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Isolation of Bioactive Natural Products from Myxomycetes

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Abstract: The Myxomycetes (true slime molds) are an unusual group of primitive organisms that may be assigned to one of the lowest classes of eukaryotes. As their fruit bodies are very small and it is very difficult to collect much quantity of slime molds, few studies have been made on the chemistry of myxomycetes. Cultivation of the plasmodium of myxomycetes in a practical scale for natural products chemistry studies is known only for very limited species. Here is described a review on the recent results on isolation of bioactive natural products from myxomycetes obtained in these two years in the laboratories. Spore germination experiments were studied of hundreds of field-collected myxomycetes collected in Japan and succeeded in laboratory culture of plasmodia of several myxomycetes in a practical scale for natural products chemistry studies. As a result, pyrroloiminoquinones, polyene yellow pigments, and a peptide lactone from cultured plasmodia of *Didymium iridis*, *Physarum rigidum* and *P. melleum*, respectively were isolated. New naphthoquinone pigments, cycloanthranilylprolines, tyrosine-kinase inhibitory bisindoles, and a cytotoxic triterpenoid aldehyde lactone were also isolated from field-collected fruit bodies of *Cribraria purpurea*, *Fuligo candida*, *Tubifera casparyi*, and *Tubifera dimorphotheca*, respectively.

Key Words: Myxomycetes, slime molds, natural products, culture, fruit body, pigment, bisindole, triterpenoid.

1. INTRODUCTION

Natural products continue to play an important role in the discovery of low-molecular weight lead compounds for the development of new drugs for the treatment of human diseases. Most parts of natural resources on the earth still remain to be explored, particularly the marine and microbial environments, which may contain many unknown bioactive substances awaiting discovery [1,2]. During the investigations on search for bioactive natural products from unexplored natural resources [3,4], studies were initiated on search for bioactive metabolites of myxomycetes in 1998. Two years ago, a review was published on natural products from myxomycetes [5]. This review describes more recent results on the studies of bioactive metabolites from myxomycetes obtained in these two years in the laboratories.

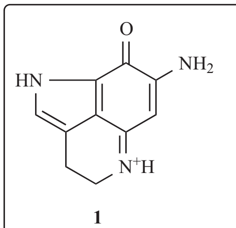
2. METABOLITES OF CULTURED MYXOMYCETES

2.1. Results of Spore-Germination Experiments

Cultivation of the plasmodium of myxomycetes in a considerable scale to carry out chemical studies has been known only for very limited species such as *Physarum polycephalum*. Cultivation of other myxomycetes for natural products chemistry studies had never been reported. The fruit bodies of the myxomycetes (222 strains) were collected in Japan after 2002, and laboratory-cultivation of these myxomycetes was investigated. Detailed procedures of cultivation experiments were described in the previous review [5]. Out of 222 strains of wild fruit bodies of myxomycetes, spore-germination was observed for 80 strains on an agar medium. Among them, 45 strains (8 *Didymium* sp., 14 *Physarum* sp., 3 *Criterium* sp., 2 *Stemonitis* sp., and 1 *Arcyria* sp.) were able to develop into the plasmodial stage, and 9 strains (2 *Didymium* sp., 2 *Physarum* sp., and 1 *Arcyria* sp.) successfully showed formation of fruit bodies.

2.2. Didymium iridis

Fruit bodies of *Didymium iridis*, which were found on a dead leaf, were collected at Ochi-san, Fukui Prefecture, Japan, in July 2002. Although chemical constituents were previously studied of the cultured myxomycetes of *Didymium squamulosum* [6], *D. bahiense* [7], and *D. minus* [8], no studies have been made on chemical examinations of the secondary metabolites of *D. iridis*. The plasmodium of this myxomycete obtained in a plate culture was mass cultured in the laboratory by agar plates with oatmeal [5]. From the extract of the harvested plasmodial cells, a green pigment, makaluvamine I (**1**), and a red pigment, damirone C (**2**) were isolated by silica gel column chromatography followed by HPLC separation [9]. These compounds were identified by comparison of the spectroscopic data including ^{1}H and/or ^{13}C NMR and FABMS with literature data [10]. These two pyrroloiminoquinone pigments had been previously isolated from a marine sponge *Zyzzya fuliginosa* [10]. Other related pyrroloiminoquinone pigments, makaluvamines A-N and damirones A-B, were also isolated from marine sponges [11-13], while makaluvamines A and B were previously isolated from the cultured myxomycete *Didymium bahiense* [7].



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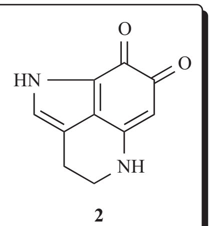

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Recently, it was reported that the makaluvamines can induce dose-dependent DNA cleavage *via* topoisomerase II, but the levels of cleavage for some of the makaluvamines are significantly below those achieved by equimolar concen-trations of etoposide [14]. Other groups also reported the synthesis of some of the pyrroloiminoquinone alkaloid derivatives and the evaluation *in vitro* for their antiproliferative activity using the murine L1210 leukemia cell line showed that they exhibited interesting antiproliferative properties with IC$_{50}$ values of less than 20 μM, while none of the pyrroloiminoquinone derivatives induced significant modi-fication of the L1210 cells cycle, thus implying that the pyrroloiminoquinone skeleton on its own is not able to exert a specific cytotoxic action [15].

2.3. Physarum rigidum

The fruit bodies of the myxomycetes *Physarum rigidum* were collected at Tokorozawa, Saitama Prefecture, Japan, in June 2001. The plasmodium of this myxomycete obtained in a plate culture was mass-cultured in the laboratory by agar plates with oatmeal. The 90% MeOH-soluble fraction of the extract of this plasmodium was revealed to contain a complex mixture of yellow pigments by TLC examinations. Separation by ODS column chromatography [50-60% aqueous MeOH with 0.1% trifluoroacetic acid (TFA)], followed by purification with silica column ($CHCl_3$/MeOH/H_2O, 8:2:1 to 7:3: 1) afforded three yellow pigments, physarigins A (**3**), B (**4**), and C (**5**) in 0.05, 0.006%, and 0.1% yield, respectively [16].

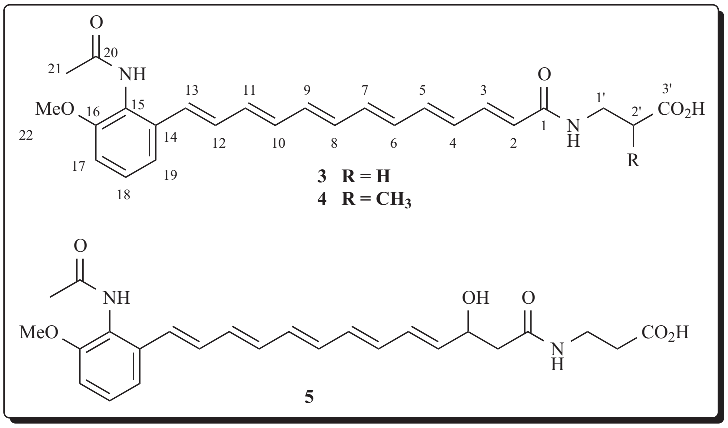
The molecular formula of physarigin A (**3**) was suggested as $C_{25}H_{28}O_5N_2$ by its positive HRESIMS data, and the UV spectrum of **3** showed absorption maxima at λ_{max} (MeOH) 403 (ε 26000) and 426 nm (22000), indicating the presence of a conjugated system. Analysis of the 2D NMR data showed that **3** consisted of a 1,2,3-trisubstituted benzene ring bearing a methoxy, an acetamide, and a polyene side-chain residue, and the polyene side-chain residue was revealed as a tridecahexanoyl group connected to a β-alanine unit through an amide bond [HMBC correlations: H_2-1'/C-1, H_2-1'/C-2', H_2-1'/C-3', H_2-2'/C-1', and H_2-1'/C-3']. Physarigin B (**4**) was revealed to possess a 3-aminoiso-butyric acid residue instead of the β-alanine residue of **3**, while physarigin C (**5**) had a β-hydroxy amide moiety at C-1 to C-3 position.

These yellow pigments of plasmodium of *Physarum rigidum* are relatively unstable and not easily dissolved in organic solvents. The HPLC analysis of physarigin A (**3**) and C (**5**) [Develosil ODS-UG-5; flow rate: 1.8 mL/min; eluent: 60% MeOH with 0.1% TFA; photodiode array detection] revealed that physarigin A (**3**, t_R 32.1 min; λ_{max} 351, 368, and 390 nm) was produced from physarigin C (**5**, t_R 18.3 min; λ_{max} 426 and 403 nm) after ODS flash column chromatography using 60% MeOH with 0.1% TFA. A β-hydroxy amide could not be isolated with 2-aminoisobutyric acid moiety, which may correspond to a precursor of physarigin B (**4**). Steglich and Steffan's group previously studied plasmodial yellow pigments of *Physarum polycephalum*, which were considered to act as photoreceptors, and they cultured the plasmodia on oat flakes and harvested after 3 days, and isolated physarochrome A [17] and other yellow pigments [18-21] from the methanol extracts of the plasmodia. They also isolated a yellow pigment fuligorubin A [22] from field-collected plasmodium of *Fuligo septica*, and this pigment was thought to be involved in photoreceptor and energy conversion processes during the lifecycle of *F. septica*. Yellow pigments of cultured myxomycetes except those of *Physarum polycephalum* had never been described before, while physarigins A-C (**3**-**5**) showed some structural similarities to physachrome A and fuligorubin A.

2.4. Physarum melleum

The fruit bodies of the myxomycetes *Physarum melleum* were collected at Tokorozawa, Saitama Prefecture, Japan, in June 2001. The plasmodium of this myxomycete obtained in a plate culture was mass-cultured in the laboratory on agar plates in the presence of *Escherichia coli*. The harvested plasmodial cells (19.5 g from 2112 plates (9 cmϕ)) were extracted with 90% MeOH and 90% acetone, and the combined extract was partitioned between ethyl acetate and water. The ethyl acetate-soluble layer was subjected to silica gel column chromatography, followed by separation with HPLC on ODS eluted with 50% MeOH to give two UV(254 nm)-positive compounds, named melleumins A (**6**) and B (**7**), in 0.007 and 0.02% yield, respectively [23].

