Constituents of the Endophytic Fungus *Annulohypoxylon boveri* var. *microspora* BCRC 34012

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A new azaphilone metabolite with a new substitution pattern, named annulohypoxylin (**1**), together with twelve known compounds, were isolated from the BuOH-soluble fraction of the 95% EtOH extract of long-grain rice (*Oryza sativa*) fermented with the endophytic fungus *Annulohypoxylon boveri* var. *microspora* (BCRC 34012). Annulohypoxylin (**1**) contains a dihydrobenzofuran-2,4-dione backbone, 1-hydroxyoctyl side chain, and one γ-lactone ring. Its structure was determined on the basis of extensive 1D- and 2D-NMR analyses in combination with HR-ESI-MS. The relative configuration of **1** was confirmed by NOESY experiment. Other known compounds were identified by comparing their spectral data with those in the literature. All known compounds were isolated from this species for the first time.

Introduction. – Endophytes, commonly present in almost all plants, are important sources of natural products with pharmaceutical potential [1][2]. Endophytic fungi, living in the intracellular spaces of the tissues of host plants without causing any apparent disease, are a rich source of functional biomolecules [1][2]. Recently, endophytes have been recognized as a fruitful source of structurally novel and biologically active secondary metabolites [3–7]. In our ongoing research on metabolites from plant endophytes, we investigated the secondary metabolites produced by the endophytic fungus *Annulohypoxylon* sp. BCRC 34012, a strain isolated from the bark of medicinal plant *Cinnamomum* sp. This strain was identified as *Annulohypoxylon boveri* var. *microspora* (family Xylariaceae) based on the cultural and anamorphic data of *Hsieh* and *Ju* [8]. They had placed the taxa assigned to *Hypoxylon* sect. *Annulata* in a new genus, for which the name *Annulohypoxylon* was coined based on the morphological characteristics and the analyses of β-tubulin and α-actin gene sequences [8]. Xylariaceae is a large family (Xylariales, Ascomycotina) of more than 36 genera. Secondary metabolites produced by representatives from at least one third of these genera have now been isolated and identified [9]. A variety of structurally diversified compounds are distributed in the *Xylaria* sp. [10–18]. Contrary to *Xylaria* sp., the secondary metabolites of the genus *Annulohypoxylon* have received

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less attention, and only a few articles had been reported [19–21]. To further understand the chemotaxonomy of the genus *Annulohypoxylon* and to continue searching for novel bioactive metabolites from Xylariaceae, *A. boveri* var. *microspora* was selected for a phytochemical investigation. Careful examination of the above mentioned fungus resulted in the isolation of a compound with a new dihydrobenzofuran-2,4-dione skeleton combined with a γ-lactone ring, named annulohypoxylin (**1**; *Fig. 1*), together with twelve known compounds, which were found in this species for the first time. The isolation and structure elucidation of the new compound **1** are described herein.

Results and Discussion. – 1. *Structure Elucidation*. The BuOH-soluble fraction of the 95% EtOH extract was fractionated by a combination of silica gel, *RP-18* columns, and prep. TLC to yield 13 compounds, the structures of which were elucidated by 1D- and 2D-NMR spectra, and comparison with literature data.

Compound **1** was obtained as an optically inactive yellowish oil. $[\alpha]_D^{25} = \pm 0$ (c = 0.04, $CHCl_3$). The molecular formula was determined as $C_{19}H_{26}O_6$ on the basis of the $[M+Na]^+$ peak at m/z 373.1627 (calc. 3732.1629 for $C_{19}H_{26}NaO_6$) in the HR-ESI-MS. The UV absorptions (λ_{max} 235 and 352 nm) confirmed that **1** structurally related to a conjugated ketone [22]. The bands at 3400, 1710, and 1652 cm^{-1} in the IR spectrum revealed the presence of a OH, a conjugated ketone, and an ester group, respectively. Seven indices of hydrogen deficiency (IHD) were determined from the molecular formula, and ^{13}C-NMR (*Table*), and DEPT spectra. Based on further spectral

Table. *^{13}C- and ^{1}H-NMR Data* ($CDCl_3$; 150 and 600 MHz, resp.) *of Compound* **1**. δ in ppm, J in Hz. Arbitrary numbering of the side chain.

