* BCC 1620

I. Taxonomy, Fermentation, Isolation and Antimalarial Activity

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Eleven bioxanthracenes and two monomers, six novel in nature, were isolated from the insect pathogenic fungus *Cordyceps pseudomilitaris* BCC 1620. Growth optimization of the strain led to the improvement of bioxanthracenes production. The bioxanthracenes were evaluated for their antimalarial activity and cytotoxicity.

Cordyceps pseudomilitaris Hywel-Jones & Sivichai is an insect pathogenic fungus that infects the lepidoptera. This fungus has recently been isolated and identified at this research center (BIOTEC) in Thailand.[1] In the course of our systematic collection and identification of insect pathogenic fungi from various parts of Thailand, the fungus *C. pseudomilitaris* has been found only at Sam Lan National Park, Central Thailand. Due to the interest in the new, locally grown species, an investigation of its chemical constituents has been conducted.

Recently, we reported the isolation and structure determination of two novel anhydrides, cordyanhydrides A and B, from a culture filtrate of *C. pseudomilitaris* strain BCC 1620.[2] Detailed studies on the fermentation and isolation of the chemical constituents of this strain led to the isolation of other types of secondary metabolites, bioxanthracenes (**1**~**11**) and two monomers (**12**, **13**). In this paper, we report the taxonomy of the strain BCC 1620, the fermentation, the isolation and the evaluation of the biological activity of its secondary metabolites **1**~**13**. Structure elucidation of these compounds is described in the accompanying paper.[3]

Materials and Methods

Fermentation

For the optimization of bioxanthracenes production, small scale cultivations were conducted in four different liquid media: potato dextrose broth (PDB: composition; potato infusion 200 g, Bacto dextrose 20 g, per 1 liter distilled water), yeast extract sucrose (YES: composition; yeast extract 20 g, sucrose 150 g, per 1 liter distilled water), M102 (composition; sucrose 30 g, malt extract 20 g, Bacto-peptone 2 g, yeast extract 1 g, KCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KH_2PO_4 0.5 g, per 1 liter distilled water) and Czapek-Dox medium (CDM: composition; sucrose 30 g, $NaNO_3$ 3 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4 \cdot$

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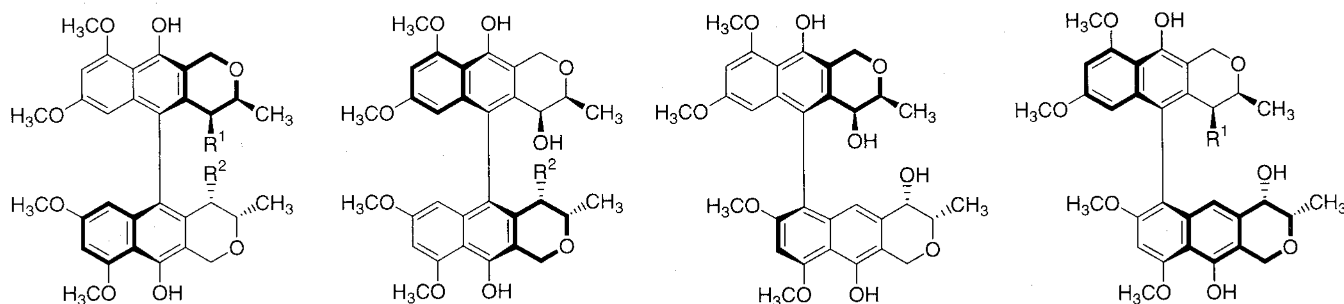
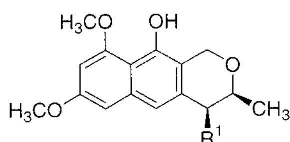


1 : R^1 = OH, R^2 = OH
2 : R^1 = OAc, R^2 = OH
3 : R^1 = OAc, R^2 = OAc
4 : R^1 = OH, R^2 = H
5 : R^1 = OAc, R^2 = H
6 : R^1 = H, R^2 = H

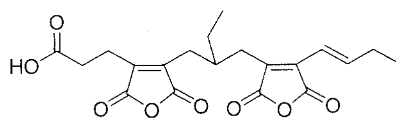
7 : R^2 = OH
8 : R^2 = H

9

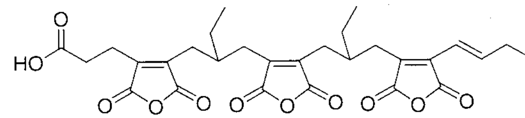
10 : R^1 = OH
11 : R^1 = H



12 : R^1 = OH
13 : R^1 = H



cordyanhydride A



cordyanhydride B

$7H_2O$ 0.1 g, per 1 liter distilled water).