Melleumin A (**6**) showed a quasi-molecular ion peak at *m/z* 500 $(M+H)^+$ and 522 $(M+Na)^+$ in its positive FAB mass spectrum. The molecular formula of **6** was revealed as

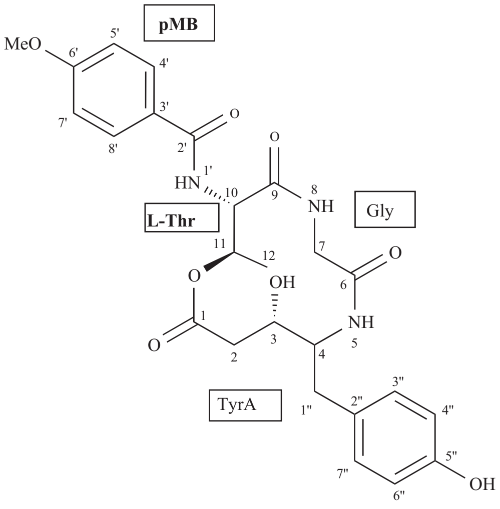


3 **R = H**
4 **R = CH$_3$**

5

$C_{25}H_{30}O_8N_3$ by the HRFABMS data [*m/z* 500.1995, $(M+H)^+$, Δ -3.9 mmu]. The UV spectrum of **6** showed absorption maxima at 254 and 225 nm, indicating the presence of conjugated or aromatic system(s). The ^{1}H NMR spectrum of **6** in DMSO-d_6 showed five signals of low-field resonances with no HMQC correlation with any carbons, which were assignable to hydroxyl or amide protons. The ^{1}H NMR also showed signals due to two *p*-substituted benzene rings, one methoxy proton, four oxygen- or nitrogen-bearing methine protons, three sp^3 methylenes, and one secondary methyl group. These observations were also corroborated by its ^{13}C NMR spectrum, aided by DEPT experiments, which gave signals assignable to four carbonyls, two *p*-substituted benzene rings, four sp^3 methines, three sp^3 methylenes, and two methyls including one methoxy group. Since twelve out of thirteen unsaturation equivalents were accounted for from the ^{13}C NMR data, compound **6** was inferred to have one ring besides the two benzene rings. Chemical shifts of four carbonyl groups (δ_C 170.7, 169.2, 169.1, and 166.6) were likely to be assigned to amides (or esters), and the nature of all these ^{1}H and ^{13}C NMR data was strongly suggestive that **6** was a peptide-related compound. The ^{1}H-^{1}H COSY spectrum of **6** showed connectivities for two amino acid residues: threonine (Thr) [H_3-12/H-11/H-10/NH-1’] and glycine (Gly) [H_2-7/NH-8], along with another proton-network included in an unusual amino acid residue [H_2-2/H-3/H-4/H_2-1”(NH-5)]. Interpretation of the HMBC spectral data of **6** revealed the presence of a *p*-methoxybenzoic acid group (pMB) [C-2’ to C-8’ position; HMBC correlations: MeO/C-6’, H-5’/C-3’(H-7’/C-3’), H-5’/C-7’(H-7’/C-5’), H-4’/C-6’ (H-8’/C-6’), H-4’/C-8’ (H-8’/C-4’), and H-4’/C-2’ (H-8’/C-2’)] and a *p*-hydroxyphenyl group [C-2” to C-7” position; HMBC correlations: H-3”/C-5” (H-7”/C-5”), H-3”/C-7” (H-7”/C-3”), H-4”/C-2” (H-6”/C-2”), H-4”/C-5” (H-6”/C-5”), and H-4”/C-6” (H-6”/C-4”)], and this *p*-hydroxyphenyl group was revealed to be connected to C-1” [HMBC correlations: H_2-1”/C-2”, H_2-1”/C-3”(C-7”), and H-3”(H-7”)/C-1”], constructing an unusual amino acid residue (C-1 to C-4 and C-1” to C-7” position), which corresponded to a tyrosine-attached acetic acid (TyrA). The HMBC correlation data clearly revealed the connectivity of these partial structural residues from TyrA to Gly (NH-5/C-6), from Gly to Thr (NH-8/C-9), and from Thr to pMB (NH-1’/C-2’), thus giving rise to the sequence of four residues as pMB-Thr-Gly-TyrA. This sequence was also corroborated by the observation of the ROESY correlations for 5-NH/H-7, 8-NH/H-10, and 1’-NH/H-4’(H-8’). The oxymethine proton on C-11 having low-field resonance (δ_H 5.63) showed HMBC cross-peak with C-1 carbonyl, which suggested the presence of a linkage between the hydroxyl group of Thr (C-11) and the carbonyl group of TyrA (C-1) through an ester bond to construct the whole molecule of melleumin A as **6**. From the analyses of spectral data, melleumin B (**7**) was deduced to be a seco acid methyl ester derivative of compound **6**. Treatment of **6** with 28% MeONa afforded **7**, which was detected on the basis of TLC examination. Since methanol was used during isolation processes, **7** was likely to be an artifact of isolation produced from **6**.

The stereochemistry of the chiral centers at C-3, C-10, and C-11 positions was examined by using compound **7**. After acid hydrolysis of **7** (6N HCl, 110 ˚C, 12 h), the



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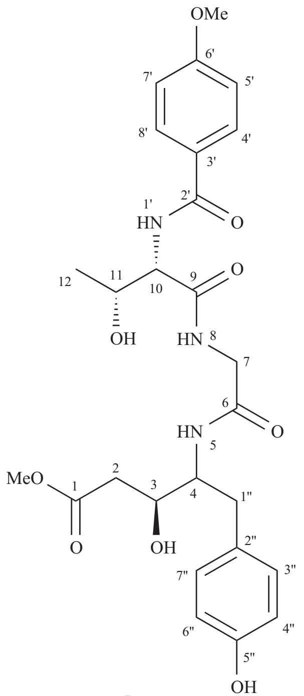

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resulting hydrolyzate was subjected to chiral TLC analysis using L-Thr and D-Thr reference samples to reveal that the threonine residue was L (10*S*, 11*R*). In addition, compound **7** was converted into its (*R*)- and (*S*)-MTPA esters, and on the basis of the modified Mosher's method, the absolute configurations of the carbons bearing secondary hydroxyl group (C-3 and C-11) were revealed as 3*S* and 11*R*. The 11*R*-configuration was also suggested by the chiral TLC analysis as above. The absolute stereochemistry of the C-4 position, however, remained undefined.

The crude extract of the cultured plasmodium of *Physarum melleum* exhibited antimicrobial activity against *Bacillus subtilis*, but the fraction containing melleumins A (**6**) and B (**7**) was inactive. The antimicrobial activity was likely to be ascribable to unstable yellow pigments, purification of which is not currently successful and is still under investigation in our laboratory. Melleumins A (**6**) and B (**7**) did not show cytotoxicity against VCR-resistant KB cell lines and TRAIL (TNF-related apoptosis inducing ligand)-resistant KOB (adult T cell leukemia) cell lines.

3. METABOLITES OF FIELD-COLLECTED MYXOMYCETES

3.1. Lindbladia tubulina

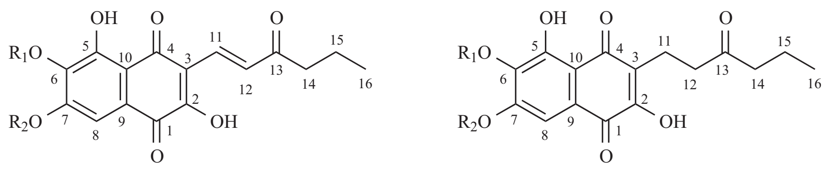
Lindbladione (**8**) and two related naphthoquinone pigments, 7-methoxylindbladione (**9**), and 6,7-dimethoxylindbladione (**10**) had been isolated from a field-collected sample of fruit bodies of *Lindbladia tubulina* collected at Takamagahara, Ohtsu, in Kochi Prefecture, Japan, in August 2001 [24]. The less polar fraction of the extract of this myxomycete we further investigated and three other naphthoquinone pigments, 6,7-dimethoxydihydrolindbladione (**11**), dihydrolindbladione (**12**), and 6-methoxydihydrolindbladione (**13**), were isolated from the EtOAc-soluble fraction of the methanol extract of this organism [25]. These three naphthaquinone pigments possessed a 11,12-saturated side chain (3-oxohexyl group) attached on C-3 position of the naphthaquinone nucleus. Lindbladione (**8**) and two other naphthaquinones (**9** and **10**) with 11,12-unsaturated side chain (3-oxo-1-hexenyl group) were previously isolated from water-soluble fraction of the methanol extract of this myxomycete.

The cytotoxic activities of three dihydrolindbladione derivatives (**11** - **13**) from the wild fruit bodies of *Lindbladia tubulina* against murine leukemia P388 cells were examined, and the IC_{50} values (µg/mL) against vincristine (VCR)-resistant P388 cells (P388/VCR) as well as those against a sensitive P388 strain (P388/S) are presented in Table **1**. Compound **12** was cytotoxic against all P388 cell lines, while compound **13** appreciably showed a reversal effect of multidrug resistance, since it was inactive against P388/S cell and P388/VCR cell in the absence of VCR, but was active against P388/VCR cell in the presence of VCR.

The pigments contained in the fruit bodies of *Cribraria intricata*, collected also at Takamagahara, Ohtsu, in Kochi Prefecture, Japan, in August 2001, were also examined, and purification of the extract of this myxomycete by chromatography using ODS eluted with acetonitrile and water afforded lindbladione (**8**) as a major pigment constituent (0.3% yield). Myxomycetes, *Lindbladia tubulina* and *Cribraria intricata*, belong to different genus but to the same family Cribrariaceae. It may be possible that lindbladione (**8**) is one of the common red pigments contained in myxomycetes of the family Cribrariaceae.