Position	**1**	
	δ(C)	δ(H)
C(2)	173.8 (*s*)	–
H–C(3)	48.6 (*d*)	2.84 (*dd*, J = 13.2, 3.0)
C(3a)	42.8 (*s*)	3.20 (*ddd*, J = 13.2, 11.4, 4.2)
CH_2(4)	24.9 (*t*)	2.63 (*dddd*, J = 18.6, 11.4, 3.6, 3.6, H_{ax}), 3.07 (*dddd*, J = 18.6, 4.2, 3.0, 1.2, H_{eq})
C(4a)	151.2 (*s*)	–
CH_2(5)	68.3 (*t*)	4.95 (*ddd*, J = 18.0, 3.6, 1.2), 5.11 (*ddd*, J = 18.0, 3.0, 3.0)
C(7)	170.7 (*s*)	–
C(7a)	143.5 (*s*)	–
C(8)	189.1 (*s*)	–
C(8a)	83.6 (*s*)	–
Me(9)	16.8 (*q*)	1.50 (*s*)
H–C(10)	69.2 (*d*)	4.27 (*dt*, J = 9.0, 3.0)
CH_2(11)	35.1 (*t*)	1.57 (*m*)
CH_2(12)	25.9 (*t*)	1.32–1.35 (*m*)
CH_2(13)	29.4 (*t*)	1.32–1.35 (*m*)
CH_2(14)	29.7 (*t*)	1.32–1.35 (*m*)
CH_2(15)	31.4 (*t*)	1.32–1.35 (*m*)
CH_2(16)	22.5 (*t*)	1.32–1.35 (*m*)
Me(17)	14.0 (*q*)	0.88 (*t*, J = 7.0)

evidences, the structure of **1** was elucidated as *rel*-(1*R*,3a*S*,8a*S*)-3a,8a-dihydro-3-(1-hydroxyoctyl)-3a-methyl-3*H*,4*H*,5*H*-benzo[1,2-*b*;4,5-*c*′]difuran-2,4,5-trione, named annulohypoxylin, which was further confirmed by ^{13}C-NMR, COSY (*Fig. 1*), NOESY (*Fig. 2*), HSQC, and HMBC (*Fig. 3*) experiments.

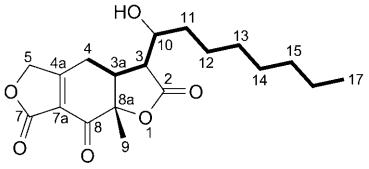

Fig. 1. *Important COSY* (—) *correlations of* **1**

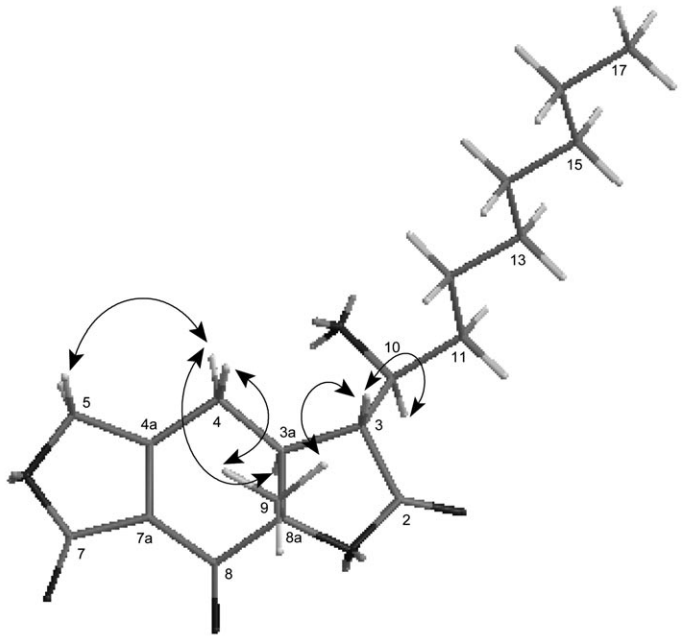

Fig. 2. *Major NOESY* (H ↔ H) *correlations and most stable conformation for* **1** *as predicted by molecular mechanics* (MM2) *calculations*