The strain BCC 1620 was maintained on potato dextrose agar at 22°C for 5 days. The inoculum was transferred as 2 pieces of culture (5×5 mm) into 16×250 ml Erlenmeyer flasks, each containing 50 ml of PDB and statically incubated at 22°C. After 4, 7, 14 and 21 days, each 4 flasks (total 200 ml broth) was harvested and subjected to extraction and analysis of the metabolites. Cultivation in three other media (YES, M102, CDM) were also carried out simultaneously, using the inoculum on the same PDA plate.

Extraction and Metabolites Analysis for Optimization

The cultures in four Erlenmeyer flasks were filtered. The combined supernatant (*ca.* 200 ml) was extracted twice with AcOEt (150 ml each). The AcOEt solution was dried over $MgSO_4$, filtered and concentrated under reduced pressure to obtain a crude extract. The wet mycelia obtained from filtration were freeze-dried to measure dry-weight, and were extracted with MeOH (40 ml, room temperature, 2 days). To the filtrate was added 5 ml of H_2O and washed with 20 ml of hexane. The aqueous MeOH layer was separated from the hexane layer, and partially concentrated under reduced pressure. The residue was dissolved in AcOEt (60 ml) and washed with H_2O (20 ml), dried over $MgSO_4$, filtered and concentrated to yield a crude mycelial extract.

Extracts from both supernatant and mycelia were analyzed for secondary metabolites by silica gel TLC (5% MeOH in CH_2Cl_2). Quantitation of the metabolites was carried out by ^{1}H-NMR (in $CDCl_3$) analysis of each of the whole extract using 20 μmol of dimethylformamide (DMF) as an internal standard. Relative integration value between the peaks at δ 9.1~9.4 due to the phenolic protons of the bioxanthracenes (2H) and a δ 8.1 singlet peak (1H) due to DMF indicated the total amount of bioxanthracenes in each of the extract.

Antimalarial Activity and Cytotoxicity

The asexual erythrocytic stage of *Plasmodium falciparum* (K1, multidrug-resistant strain) were maintained continuously *in vitro* following the method of TRAGER and JENSEN.[4] Quantitative assessment of antimalarial activity *in vitro* was determined using the microculture radioisotope technique based upon the method described by DESJARDINES.[5] The crude extracts were initially dissolved

in DMSO (20 mg/ml) and diluted with the culture media. Aliquots (25 μl) of the compound having different concentrations were dispensed in a 96-well plate and a 1.5% cell suspension of parasitized erythrocytes with 1~2% parasitemia (200 μl) were added. The final concentration of DMSO (0.1%) did not affect the growth of the parasite. The mixtures were incubated in a 3% CO_2 incubator at 37°C. After 24 hours of incubation, 25 μl (0.25 μCi) of [^{3}H]-hypoxanthine were added to each well and the parasite cultures were further incubated under the same conditions for an additional 18~24 hours prior to harvesting the parasite DNA onto 96-well microplates with built-in glass fiber filters (Unifilter™ Plate, Packard, USA). The filters in the plates were dried and 22 μl of liquid scintillation fluid (Microscint™, Packard, USA) was added. The radioactivity on the filters was then measured using a microplate scintillation counter (TopCount™, Packard, USA). The 50% inhibitory concentrations (IC_{50}) were determined from the dose-response curves.

Cytotoxicity of the purified compounds against human epidermoid carcinoma (KB) and human breast cancer (BC-1) and vero cell lines were tested using the protocol described by SKEHAN *et al.*[6] IC_{50} values of a standard compound ellipticine are 0.46 μg/ml for KB, and 0.60 μg/ml for BC-1.

Results

Taxonomic Identification

Cordyceps pseudomilitaris BCC 1620 was described from a collection of Lepidoptera larvae in deciduous forest at Sam Lan National Park, Thailand (HYWEL-JONES, 1994). The specimens were compared with *Cordyceps militaris* (L.: Fr.) Link which is the Type Species for the genus. The major difference between the two species is that *C. pseudomilitaris* produces and discharges whole ascospores whereas *C. militaris* produces and discharges part-spores. *C. pseudomilitaris* is a stable part of the Lepidoptera population at Sam Lan and has been recorded every year from 1992 to the present in the latter part of the wet season (August to October). PETCH (1931) considered the production of whole ascospores a character that warranted the erection of a new genus-*Ophiocordyceps*. This was not accepted (MAINS, 1958) and recent molecular work (ARTJARIYASRIPONG, MITCHELL & HYWEL-JONES unpublished) indicates that the production of whole ascospores has evolved several times within the genus *Cordyceps*. This molecular work also confirms that *C. pseudomilitaris* is distinct from *C. militaris* and is ancestral to the Type Species and other species from Lepidoptera.