3.2. Cribraria purpurea

The fruit bodies of *Cribraria purpurea* (0.85 g) collected at Mt. Shiraga, Monobe-mura, in Kochi Prefecture, Japan, in November 2001, were extracted with 90% MeOH and 90% acetone. The combined extract (0.12 g) having antimicrobial activity against *B. subtilis* was subjected to chromatographies



8 $R_1=R_2=$ H
9 $R_1=$ H, $R_2=$ Me
10 $R_1=R_2=$ Me

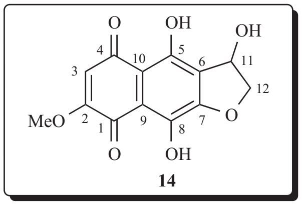
11 $R_1=R_2=$ Me
12 $R_1=R_2=$ H
13 $R_1=$Me, $R_2=$ H

Table 1. Cytotoxic Activity (IC_{50} Values, µg/mL) of Compounds 11 ~ 13[a)]

compound	P388/S	P388/VCR(-)	P388/VCR(+)
11	>50	>50	>50
12	12.1	14.0	12.6
13	>50	>50	13.8

[a)] P388/VCR is a vincristine-resistant P388 cell line, while P388/S is a sensitive P388 cell line. Tests towards P388/VCR cell line were carried out in the absence (-) and presence (+) of 0.004 µg/mL of VCR, which did not affect the growth of the cells.

on silica gel and Sephadex LH-20 to give cribrarione A (**14**) in 0.8% yield [26]. The molecular formula of **14** was revealed as $C_{13}H_{10}O_7$ by the HRFABMS data (*m/z* 279.0494, $[M+H]^+$, Δ–1.1 mmu). The UV spectrum of **14** showed absorption maxima at 275, 316, and 510 nm, which were shifted to 310, 537, and 568 nm, respectively, on addition of alkali (NaOH), indicating the presence of phenol group(s). By interpreting the ^1H, ^{13}C and 2D NMR spectral data, a naphthoquinone nucleus with two hydroxyl groups at C-5 and C-8 and a methoxy group on C-2 was constructed for compound **14**. It should be noteworthy that in the HMBC spectrum of **14** ($J_{C\text{-}H}$=8 Hz), cross-peaks were clearly observed from OH-5 (δ_H 13.2) to C-4 (δ_C 180.9) and C-3 (δ_C 110.1). These HMBC correlations may be attributable to 2- and 3-bond $J_{C\text{-}H}$ couplings, respectively, through a rigid intramolecular hydrogen bond between the hydroxyl proton (OH-5) and the oxygen of the C-4 carbonyl group, which further corroborated the proposed naphthoquinone structure. The ^{13}C chemical shift of C-7 (δ_C 158.0) implied that this carbon bore an oxygen atom, and the unsaturation degree of **14** had suggested the presence of one more ring other than the naphthoquinone. The C-7, therefore, had to be connected with the sp^3 methylene carbon (C-12) through an ether-oxygen atom to give rise to a dihydrofuran ring moiety. The oxymethine proton (H-11) exhibited a clear NOE to one of the oxymethylene proton (δ_H 4.81; H-12a), while the NOE between H-11 and the other oxymethylene proton (δ_H 4.71; H-12b) was obscure, thus suggesting that H-12a (δ_H 4.81) was *cis* and H-12b (δ_H 4.71) was *trans* to H-11. The coupling constant for H-11/H-12a (*cis*) was 7.3 Hz and that for H-11/H-12b (*trans*) was 3.1 Hz; these *J*-values may be consistent for the vicinal protons of a five-membered ring. Cribrarione A (**14**) was shown to be optically active from the CD spectrum. The absolute stereochemistry of the C-11 chiral center of cribrarione A (**14**), however, remained undefined, since MTPA esterification of **14** easily led to dehydration of 11-hydroxyl group to give a furan.



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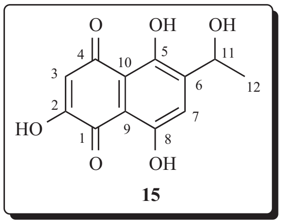
Chemical studies on the constituents of the myxomycetes of the genus *Cribraria* had never been described in the literature, and cribrarione A (**14**) was the first chemical constituent of the genus *Cribraria* sp., although the genus *Lindbladia* belongs to the same family (Cribrariaceae) as *Cribraria*. Crude extract of *Cribraria purpurea* exhibited antimicrobial activity against *Bacillus subtilis*, and this activity was revealed to be ascribable to cribrarione A (**14**), since **14** was substantially active against *B. subtilis* with a diameter of inhibition zone 11 mm at 5 µg per paper disc (8 mm in diameter).

3.3. Cribraria cancellata

The fruit bodies of *Cribraria cancellata* (1.44 g), collected at Seki, Ohtsu, Kochi-shi, in Kochi Prefecture, Japan, in August 2001, were extracted subsequently with 90% MeOH and 90% acetone. The residue of the combined extracts (0.22 g) containing brown-red pigments was subjected to chromatographies on ODS (0-100% MeOH in H_2O) and Sephadex LH-20 (50% MeOH in H_2O) to give cribrarione B (**15**) in 0.14% yield [27]. The molecular formula of cribrarione B (**15**) was revealed as $C_{12}H_{10}O_6$ by the HRFABMS data [*m/z* 249.0404, $(M-H)^-$, Δ +0.5 mmu]. The UV spectrum of **15** showed absorption maxima at 254, 299, and 484 nm, indicating the presence of conjugated system(s). The ^1H NMR spectrum of **15** in CD_3OD showed only four signals due to two aromatic (or olefinic) protons [δ_H 7.17 (1H, d, *J*=0.8 Hz) and 5.67 (1H, s)], one oxymethine [δ_H 5.13 (1H, qd, *J*=6.6 and 0.8 Hz)], and a secondary methyl [δ_H 1.43 (3H, d, *J*=6.6 Hz)] group. The ^{13}C NMR data of **15** showed twelve signals assignable to two carbonyls (δ_C 189.0 and 188.6), eight other sp^2 carbons, and one sp^3 oxymethine (δ_C 63.9) and methyl (δ_C 22.3) carbon. Since six out of eight unsaturation equivalents were accounted for from the ^{13}C NMR data, **15** was inferred to have two rings. The ^1H-^1H COSY spectrum of **15** showed that the oxymethine proton (δ_H 5.13; H-11) was adjacent to the secondary methyl group (δ_H 1.43; H_3-12). By interpreting the spectral data, a naphthoquinone nucleus was constructed for compound **15** and a 1-hydroxyethyl group was attached on the C-6 position. The presence of three other hydroxyl groups was suggested from the molecular formula of **15**, and these hydroxyl groups were assigned to be on C-2, C-5, and C-8 on the naphthoquinone nucleus from their ^{13}C NMR chemical shifts (δ_C 173.7, 151.2, and 157.4, respectively). The low-field resonance of C-2 (δ_C 173.7) was consistent to the ^{13}C chemical shift of the hydroxy-bearing α-carbon of *p*-quinones. From these results, the structure of cribrarione B was concluded as 2,5,8-trihydroxy-6-(1-hydroxyethyl)-[1,4]-naphthoquinone (**15**). Crude extract of *Cribraria cancellata* exhibited antimicrobial activity against *Bacillus subtilis*, but cribrarione B (**15**) proved inactive against *B. subtilis*.



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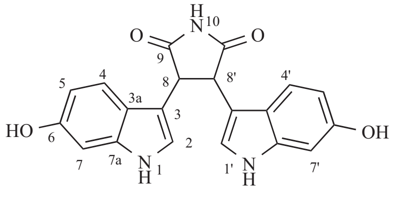
3.4. Arcyria ferruginea

The fruit bodies of *Arcyria ferruginea*, collected at Hao, Yasu-cho, in Kochi Prefecture, Japan, in May 2001, were extracted with 90% MeOH and 90% acetone. The combined extracts were subjected to chromatographies on silica gel and Sephadex LH-20 to give a new compound, dihydroarcyriarubin C (**16**, 0.07% yield), together with arcyriarubin C (**17**, 0.9% yield) and arcyriaflavin C (**18**, 0.14% yield) [28]. Compounds **17** and **18** were previously isolated from *Arcyria denudata* [29] and **18** was also obtained from *Metatrichia vesparium* [30].

Dihydroarcyriarubin C (**16**) was obtained as a colorless solid, and its ^1H NMR spectrum (in acetone-d_6) showed eight



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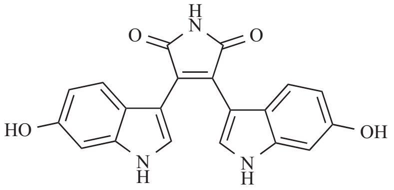

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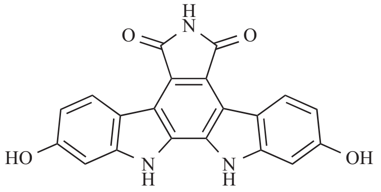

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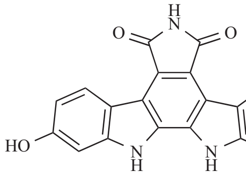
signals for four aromatic (or olefinic) protons, one sp^3 methine proton, and three OH or NH signals. The ^{13}C NMR data of **16** gave ten signals including eight aromatic (or olefinic) carbons, one sp^3 methine carbon, and one carbonyl carbon. These ^{1}H and ^{13}C NMR signals of sp^2 region of **16** suggested the presence of a 6-hydroxyindole moiety from comparison with those signals of arcyriarubin C (**17**). The H-5 (δ_H 6.61) and H-7 (δ_H 6.85) were coupled to each other by *J*=2.2 Hz, implying that these two hydrogens were *meta*. The H-5 was also coupled with H-4 (δ_H 7.25) by *J*=8.5 Hz, suggesting that H-4 and H-5 were *ortho*. The H-4 showed an HMBC correlation to C-3 (δ_C 111.3) and C-6. From these observations, the hydroxyl group was confirmed to be located on C-6. The sp^3 methine signal (δ_H 4.44) was observed as a singlet, which showed HMBC correlations to C-3 and C-3a (δ_C 120.4) of the indole nucleus, and also to the carbonyl carbon at δ_C 177.9. These observations along with comparison with spectral data of arcyriarubin C (**17**) suggested that the sp^3 methine was assignable to C-8. The HMBC spectrum clearly showed a cross-peak from H-8 to C-8 itself (δ_C 48.2), and this HMBC correlation may be assigned to H-8 to C-8' (or H-8' to C-8), thus implying that compound **16** is also dimeric. From these results, the structure of compound **16** was concluded as 8,8'-dihydroarcyriarubin C.