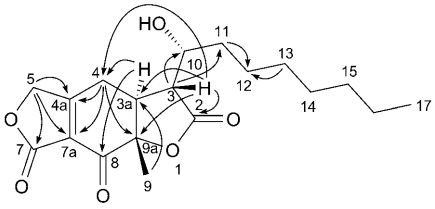

Fig. 3. *Key HMBC* (H → C) *correlations of* **1**

Four of the seven degrees of unsaturation inherent in the formula were accounted by ^{13}C-NMR as one conjugated CO group, two ester CO groups (γ-lactone rings), and two olefinic C-atoms. Accordingly, the compound **1** contained a dihydrobenzofuran-2,4-dione skeleton combined with a γ-lactone ring. The ^{1}H- and ^{13}C-NMR spectra (*Table*) indicated the presence of six quaternary C-atoms, and three CH, eight CH_2, and two Me groups. In the ^{1}H- and ^{13}C-NMR data, there were typical signals for one aliphatic terminal Me group (δ(H) 0.88 (*t*, *J* = 7.0, Me(17)); δ(C) 14.0 (C(17))), one Me group attached to a quaternary C-atom (δ(H) 1.50 (*s*, Me(9)); δ(C) 16.8 (C(9))), one CH_2 moiety (δ(H) 2.63 (*dddd*, *J* = 18.6, 11.4, 3.6, 3.6, H_{ax}–C(4)), 3.07 (*dddd*, *J* = 18.6, 4.2, 3.0, 1.2, H_{eq}–C(4)); δ(C) 24.9 (C(4))), one O-bearing CH_2 moiety (δ(H) 4.95 (*ddd*, *J* = 18.0, 3.6, 1.2, H–C(4a)), 5.11 (*ddd*, *J* = 18.0, 3.0, 3.0, H–C(4b)); δ(C) 68.3 (C(5))), and two mutually coupling CH groups (δ(H) 2.84 (*dd*, *J* = 13.2, 3.0, H–C(3)); δ(C) 48.6 (C(3)), 3.20 (*ddd*, *J* = 13.2, 11.4, 4.2, H–C(3a)); δ(C) 42.8 (C(3a))). In addition, sequential cross-peaks for one 1-hydroxyoctyl side chain (δ(H) 0.88 (*t*, *J* = 7.0, Me(17)), 1.32 – 1.35 (*m*, H–C(12 – 16)), 1.57 (*m*, H–C(11)), and 4.27 (*dt*, *J* = 9.0, 3.0, H–C(10)); δ(C) 22.5 (C(16)), 25.9 (C(12)), 29.4 (C(13)), 29.7 (C(14)), 31.4 (C(15)), 35.1 (C(11)), 69.2 (C(10))) were observed in the COSY spectrum (*Fig. 1*). In the ^{13}C-NMR spectrum, besides the signals corresponding to the above-mentioned H-atoms, there are still signals of six tertiary C-atoms corresponding to three C=O functions (δ(C) 189.1 (C(8)), 170.7 (C(7)), 173.8 (C(2))), two C=C bonds (δ(C) 143.5 (C(7a)), 151.2 (C(4a))), and one O-bearing quaternary C-atom (δ(C) 83.6 (C(8a))) constructing the remaining part of structure **1**. By analyzing the above data and comparing them with those in the literature reported for related systems [22][23], the structure of **1** was deduced as a dihydrobenzofuran-2,4-dione with a γ-lactone moiety.