Optimization of Bioxanthracenes Production

In the preliminary studies, we isolated only small amount of major bioxanthracenes **1**~**5** from a total 2 liters culture broth statically cultivated in potato dextrose broth (PDB). Compounds **1**~**3** were isolated from AcOEt extract of the culture filtrate, while compounds **4** and **5** were obtained, along with **1**~**3**, from MeOH extract of mycelia. Cordyanhydrides A and B were also isolated from culture filtrate, but, not detected in the mycelia. Incubation on a rotary shaker led to the selective production of cordyanhydrides, where bioxanthracenes were not detected neither in culture filtrate nor in mycelia. For optimization of the bioxanthracenes production, quantitation of the metabolites was conducted by using four liquid media, PDB, YES, M102 and CDM. Amount of bioxanthracenes (total from culture filtrate and mycelia) and cordyanhydrides (in culture filtrate) from total 200 ml culture (4×50 ml), after 14 days' statical cultivation, are shown in Fig. 1, along with dry cell weights. Bioxanthracenes were best produced in YES, that is in parallel with the efficiency of cell growth. Incubation in M102 also gave better production of bioxanthracenes than in PDB. In CDM medium, neither the bioxanthracenes nor cordyanhydrides were produced. In YES cultures, bioxanthracenes were present mainly in mycelial cake and partially dissociated in the liquid medium (Fig. 2).

Large Scale Fermentation and Isolation

Large scale cultivation was carried out under the conditions optimized for bioxanthracenes production. The strain BCC 1620 in 40×1 liter Erlenmeyer flasks each containing 250 ml of YES medium were incubated for 14 days. The cultures were separated by filtration into mycelial cake and supernatant. Wet mycelia were extracted twice with each 1 liter of MeOH. After partial concentration of the MeOH solution into *ca.* 1 liter, H_2O (100 ml) was added and washed with hexane (600 ml). The aqueous MeOH layer was separated and concentrated under reduced pressure. The residue was dissolved in AcOEt (1 liter) and washed with H_2O (500 ml), dried over $MgSO_4$, and concentrated under reduced pressure to obtain a brown gum (2.4 g). The crude extract was passed through a silica gel column ($MeOH/CH_2Cl_2$, step gradient elution from 0 : 100 to 10 : 90) and was separated into seven fractions (Scheme 1). Compounds **1**~**11** and **13** were present in the fractions. The order of elution of compounds is as follow: (a mixture

Fig. 1. Comparison of liquid media type on the production of metabolites.