3.5. Tubifera casparyi

The fruit-bodies of *Tubifera casparyi*, collected at Mt. Mitsumine, Monobe-mura, in Kochi Prefecture, Japan, in November 1997, were extracted with 90% MeOH and 90% acetone. The combined extracts were partitioned between ethyl acetate and water, and the ethyl acetate-soluble fraction was subjected to chromatographies on silica gel and Sephadex LH-20 to give arcyriaflavin C (**18**, 0.2% yield) and arcyriaflavin B (**19**, 0.02% yield) [28]. Compounds **18** and **19** were previously isolated from *Arcyria denudata* [29].

Recently, considerable attention has been focused on the metabolites belonging to bisindolylmaleimides such as staurosporine (**20**) [31] and UCN-01 (**21**) [32], which were produced by the family of *Streptomyces*, *Actinomycetes*, and *Saccharothrixes*. The cell cycle inhibition effects of arcyriarubin C (**17**) and arcyriaflavin C (**18**) were examined by flow cytometry studies on HeLa cells. Compound **18** showed considerable increase of subG1 phase at more than 1 µg/mL, implying that this compound was cytotoxic at these concentrations. Arcyriaflavin C (**18**), however, exhibited significant increase of G2/M and G1 phases at 100 ng/mL and 10 ng/mL, respectively, suggesting that at these concentrations of **18**, the cell cycle was inhibited at these stages, respectively. The cell cycle inhibition effect of arcyriarubin C (**17**) was weak, compared with **18**; no significant change in flow cytometry study was observed at 10 µg/mL of **17**, while appreciable increase of subG1 phase and decrease of G1 phase was observed at 50 µg/mL of **17**. This finding suggested that the presence of a C-C bond between C-2 and C-2' positions appears to be important for the cell cycle inhibition effect.



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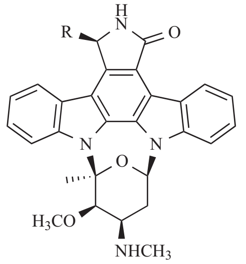


20 **R = H**
21 **R = OH**

3.6. Lycogala epidendrum

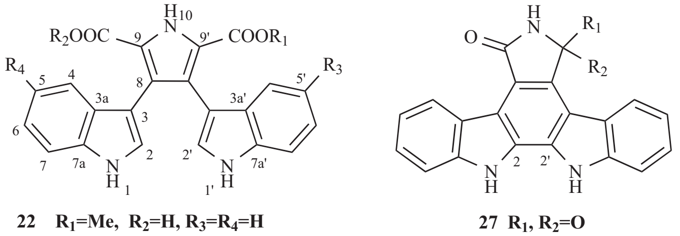
The fruit bodies of *Lycogala epidendrum*, collected in Kochi Prefecture, Japan, in January 2002, were also extracted with 90% MeOH and 90% acetone. The combined extracts were partitioned between EtOAc and water, and the EtOAc layer was then subjected to chromatographies on silica gel, ODS, and Sephadex LH-20 to give three new compounds **22** - **24** as well as six known bisindoles, which were identified as lycogarubins B (**25**) and C (**26**) [33], arcyriaflavins A (**27**) and B (**19**) [34], staurosporinone (**28**) [35], and lycogaric acid A (**29**) [34] on the basis of comparison of the spectral data [36].

The molecular formula of compound **22** was revealed as $C_{23}H_{17}N_3O_4$ by the HRFABMS data (*m/z* 399.1184, $[M]^+$, Δ -3.5 mmu). The ^1H NMR spectral data of **22** were similar to those of lycogarubin C (**26**), but **22** had only one methoxy group, while lycogarubin C (**26**) had two. Lycogarubin C (**26**) was a symmetrical bisindole with two methyl ester groups, whereas the ^1H and ^{13}C NMR data of **22** showed that **22** was unsymmetrical. Thus, compound 3 was deduced to have one carboxylic acid and one methyl ester, corresponding to monodemethyl derivative of lycogarubin C (**26**). Compounds **23** and **24** had the same molecular formula of $C_{23}H_{17}N_3O_5$ as revealed by the HRFABMS data (**23**: *m/z* 415.1179, $[M]^+$, Δ +1.1 mmu; **24**: *m/z* 415.1179, $[M]^+$, Δ +1.1 mmu). The ^1H and ^{13}C NMR spectra of **23** and **24** were similar to each other and both of them also resembled those of lycogarubin B (**25**), which was an asymmetric molecule with one indole and one 5-hydorxyindole moieties with two methoxycarbonyl groups at C-9 and C-9' positions. The ^1H and ^{13}C NMR data showed that both compounds **23** and **24** had only one methoxy groups. This fact as well as interpretation of the ^1H-^1H COSY and HMBC spectral data suggested that compounds **23** and **24** were monodemethyl derivatives of lycogarubin B (**25**). The NOE experiment using compound **23** showed an NOE correlation observed between the methoxy protons and H-4', which was on the 5-hydroxyindole group. This result suggested that compound **23** had the 5-hydroxyindole on the adjacent position (C-8') to the methoxycarbonyl group on C-9', while compound **24** was an isomer of **23** possessing the indole group on C-8'.

Arcyriaflavins A (**27**) and B (**19**) and staurosporinone (**28**), which had a C-C bond connection between C-2 and C-2' positions, showed cytotoxic activity against HeLa cells with IC_{50} values of 47.6, 8.9, and 4.4 µg/mL, respectively, while compound **22**, lycogarubins B (**25**) and C (**26**), and lycogaric acid A (**29**) without C-2/C-2' bond were almost inactive with IC_{50} values of 93.3, >100, 24.0, >100, respectively. Arcyriaflavin B (**19**) showed cytotoxicity (IC_{50} value, 2.3 mg/mL) against vincristine (VCR)-resistant KB cells, but did not show the reversal effect of VCR resistance. Furthermore, arcyriaflavin B (**19**) was evaluated in the Japanese Foundation for Cancer Research 39 cell line panel assay (Table **2**) [37]. Although arcyriaflavin B (**19**) showed low differential cellular sensitivities, NCI-H522 and NCI-H226 lung cells, BSY-1 breast cancer cells, SK-OV-3 ovary cells, and MKN7 stomach cells were relatively sensitive to **19** with $LogGI_{50}$ values of –6.66, -6.07, -6.24, -6.15, and –6.10, respectively, while HCT-15 and HT-29 colon cells, SF-295 CNS cancer cells, and HBC-5 breast cancer cells were relatively resistant with $LogGI_{50}$ values of –5.36, -5.45, -5.53, and –5.57, respectively. The average logarithm of the GI_{50} (MG-MID) across all cell lines tested was –5.80. The fingerprints of compound **19** appreciably showed differential growth inhibition with the delta and range values of 0.86 and 1.3, respectively (effective value: delta >0.5 as well as range >1.0), indicating that compound **19** had selective cytotoxic activity. Evaluation of the pattern of differential cytotoxicity using the COMPARE program [38] suggested the possibility that the mode of action for **19** might be different from that shown by any other anticancer drug developed to date.

Another collection of the fruit bodies of *L. epidendrum*, collected at Ohtsu, Kochi-shi in Kochi Prefecture, Japan, in February 2004, were extracted with 90% MeOH and 90% acetone successively, and the combined crude extract was partitioned between H_2O and EtOAc. The EtOAc-soluble fraction was subjected to silica gel, ODS or/and Sephadex LH-20 column chromatography, and further purification by reversed-phase HPLC gave two new bisindole alkaloids (**30** and **31**), as well as eight known compounds, which were identified as lycogarubins B (**25**) and C (**26**) [33], arcyriaflavins A (**27**) and B (**19**) [34], staurosporinone (**28**) [35], lycogaric acid (**29**) [34], arcyriarubin A (**32**) [34], and lycogarubin A (**33**) [33], on the basis of comparison with their spectral data in the literatures. Two new bisindoles (**30** and **31**), as well as two known ones (**32** and **33**) were not obtained from the extracts of *L. epidendrum* of collection in 2002 [39].

Compound **30** was obtained as light brown amorphous solid, and its molecular formula was determined as $C_{20}H_{13}O_2N_3$ by the HRFABMS data (*m/z* 327.0981, M^+, Δ –2.7 mmu). The IR absorptions were indicative of the presence of hydroxyl group (3282 cm^{-1}) and aromatic ring (1646 and 1583 cm^{-1}), and the UV absorption maxima were observed at 342, 294 and 226 nm, suggesting the presence of conjugating or aromatic system(s). The ^1H NMR spectral data in acetone-d_6 showed the signals for seven aromatic



22 R$_1$=Me, R$_2$=H, R$_3$=R$_4$=H
23 R$_1$=Me, R$_2$=H, R$_3$=OH, R$_4$=H
24 R$_1$=Me, R$_2$=H, R$_3$=H, R$_4$=OH
25 R$_1$=R$_2$=Me, R$_3$=OH, R$_4$=H
26 R$_1$=R$_2$=Me, R$_3$=R$_4$=H
29 R$_1$=R$_2$=H, R$_3$=R$_4$=H

27 R$_1$, R$_2$=O
28 R$_1$=H, R$_2$=H

Table 2. Results of Human Cancer Cell Line Panel Assay of Arcyriaflavin B (19)

origin of cancer	cell line	Arcyriaflavin B (19)	
		Log GI_{50} (M)[a)]	(Log GI_{50}) - (MG-MIG)
breast	HBC-4	-5.77	-0.03
	BSY-1	-6.24	0.44
	HBC-5	-5.57	-0.23
	MCF-7	-5.77	-0.03
	MDA-MB-231	-5.69	-0.11
central nervous system	U251	-5.76	-0.04
	SF-268	-5.70	-0.10
	SF-295	-5.53	-0.27
	SF-539	-5.87	0.07
	SNB-75	-5.73	-0.07
	SNB-78	-5.80	0.00
colon	HCC2998	-5.81	0.01
	KM-12	-5.85	0.05
	HT-29	-5.45	-0.35
	HCT-15	-5.36	-0.44
	HCT-116	-5.90	0.10
lung	NCI-H23	-5.73	-0.07
	NCI-H226	-6.07	0.27
	NCI-H522	-6.66	0.86
	NCI-H460	-5.70	-0.10
	A549	-5.62	-0.18
	DMS273	-5.75	-0.05
	DMS114	-5.82	0.02
melanoma	LOX-IMVI	-5.87	0.07
ovary	OVCAR-3	-5.75	-0.05
	OVCAR-4	-5.80	0.00
	OVCAR-5	-5.76	-0.04
	OVCAR-8	-5.70	-0.10
	SK-OV-3	-6.15	0.35
kidney	RXF-631L	-5.86	0.06
	ACHN	-5.97	0.17
stomach	St-4	-5.64	-0.16
	MKN1	-5.66	-0.14
	MKN7	-6.10	0.30
	MKN28	-5.64	-0.16
	MKN45	-5.83	0.03
	MKN74	-5.79	-0.01

(Table 2. Contd….)

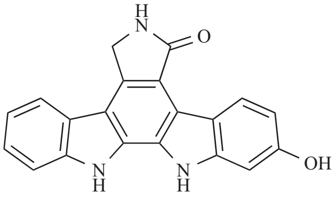
origin of cancer	**cell line**	**Arcyriaflavin B (19)**	
		Log GI_{50} (M)$^{a)}$	**(Log GI_{50}) - (MG-MIG)**
prostate	DU-145	-5.85	0.05
	PC-3	-5.83	0.03
MG-MID$^{b)}$		-5.80	
delta$^{c)}$		0.86	
range$^{d)}$		1.30	

$^{a)}$Log concentration of compound for inhibition of cell growth at 50% compared to control.

$^{b)}$Mean value of log GI_{50} over all cell lines tested.

$^{c)}$The difference in log GI_{50} value of the most sensitive cell and MG-MID value.

$^{d)}$The difference in log GI_{50} value of the most sensitive cell and the least sensitive cell.



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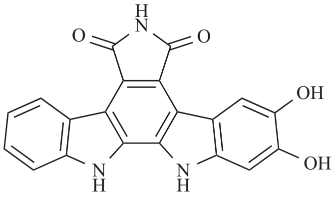

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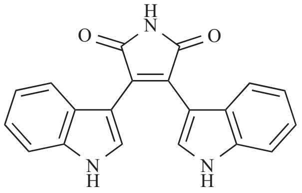

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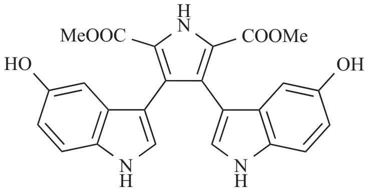


33

protons, sp^3 methylene protons (δ_H 5.02, 2H, s), and two NH signals (δ_H 11.08 and 10.76). The ^{13}C NMR spectrum displayed signals assignable to eighteen aromatic (or olefinic) carbons, one sp^3 methylene carbon (δ_C 46.2) and one carbonyl carbon (δ_C 173.6). These ^{1}H and ^{13}C NMR data were similar to those of staurosporinone (**28**) [35], while compound **30** possessed one more oxygen atom in its molecular formula than **28**. The ^{1}H-^{1}H COSY spectrum showed the connectivity between two aromatic protons of H-4 (δ_H 9.17) and H-5 (δ_H 6.80), and also showed the presence of four consecutive aromatic protons of H-4' (δ_H 8.00), H-5' (δ_H 7.27), H-6' (δ_H 7.40), and H-7' (δ_H 7.64). The H-5 showed *meta*-coupling (*J*=1.8 Hz) with an aromatic proton resonating at δ_H 7.64, which was assignable to H-7. This observation suggested that a hydroxyl group was attached on C-6, which was consistent with the low field resonance of C-6 (δ_C 157.3). These assignments were also suggested by the HMBC correlations observed for H-4/C-2, H-4/C-3, H-4/C-6, H-5/C-3a, H-5/C-7, H-7/C-3a, and H-7/C-5. In addition, the NOESY spectrum showed correlations from the methylene protons on C-9' (H_2-9', δ_H 5.02, 2H, s) to H-4' (δ_H 8.00) and H-5' (δ_H 7.27), suggesting that the sp^3 methylene (C-9') was present on the side of the benzene ring without hydroxyl group. Accordingly, the structure of **30** was concluded to be 6-hydroxystaurosporinone.

Compound **31**, obtained as yellow amorphous solid, had the molecular formula $C_{20}H_{11}O_4N_3$ as determined by the HRFABMS data (*m/z* 357.0762, M$^+$, Δ +1.2 mmu). Analysis of the ^{1}H and ^{13}C NMR spectra of **31** and comparison of these data with those of arcyriaflavin A (**27**) or B (**19**) implied that they had the same bisindole backbone-skeleton. Moreover, its molecular weight was 32 amu bigger than that of **27** in FABMS analysis (*m/z* 357 [M]$^+$ for **31** and *m/z* 325 [M]$^+$ for **27**); 32 amu corresponded to two oxygen atoms, thus suggesting that **31** possessed two hydroxyl groups (IR, 3310 cm^{-1}) attached on the bisindole nucleus of **27**. The ^{1}H NMR and ^{1}H-^{1}H COSY of **31** showed signals due to four successive aromatic protons [δ_H 9.10 (H-4'), 7.31 (H-5'), 7.48 (H-6'), and 7.64 (H-7')] and two other aromatic protons observed as singlets [δ_H 8.56 (1H, s) and 7.12 (1H, s)]. These two singlets were assignable to H-4 and H-7, respectively, from the HMBC correlations observed for H-4/C-3, H-4/C-5, H-4/C-6, H-4/C-7a, H-7/C-3a, H-7/C-5, and H-7/C-7a. From these findings, two hydroxyl groups were inferred to be located vicinally at C-5 and C-6 positions, the ^{13}C chemical shifts of which (δ_C 141.4 and 136.9, respectively) were consistent with those of corresponding positions of 5,6-dihydroxyindole (δ_C 142.4 and 140.3, respectively). From these results, the structure of **31** was revealed as 5,6-dihydroxyarcyriaflavin A.

Two new compounds (**30** and **31**) exhibited cytotoxicity against HeLa cells with IC_{50} values of 5.4 µg/mL and 2.1 µg/mL, respectively. The new compounds (**30** and **31**) as well as known compounds lycogarubins B (**25**) and C (**26**), and arcyriarubin A (**32**) also exhibited cytotoxicity against Jurkat cells as shown in Table **3**, while compounds **30** and **31** showed slightly weak cytotoxicity against vincristine resistant KB/VJ300 cells, as compared with that on Jurkat cells, and compounds **25**, **26**, and **32** were almost inactive (Table **3**).

The effects on protein kinase activities of these bisindole alkaloids (**30**, **31**, **25**, **26**, and **32**) as well as arcyriaflavins B (**19**) and C (**18**), which were previously obtained from *Tubifera casparyi* [26], were examined using a method that allows detection of activities of several protein kinases simultaneously [40]. When 6-hydroxystaurosporinone (**30**) was added at 1 µg/mL to a postnuclear supernatant of v-Src expressing NIH3T3 cells and phosphorylation was allowed to proceed, the intensities of band of proteins phosphorylated by protein tyrosine kinase (PTK) markedly decreased. Compound **30**, added at a higher concentration (10 µg/mL), also induced the decrease of the band intensities of proteins phosphorylated by protein kinase C (PKC), protein kinase A (PKA), eukaryotic elongation factor-2 kinase (eEF2K), and vascular endothelial growth factor receptor-1 (VEGFR-1 or Flt-1) kinase (results not shown), while the phosphorylation by epidermal growth-factor receptor (EGFR) kinase was not altered as summarized in Table 4. Arcyriaflavins B (**19**) and C (**18**) more strongly inhibited PKC, PTK, and PKA than 6-hydroxystaurosporinone (**30**). 5,6-Dihydroxyarcyriaflavin A (**31**) and arcyriarubin A (**32**) added at 10 µg/mL also induced decrease of the band intensity due to phosphorylation by PTK and PKC, while lycogarubins B (**25**) and C (**26**) added at 10 µg/mL showed almost no effects on phosphorylation by these protein kinases. The order of the potency of cytotoxicity appears almost parallel to that of protein kinase inhibition activity (Tables **3** and **4**).

3.7. Arcyria cinerea

The wild fruit bodies of *Arcyria cinerea*, collected in Kochi Prefecture, Japan, in January 2003, were extracted with 90% MeOH and 90% acetone. The combined extracts were separated by chromatographies on silica gel, ODS, and Sephadex LH-20 to give two new bisindole alkaloids, named cinereapyrrole A (**34**) and B (**35**) [36], together with a known compound, arcyriarubin A (**32**), which was previously isolated from *Arcyria denudata* [29] and *Lycogala epedendrum* [34].