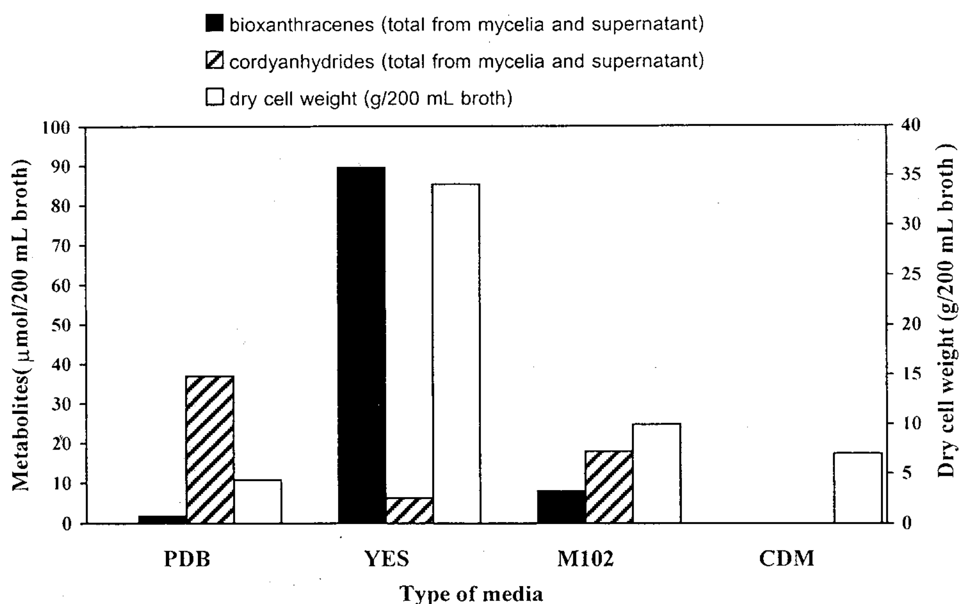
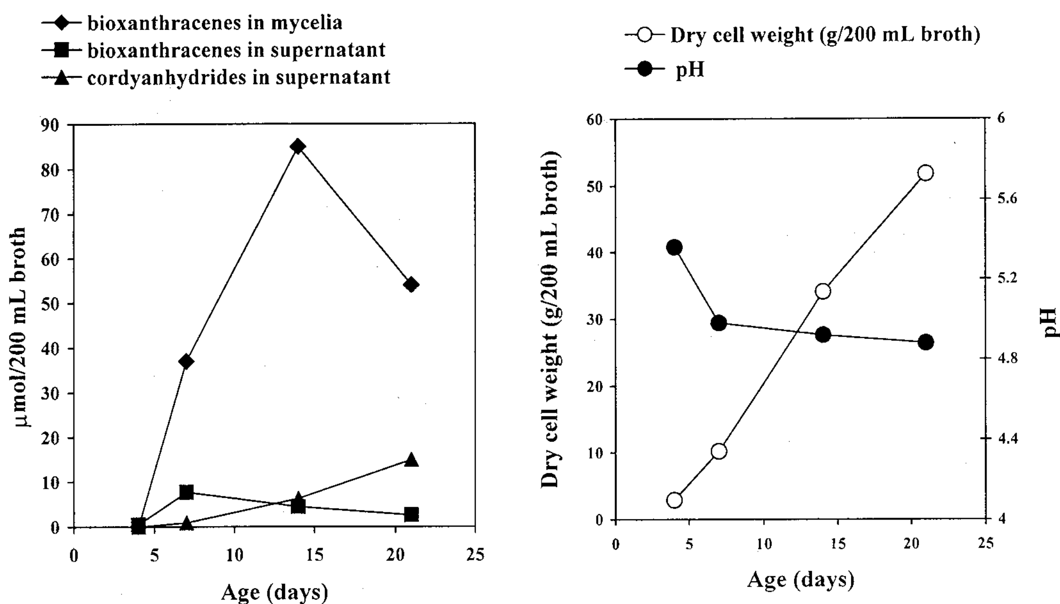
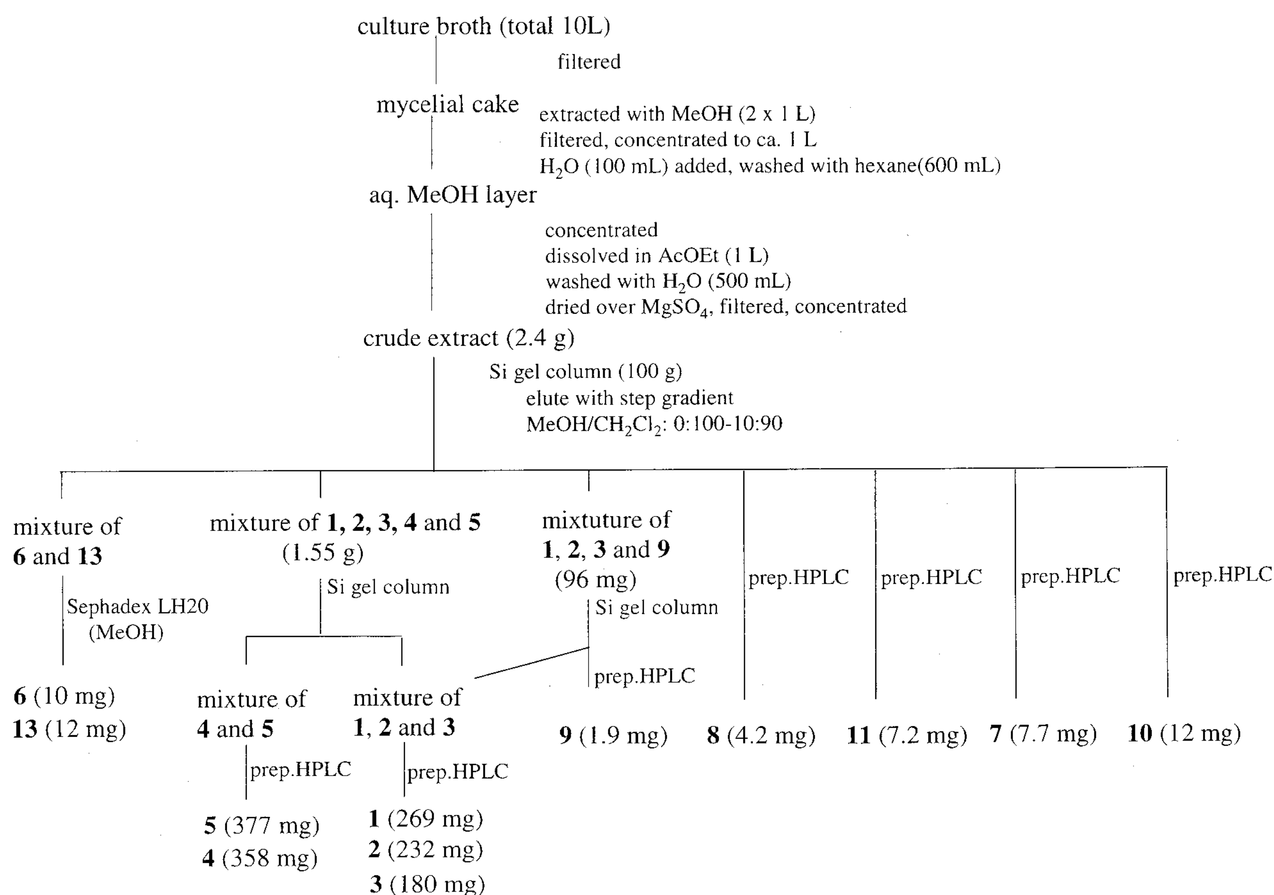