Cinereapyrrole A (**34**) was shown to have the molecular formula $C_{22}H_{17}N_3O_4$ by the HRFABMS data (*m/z* 387.1208, $[M+H]^+$, Δ -1.1 mmu). The UV spectrum of **34** showed absorption maximum at 283 nm, indicating the presence of conjugated system(s), while the IR absorption bands observed at 3396 and 1695 cm^{-1} implied the presence of hydroxy and conjugated carbonyl groups. The ^1H NMR spectrum of **34** in CD_3OD showed ten signals for nine aromatic protons and one methoxy group. The analysis of the ^{13}C NMR and HMQC spectra of 34 clarified the presence of twenty two carbons including nine sp^2 methines, eleven sp^2 quaternary carbons, one methoxy (δ_C 51.3), and one carboxy or carboxy ester carbon (δ_C 163.6). By analysis of the ^1H-^1H COSY and HMBC spectra of compound **34** suggested the presence of two sets of 5-hydroxyindole moieties by observation of the following cross-peaks [^1H-^1H COSY: H-6/H-7 and H-6'/H-7'; HMBC: H-2/C-3, H-2/C-3a, H-2/C-7a, H-4/C-3, H-4/C-5, H-4/C-7a, H-6/C-7a, H-7/C-3a, H-7/C-5, H-2'/C-3', H-2'/C-3a', H-2'/C-7a', H-4'/C-7a', H-6'/C-7a', H-7'/C-3a', and H-7'/C-5']. The ^{13}C NMR chemical shifts of C-5 (δ_C 151.0) and C-5' (δ_C 151.4) suggested these carbons bore hydroxy groups. These NMR data were reminiscent of those of lycogarubin A (**33**), previously isolated from *Lycogala epidendrum* [33]. Lycogarubin A (**33**) was symmetrical compound with one set of NMR signals, while NMR data of **34** implied that compound **34** was an unsymmetrical molecule. Another difference was the observation of an additional sp^2 proton observed at δ_H 7.29 (1H, s) in the ^1H NMR of **34**, which showed HMBC correlations to C-8, C-8' and C-9'. Thus, this additional hydrogen was placed on the C-9 position, while a methoxycarbonyl group was inferred to be attached on the C-9' position as in case of lycogarubin A (**33**); the methoxy protons at δ_H 3.65 (3H, s) showed an HMBC correlation to the ester carbonyl carbon (δ_C 163.6). An NOE correlation observed between H-9 and H-4 further confirmed the presence of a hydrogen atom at the C-9 position. The structure of cinereapyrrole A was therefore concluded as **34**.

Cinereapyrrole B (**35**) had a molecular formula of $C_{22}H_{17}N_3O_3$ as shown by the HRFABMS data (*m/z* 371.1278, $[M+H]^+$, Δ +0.8 mmu). The UV and IR spectral data were paralle to those of **34**, and the ^1H and ^{13}C NMR data were also similar to those of compound **34**. The difference was

Table 3. Cytotoxicity Against Jurkat Cells, KB/VJ300 Cells, and HeLa Cells (IC_{50}, µg/mL)

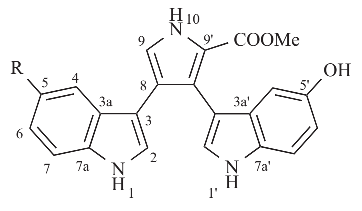
	Jurkat	KB/VJ300	HeLa
6-hydroxystaurosporinone (**30**)	1.34	7.6	5.4
5,6-dihydroxyarcyriaflavin A (**31**)	0.84	25	2.1
lycogarubin B (**25**)	18.0	>25	>100
lycogarubin C (**26**)	15.3	>25	24.0
arcyriarubin A (**32**)	6.3	>25	-

Table 4. Inhibition of Protein Kinase Activities of Bisindoles (30, 31, 25, 26, 32, 19, and 18)[a]

compounds	μg/mL	eEF2K	PKC	PTK	PKA	EGFR	Flt-1
6-hydroxystaurosporinone (30)	10	+	+	++	+	-	+
	1	-	-	+	-	-	-
	0.1	-	-	-	-	-	-
5,6-dihydroxyarcyriaflavin A (31)	10	-	+	+	-	-	+
	1	-	-	-	-	-	-
	0.1	-	-	-	-	-	
lycogarubin B (25)	10	-	-	-	-	-	-
	1	-	-	-	-	-	-
	0.1	-	-	-	-	-	
lycogarubin C (26)	10	-	-	-	-	-	-
	1	-	-	-	-	-	-
	0.1	-	-	-	-	-	
arcyriarubin A (32)	10	-	+	+	+	-	-
	1	-	-	-	-	-	-
	0.1	-	-	-	-	-	
arcyriaflavin B (19)	10	-	++	++	++	-	-
	1	-	+	+	+	-	-
	0.1	-	-	-	-	-	
arcyriaflavin C (18)	10	-	++	++	++	-	++
	1	-	+	++	++	-	-
	0.1	-	-	-	-	-	

[a]The indications "++", "+", and "-" mean >80%, 50-80%, and <50% kinase inhibition in each kinase assay, respectively.

observed in the ^{1}H NMR spectrum of **35**, showing four consecutive aromatic proton signals [δ_H 7.64 (H-4), 6.95 (H-5), 7.04 (H-6), and 7.31 (H-7)], which was supported by the ^{1}H-^{1}H COSY spectrum of **35** [cross peaks: H-4/H-5, H-5/H-6, and H-6/H-7]. These aromatic proton signals were assigned to a part of an indole nucleus without any substituents on the benzene ring part, while the ^{1}H NMR spectrum of 2 also showed signals assignable to 5-hydroxyindole moiety [δ_H 6.62 (H-4'), 6.63 (H-6'), and 7.19 (H-7')], as possessed by compound **34**, which was confirmed by the HMBC spectrum of 2 in acetone-d_6 observed for H-2'/C-3a', H-2'/C-7a', H-4'/C-3', H-4'/C-6', H-6'/C-4', H-6'/C-7a', H-7'/C-5', H-7'/C-3a', OH-5'/C-4', OH-5'/C-5', and OH-5'/C-6'. Thus, compound **35** was suggested to possess one indole and one 5-hydroxyindole moieties. As in case of compound **34**, compound **35** bore a hydrogen atom on C-9 (δ_H 7.44, H-9), which was indicated by the HMBC correlations observed for H-9/C-8, H-9/C-9, and H-9/C-9' and the ^{1}H-^{1}H COSY cross peak between H-9 and NH-10 in acetone-d_6 solution. An NOE was observed between H-9 (δ_H 7.44) and H-4 (δ_H 7.64), thus suggesting that the indole ring without 5-OH group was attached on the adjacent position (C-8) to H-9 and the 5-hydroxyindole ring was therefore present at the neighboring position (C-8') to the methoxycarbonyl group. Thus, structure of cinereapyrrole B was revealed as **35**, which corresponded to cinereapyrrole A (**34**) without 5-hydroxy group, and also corresponded to lycogarubin B (**25**) [33] without 9-methoxycarbonyl group.



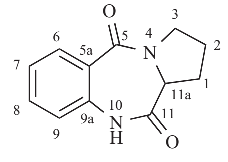
34 R=OH
35 R=H

Recently, further studies on the bioactivities of bisindole alkaloid derivatives were described. Several unsymmetrical derivatives of arcyriaflavin A (**27**) were synthesized and their antiviral activity was evaluated, and one of the derivatives having an ethyl group on N-1 position exhibited potent inhibitory activity against human cytomegalovirus (HCMV) replication in cell culture [41]. A series of macrocyclic bisindolylmaleimides containing linkers with multiple heteroatoms were prepared and several compounds effectively blocked interleukin-8 release induced by PKC-βII and increased glycogen synthase activity by inhibiting GSK (glycogen synthase kinase)-3β in cell-based functional assays [42].

3.8. Fuligo candida

The fruit bodies of *Fuligo candida*, collected at Motoyama-machi in Kochi Prefecture, Japan, in August 2001 and 2002, were extracted with 90% MeOH and 90% acetone. The combined extracts were partitioned between EtOAc and water, and the EtOAc-soluble fraction was then subjected to chromatographies on silica gel, ODS, and Sephadex LH-20 to give cycloanthranilylproline (**36**) and its derivatives (**37** and **38**). From the water-soluble fraction, separation by ODS and silica gel flash chromatographies along with reversed-phase HPLC (Develosil C30-UG-5) afforded an unstable polar cycloanthranilylproline-derivative (**39**), together with 4-aminobenzoyltryptophan (**40**) [43]. Compound **36** (= cycloanthranilylproline) was previously known, and was shown to contain L-proline from the comparison of the sign of optical rotation of **36** in the literature, which was previously obtained from a Cruciferous plant *Isatis indigotica* [44].

Compound **37**, which was the major constituent of the EtOAc-soluble fraction and was positive on the Ehlrich-reagent test on TLC, was obtained as a colorless plate, and shown to have the molecular formula $C_{15}H_{16}O_2N_2$ by the HRFABMS data (*m/z* 257.1289, $[M+H]^+$, Δ –0.1 mmu). The ^{13}C NMR spectrum of **37** showed signals for two carbonyls (δ_C 198.2 and 165.5), eight sp^2 olefinic or aromatic carbons, one nitrogen-bearing sp^3 methine (δ_C 55.2), and three *sp*^{3} methylenes (δ_C 47.0, 23.3, and 26.9), one of which was suggested to be attached to a nitrogen atom from its chemical shift (δ_C 47.0). The ^{1}H NMR spectrum of **37** showed signals due to four aromatic protons (δ_H 7.02-7.96), and *sp*^{3} methine and methylene protons (δ_H 2.1-3.8). These NMR spectral data were similar to those of cycloanthranilylproline (**36**), and the ^{1}H-^{1}H COSY (H-6/H-7, H-7/H-8, and H-8/H-9; H-11a/H$_2$-1 and H$_2$-2/H$_2$-3) and HMBC (H-6/C-5, H-6/C-9a, H-6/C-8, H-7/C-5a, H-7/C-9, H-8/C-6, H-8/C9a, H-9/5a, H-9/C-7, NH-10/C-9a, NH-10/C-5a, and NH-10/C-9; H-11a/C-1, H-11a/C-2, H-11a/C-3, H$_2$-1/C-11a, H$_2$-1/C-2, H$_2$-1/C-3, H$_2$-2/C-1, H$_2$-3/C-1, and H$_2$-3/C-2) spectra of **37** also suggested the presence of anthranilic acid and proline residues. Difference in spectral data of **37** from **36** was the observation of signals due to a conjugated methyl ketone [δ_H 2.19 (3H, s) and 5.29 (1H, s); δ_C 30.0, 198.2, 91.0, and 158.7], which was deduced to be attached to the C-11 position from the HMBC correlations (H$_3$-14/C-13, H$_3$-14/C-12, H-12/C-13, H-12/C-11, H-12/C-11a; H-11a/C-11, H-11a/C-12; NH-10/C-12, and NH-10/C-11a). NOE correlation was observed between H-12 and one of H$_2$-1, implying the 11*Z*-configuration. Thus, structure of compound **37** corresponded to that derived from condensation of acetone with compound **36** at C-11 position.



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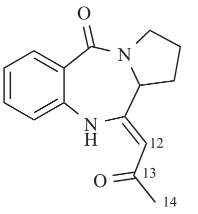

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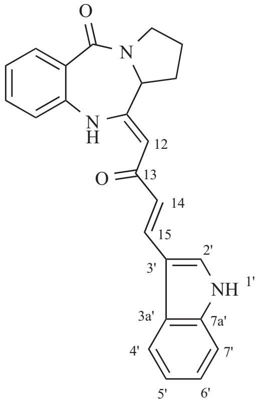

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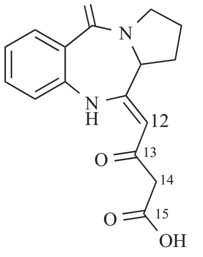

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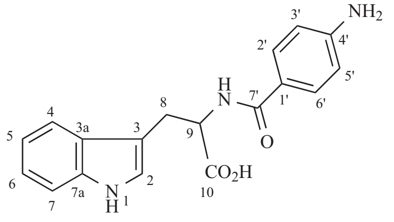

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Compound **38** was a yellow pigment, having an absorption maximum at λ_{max} 415 nm, and compound **38** also contained the cycloanthranilylproline moiety, which was revealed from its ^{1}H and ^{13}C NMR data. In place of the methyl ketone group which was embraced by compound **37**, compound **38** was shown to have an indole moiety as well as cross-conjugated ketone group [δ_C 160.4 (C-11), 93.4 (C-12), 190.2 (C-13), 123.8 (C-14), and 134.9 (C-15); δ_H 5.81 (1H, s; H-12), 7.02 (1H, d, *J*=15.2 Hz; H-14), and 7.92 (1H, d, *J*=15.2 Hz; H-15)] by its ^{1}H and ^{13}C NMR data aided by the HMBC spectrum (indole moiety: H-2'/C-3', H-2'/C-3'a, H-2'/C-7'a, H-5'/C-3'a, H-5'/C-7', H-6'/C-4', H-6'/C-7'a, H-2'/C-3', H-7'/C-5', and H-7'/C-3'a; cross-conjugated ketone group: H-12/C-13, H-14/C-13, and H-15/C-13). The HMBC spectrum also indicated that the cross-conjugated ketone group was attached to C-11 of the cycloanthranilylproline moiety and C-3' of the indole moiety (H-12/C-11, H-12/C-11a, and H-11a/C-11; H-15/C-2', and H-15/C3a'). Therefore, compound **38** was considered to be derived from condensation of a cycloanthranilic acid, an acetone, and an indole-3-carbaldehyde.

Compound **39**, which was positive on the Ehlrich- and Fast Red B-reagent tests on TLC, was isolated from the water-soluble fraction of the extract of this myxomycete. This compound was unstable and proved to be easily converted into compound **37**. The water-soluble fraction of this extract did not initially contain compound **37**. However, after ODS flash chromatography of the water-soluble fraction, compound **37** was obtained substantially. Isolation of compound **39** was carried out carefully by HPLC separation using Develosil C30-UG-5 eluted with 30% MeOH. In DMSO-d_6 solution, compound **39** was almost totally changed into compound **37** during the ^{1}H NMR experiment. Since compound **39** was not dissolved in chloroform or acetone, NMR studies of compound **39** were carried out in CD_3OD solution, in which conversion from **39** to **37** was slow and not significantly observed. The ^{1}H and ^{13}C NMR spectra of compound **39** were almost similar to those of compound **37**, but no signal due to a methyl group was observed for **39**. The EIMS analysis of compound **39** showed prominent ion peak at *m/z* 256, which corresponded to the molecular ion of compound **37**, thus implying that compound **39** was quite susceptible to fragmentation to yield compound **37** during the EIMS measurement. In the ^{13}C NMR spectrum of **39**, a signal of low-field resonance (δ_C 175.8) was observed, which was assignable to a carboxyl group (C-15). The presence of a carboxyl group may be consistent with the fact that compound **39** was polar and contained in the water-soluble fraction. The HMBC spectrum of compound **39** in CD_3OD showed connectivities from δ_H 3.30 to δ_C 92.6 (C-12), 198.1 (C-13), and 175.8 (C-15), suggesting that the signal at δ_H 3.30 was assignable to the hydrogens on C-14 methylene group and compound **39** possessed an acetic acid moiety (CH_2COOH) in place of the methyl group of compound **37**. It was therefore reasonably accounted for that compound **39** possessing a β-keto carboxylic acid moiety may be easily subject to decarboxylation to afford compound **37** possessing a methyl group. This explanation was further corroborated by the LC-MS study. A crude fraction mainly containing compound **39** was subjected to LC-MS analysis (Develosil C30-UG-5, 30% MeOH, 1.8 mL/min; t_R 6.8 min; Waters ZQ 2000), which clearly showed intense quasi-molecular ions for **39** at *m/z* 301 $(M+H)^+$ and 323 $(M+Na)^+$.

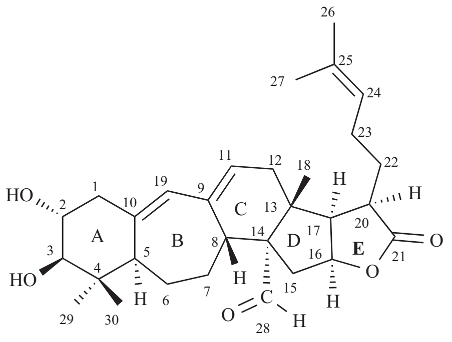
Compound **40** was another constituent isolated from water-soluble fraction. The molecular formula of compound **40** was revealed as $C_{18}H_{17}N_3O_3$ from the HRFABMS data (*m/z* 324.1349, $[M+H]^+$, Δ –2.4 mmu). In the ^{1}H and ^{13}C NMR spectra of **40**, signals due to a tryptophan residue and a *p*-substituted benzoic acid residue were observed. The ^{13}C NMR chemical shift of the C-4' position (δ_C 153.2) implied that an amino group was attached to this position. The ^{1}H-^{1}H COSY and other spectral data also suggested the structure of compound **40** to be 4-aminobenzoyltryptophan, which was first isolated as a natural product and its full characterization was first described here, although it was previously reported as a reaction product [45].

Cycloanthranilylproline derivatives (**37** – **39**) were considered to contain L-proline since compound **36**, which was co-isolated from the same organism, had L-proline residue. The possibility could not be excluded that compound **37** was an artificial product. Although acetone was used for extraction, it was likely that compound **37** was not produced through condensation of acetone with compound **36**, but it was produced through decarboxylation from compound **39**. Treatment of compound **36** with acetone did not afford compound **37**. Compounds **36** and **37** were cytotoxic against murine leukemia P388 cells *in vitro* with IC_{50} values of 2.9 μg/mL and 13.0 μg/mL, respectively, while compounds **39** and **40** were inactive (IC_{50} >25 μg/mL).

3.9. Tubifera dimorphotheca

The fruit bodies of *Tubifera dimorphotheca*, collected at Ohtsu, Kochi-shi in Kochi Prefecture, Japan, in August 2001, were extracted with 90% MeOH and 90% acetone. The combined extracts were subjected to flash chromato-graphy on ODS, followed by preparative HPLC on ODS eluted with 65 - 75% MeOH to give tubiferals A (**41**) and B (**42**) in 0.3 and 0.4% yield of the extract, respectively [46].

Tubiferal A (**41**), a colorless amorphous solid, $[\alpha]_D^{22}$ –87 (c 0.12, MeOH), showed a quasi-molecular ion peak at *m/z* 483 $(M+H)^+$ in its positive ion FAB mass spectrum. The molecular formula of **41** was revealed as $C_{30}H_{42}O_5$ by the HRFABMS data [*m/z* 483.3101, $(M+H)^+$, Δ -0.9 mmu]. The UV spectrum of **41** showed absorption maxima at 239 and 246 nm, indicating the presence of conjugated system(s). The IR absorption bands at 1770 and 1715 cm^{-1} were suggestive of the presence of two carbonyl groups. The ^{1}H NMR spectrum of **41** in $CDCl_3$ showed a characteristic low-field resonance at δ_H 9.65 (1H, s), assignable to an aldehyde group. The ^{1}H NMR also showed signals due to three olefinic protons (δ_H 5.07, 5.78, and 6.10), three oxymethine protons (δ_H 3.13, 3.59, and 4.85), and five singlet methyls (δ_H 0.77, 1.04, 1.07, 1.60, and 1.70). These observations were also corroborated by its ^{13}C NMR spectrum, aided by DEPT experiments, which gave thirty signals assignable to two carbonyls, six olefinic carbons (three methines and three quaternary), three oxymethines, seven sp^3 methylenes, four non-oxygenated sp^3 methines, three sp^3 quaternary carbons, and five methyl groups. Since five out of the ten unsaturation



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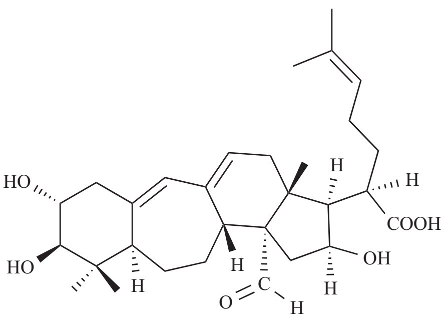

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equivalents were accounted for from the ^{13}C NMR data, **41** was inferred to have five rings. The ^1H-^1H COSY spectrum of **41** showed the connectivities for five proton-networks: H_2-1/H-2/H-3, H-5/H_2-6/H_2-7/H-8, H-11/H_2-12, H_2-15/H-16/H-17/H-20/H_2-22, and H_2-23/H-24. Interpretation of the HMBC spectral data of **41** led to assemblage of these five proton-networks and the remaining hydrogens and quaternary carbons to construct the whole structure of **41** consisting of four consecutive 6/7/6/5 carbocycles with a -lactone and a side chain as described below.