Fig. 2. Time course of cultivation in YES.



of **6** and **13**), (a mixture of **1**~**5**), (a mixture of **1**, **2**, **3** and **9**), **8**, **11**, **7**, **10**. Compound **6** and the monomer, **13**, were separated by Sephadex LH-20 column with MeOH as eluent, where **13** eluted after **6**. A mixture of **1**~**5** was separated into two mixtures (**4**, **5**) and (**1**, **2**, **3**) by repeated silica gel column chromatography ($MeOH/CH_2Cl_2=1:99$ to 2:98). Compounds **4** and **5** were separated and purified by continuous preparative HPLC (Nova-Pak HR C_{18}, 6 μm, 40×100 mm, $MeCN/H_2O=60:40$, 20 ml/minute). A mixture of compounds **1**~**3** was also separated by prep. HPLC ($MeCN/H_2O=40:60$). Compound **9** was separated from a silica gel column fraction mainly containing **1**~**3** by

Scheme 1. Isolation preocedure for bioxanthracenes **1**~**11** and a monomer **13**.



repeated silica gel column ($MeOH/CH_2Cl_2$=2:98) and further purified by prep. HPLC ($MeCN/H_2O$=40:60). Other four silica gel column fractions mainly containing **8**, **11**, **7** and **10**, respectively were also purified further by prep. HPLC ($MeCN/H_2O$).

Supernatant (*ca.* 10 liters) was extracted with an equal volume of AcOEt to obtain a brown gum (1.3 g) which mainly consisted of cordyanhydrides A and B, bioxanthracenes **1**, **2** and **3**, and fatty acids. A small amount of the mixture of the two monomers **12** and **13** was separated from the major metabolites by Sephadex LH-20 column (100 g, elution with MeOH), where the order of elution is in the following: fatty acids, cordyanhydrides, compounds **1**~**3**, monomers. The two monomers were separated by silica gel column chromatography (MeOH/CH_2Cl_2=1:99 then 3:97). The low Rf compound was further purified by preparative HPLC ($MeCN/H_2O$=30:70) and identified as monomer **12** (yield, 1.6 mg). The high Rf compound (1.5 mg) was spectroscopically consistent with monomer **13** isolated from the mycelial extract.

Comparison between the Isolates of *Cordyceps pseudomilitaris*

Since several different isolates of *C. pseudomilitaris* are deposited at the Thailand BIOTEC Culture Collection (BCC) and are available, the production of the metabolites was tested for each of the isolates. Seven isolates were statically cultivated in YES, and ethyl acetate extracts from culture filtrates were analyzed by TLC (silica gel) and ^{1}H-NMR to detect the metabolites (Table 1). Among the seven isolates tested, four produced bioxyanthracenes, while six produced cordyanhydrides. The production of bioxanthracenes and cordyanhydrides are independent of each other. However, the detection from four or six isolates indicates that both the bioxanthracenes and cordyanhydrides are common secondary metabolites of the fungus *C. pseudomilitaris*.