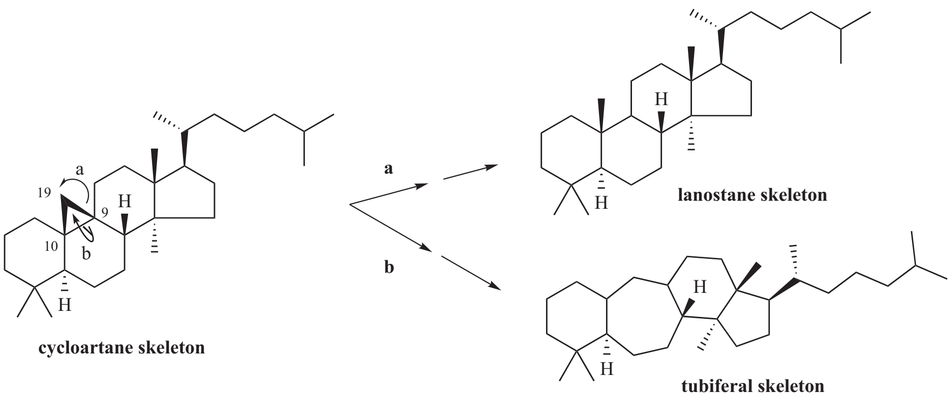
The HMBC correlation data provided evidence for the following connections: (1) Two singlet methyls of H_3-29 (δ_H 1.04) and H_3-30 (δ_H 0.77) were attached to the C-4 quaternary carbon (δ_C 40.8), which were located between the two sp^3 methines C-3 (δ_C 82.5) and C-5 (δ_C 50.1); (2) Two trisubstituted double bonds (δ_C 134.8, 130.2, 137.7, and 129.1; δ_H 6.10 and 5.78) were conjugated and placed at the C-10/C-19/C-9/C-11 positions to construct 6- and 7-membered rings for the A- and B-rings, respectively; (3) A tertiary methyl (δ_H 1.07 and δ_C 18.9; C-18) and the aldehyde group (δ_H 9.65 and δ_C 208.1; C-28) were attached at the C-13 and C-14 positions, respectively, to give rise to 6- and 5-membered rings for the C- and D-rings, respectively; (4) An ester (or lactone) carbonyl group (δ_C 178.4; C-21) was connected to the C-20 methine carbon, and the low-field resonance of the oxymethine proton on C-16 (δ_H 4.85) implied that the C-16 oxymethine was connected with the C-21 carbonyl group to form a -lactone ring, which was consistent with the fact that **41** was inferred to have five rings (*vide supra*); (5) A side chain consisting of a 4-methyl-3-pentenyl group (C-22 ~ C-27) was also attached to the C-20 methine carbon.

The ^1H NMR chemical shifts of H-2 (δ_H 3.59) and H-3 (δ_H 3.13) implied that two hydroxyl groups were located vicinally at the C-2 and C-3 positions, and the coupling constant ($J_{2,3}$=9.2 Hz) was typical for *trans*-diaxial protons, thus indicating that both hydroxyl groups were equatorial. The stereochemical assignment of the angular hydrogens (H-5, H-8, H-16, H-17, and H-20), the angular methyl group (C-18), and the aldehyde group (C-28) were made on the basis of a NOESY spectrum. The key correlations were: H-1α/H-3, H-1α/H-5, H-1β/H-2, H-2/H_3-30, H-3/H-5, H-3/H_3-29, H-6β/H_3-30, H-6β/H-8, H-7α/H-28, H-8/H-15β, H-8/H_3-18, H-12β/H_3-18, H-12α/H-28, H-15α/H-16, H-16/H-17, H-17/H-20, and H_2-22/H_3-18. These ring-juncture configurations of **41** were consistent with those of the cycloartane-triterpene nucleus, although **41** possessed no cyclopropane moiety.

The molecular formula of tubiferal B (**42**) was revealed as $C_{30}H_{44}O_6$ on the basis of HRFABMS data [*m/z* 501.3179, $(M+H)^+$, Δ –3.7 mmu], having one H_2O molecule more than tubiferal A (**41**). TLC examination implied that compound **42** was more polar than **41** (R_f values on ODS TLC developed with 90% MeOH; **41**: 0.39 and **42**: 0.64). The ^1H and ^{13}C NMR spectral data of **42**, as well as its UV absorption, paralleled those of compound **41**, although the oxymethine proton on C-16 and the C-17 methine proton resonated at relatively higher field for **42** (H-16, δ_H 4.28; H-17, δ_H 1.50). Thus, compound **42** was suggested to be the seco acid derivative of the -lactone moiety of compound **41**, which was supported by the detailed interpretation of ^1H-^1H COSY and HMBC spectra of **42**. Treatment of **41** with dilute KOH afforded **42**, which was detected on the basis of TLC examination.

The basic skeleton of tubiferal A (**41**) may be classified as a 9,10-secocycloartan-16,21-olide skeleton, which was conceived to be biogenetically produced through (i) ring opening of the cyclopropane of a cycloartane triterpene by fission of the 9,10-bond to give a 7-membered B-ring (Scheme **1**), and (ii) oxidation of the C-21 methyl group to a carboxyl group. Reopening of the cyclopropane ring of cycloartane triterpene occurs by fission of the 9,19-bond in the biosynthesis of plant sterols to give the methyl group at C-10, whereas cyclopropane ring-opening in a different direction may take place in the case of this myxomycete metabolite **41**.

Acerinol (**43**) [47] and its related compounds are known to possess a related 3,10-epoxy-9,10-secocycloartane skeleton, which were first obtained from the hydrolysis products of the glycoside fraction of *Cimicifuga acerina.* Acerinol (**43**) was therefore artificially generated from a cycloartane-triterpene, such as cimigenol (**44**), by treatment with mineral acid. Although an acerinol-related compound was once reported to have been isolated from *Cimicifuga heracleifolia* without acid treatment [48], in case of acerinol-



Scheme 1. Hivable backbone rearrangements from the cycloartane skeleton.

related compounds, the fission of cyclopropane ring is likely to be assisted by the formation of the 3,10-epoxy ring.

The cytotoxic activity of tubiferals A (**41**) and B (**42**) was examined against three human tumor cell lines, and IC_{50} value (µg/mL) data are presented in Table **5**. Tubiferal A (**41**) showed weak or no cytotoxicity against these cell lines at concentrations used in this experiment. Interestingly, in the presence of 100 ng/mL of VCR in the culture of VCR-resistant KB cells [49], compound **41** possessed cytotoxicity (IC_{50} value: 2.7 µg/mL). This finding suggests that compound **41** overcame multidrug resistance of tumor cells. On the other hand, the seco acid (**42**) was inactive against all tested cell lines. Both compounds **41** and **42** did not enhance TRAIL (TNF-related apoptosis inducing ligand) induced apoptosis in TRAIL resistant KOB (adult T cell leukemia) cells.

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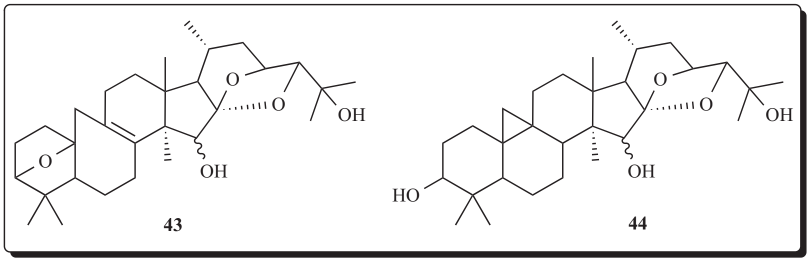

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Table 5. Cytotoxicity of tubiferals A (41) and B (42) (IC_{50} values, µg/mL)

–	KB/VJ-300		LNCaP	KOB	
	VCR(+)	VCR(-)		TRAIL(+)	TRAIL(-)
41	2.7	>12.5	13.2	>6.3	>6.3
42	>12.5	>12.5	>12.5	>6.3	>6.3
Verapamil[a]	1.1	>25	-	-	-
Curcumin[b]	-	-	-	20	>25
Cisplatin[c]	-	-	1.5	-	-

Tests toward each cell line were carried out in the absence (-) and presence (+) of 100 ng/mL of VCR and 500 ng/mL of TRAIL, respectively, which did not affect the growth of the cells.

[a, b, c]: positive controls

Chemotherapy Center, Japanese Foundation for Cancer Research, for human cancer cell line panel assay, and Dr. Yoshimasa Uehara, Department of Bioactive Molecules, National Institute of Infectious Diseases, for protein kinase inhibition activity tests. This work was partly supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by a Grant-in-Aid from the Fujisawa Foundation, and by a Grant-in-Aid from the Keimeikai Foundation.

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