Table 1. Production of bioxanthracenes and cordyanhydrides in each isolate of *C. pseudomilitaris* collected at Sam Lan National Park.

registration code	date of collection	bioxanthracenes	cordyanhydrides
BCC 1919	12 Oct 1992	−	+
BCC 1512	15 Oct 1992	−	−
BCC 1888	02 Oct 1993	+	+
BCC 1620	30 Aug 1994	+	+
BCC 1472	7 Aug 1995	+	+
BCC 1784	26 Sep 1996	+	+
BCC 1979	26 Sep 1996	−	+

+ : detected by TLC and ^{1}H NMR analysis

− : not detected

Table 2. Antimalarial activity and cytotoxicity of the compounds isolated from *C. pseudomilitaris* BCC 1620.

compound	antimalarial activity (IC_{50}, μg/mL) *P. falciparum* (K1)	cytotoxicity (IC_{50}, μg/mL)		
		KB[a]	BC-1[b]	vero[c]
1	4.7	30	63	36
2	8.1	>100	>100	>100
3	2.2	>100	>100	>100
4	3.7	>100	>100	44
5	5.2	>100	>100	>100
6	8.4	6.3	>100	37
7	1.1	28	14	20
8	5.9	17	18	33
10	7.1	>100	47	>100
11	18	>100	>100	>100
13	64	>100	63	64
cordyanhydride A	>100	>100	>100	>100
cordyanhydride B	>100	>100	>100	>100
chloroquin diphosphate[d]	0.16	16	25	46

[a] Human epidermoid carcinoma in the mouth (oral cavity). [b] Human breast cancer cells. [c] African monkey kidney fibroblast. [d] Standard antimalarial compound.

Antimalarial Activity and Cytotoxicity

Bioxanthracenes **1**~**8**, **10** and **11**, isolated from the strain BCC 1620, exhibited *in vitro* antimalarial activity in the range of IC_{50} 1~18 μg/ml (Table 2). On the other hand, the monomer **13** showed much weaker activity. This indicates that the dimer structure is important for the antimalarial activity for this series of aromatic polyketide oxanthracenes. Cordyanhydrides A and B, another secondary metabolites of the strain BCC 1620, were inactive at a concentration of 100 μg/ml. For comparison, the compounds were also screened for cytotoxicity against three cell lines (Table 2). The bioxanthraces exhibited weak or no cytotoxicity. It should be noted that the cytotoxicity of the bioxanthracenes was not in parallel with their *in vitro* antimalarial activity. For example, the potent antimalarial **3** (IC_{50} 2.2 μg/ml) showed no cytotoxicity against all the three cell lines tested, while compound **1** and its atropisomer **7** were weakly cytotoxic.

Discussion

Compounds **1**~**6** and **11** were previously isolated from *Verticillium* sp. and designated as ES-242s (ES-242-4, -3, -2, -5, -1, -6 and -8, respectively),[7,8] while other four dimers **7**~**10** and two monomers **12**, **13** are naturally novel. The isolation of monomers indicates that dimerization occurs at the late stage of the bioxanthracene biosynthesis, after construction of the tricyclic oxanthracene unit. Compounds **1**~**6** have been proven to bear same axial stereochemistry (see accompanying paper). We also isolated two corresponding atropisomers **7** and **8** from *C. pseudomilitaris*. Taken together with the evidence of the isolation of an another type of dimers, **9**, **10** and **11**, it is suggested that the enzyme-monomers complex may be rather conformationally flexible for the transition state of the dimerization process.

ES-242s are reported to act as *N*-methyl-D-aspartate receptor antagonists. The most potent isomer is ES-242-1 (**5**) inhibiting [^{3}H]TCP binding to rat crude synaptic membrane with an IC_{50} value of 0.12 μM,[7~9] while the activity of other ES-242s are much lower, for example, IC_{50} 25 μM for ES-242-4 (**1**). In contrast, in our antimalarial activity assay, there observed no clear activity difference between the bioxanthracene isomers. This suggests that the action of the bioxanthracenes as NMDA receptor antagonist and the mechanistic origin of the antimalarial activity should be independent.

Acknowledgments

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