HMBC Data (*Fig. 3*) allowed establishing the full connectivity within the molecule. HMBCs between the H-atom signal at δ(H) 4.95 (CH_2(5)) and the C-atom signals at δ(C) 24.9 (C(4)) and 151.2 (C(4a)) revealed that CH_2(4) is connected to C(4a) of the γ-lactone ring, and the correlations from δ(H) 3.20 (H–C(3a)) to δ(C) 189.1 (C(8)), and from δ(H) 2.63, 3.07 (CH_2(4)) to δ(C) 143.5 (C(7a)) indicate that the C(8)=O is attached to C(7a) in the same ring. This could be corroborated by a weak HMBC ^{4}J correlation between the CH_2(5) H-atoms with signals at δ(H) 4.95 and 5.11, and C(8). On the basis of the HMBC cross-peak between the *singlet* Me group at δ(H) 1.50 (Me(9)) and the C(8) signal, we can conclude that these H-atoms are located in three-bonds (^{3}J) distant from C(8), and HMBC correlation Me(9)/C(8a) (δ(C) 83.6) revealed that the Me(9) group was at an O-bearing quaternary C-atom, *i.e.*, C(8a). The Me(9) signals correlate with one CH signal (δ(C) 42.8), and, in this way, the signal at δ(C) 42.8 can be assigned to C(3a). The signal at δ(H) 2.84 (H–C(1)) correlates with the signals of C(4), C(3a), and with of the lactone CO group, C(2). This allows the determination of the position of another γ-lactone ring. That a $C_7H_{15}CH(OH)$ group was located at C(3) was confirmed by the HMBCs from H–C(1) (δ(H) 2.84) to C(10) (δ(C) 69.2) and C(11) (δ(C) 35.1), and from H–C(3a) (δ(H) 3.20) to C(10) (δ(C) 69.2).

The relative configuration of **1** was deduced from a NOESY spectrum (*Fig. 2*) in combination with those of similar compounds [22][23]. According to the NOESY spectrum, the H–C(1) was β-oriented, which was confirmed by the NOE Me–C(9)/H–C(3). NOEs Me–C(9)/H_{ax}–C(4), H–C(1)/H_{ax}–C(4), and H–C(3)/H–C(10) indi-

cated that H–C(3), H_{ax}–C(4), Me–C(9), and H–C(10) were on the same side of the molecular plane, tentatively assumed β, and thus HO–C(10) was in α-orientation. In addition, the coupling-constant values (13.2 and 11.4 Hz) of ^3J(H–C(3), H–C(3a)) and ^3J(H–C(3a)/H_{ax}–C(4)) established the *trans*-antiperiplanar positions of these H-atoms. Due to its optical inactivity ($[\alpha]_D^{22} = \pm 0$ ($c = 0.04$, $CHCl_3$)), **1** was considered to be racemic. The absolute configuration at C(10) of **1** could not be further determined due to only minute amounts of material isolated and its rapid decomposition.

To further confirm the relative configuration of **1**, a computer-assisted 3D structure was obtained by using the molecular-modeling program CS CHEM 3D Ultra 10.0, with MM2 force-field calculations for energy minimization (*Fig. 2*). The calculated distances between H–C(3) and Me–C(9) (2.250 Å), H–C(3) and H_{ax}–C(4) (2.635 Å), and Me–C(9) and H_{ax}–C(4) (2.416 Å) were all less than 4 Å. This is consistent with the above mentioned NOESY interactions between each of these H-atom pairs.

From the above data, compound **1** was unambiguously characterized as *rel*-(3*R*,3a*S*,8a*S*)-3a,8a-dihydro-3-[(1*R*)-1-hydroxyoctyl]-8a-methylbenzo[1,2-*b*:4,5-*c*′]difuran-2,7,8(3*H*,4*H*,5*H*)-trione, named annulohypoxylin, and its structure was further confirmed by COSY (*Fig. 1*), NOESY (*Fig. 2*), HSQC, and HMBC (*Fig. 3*) experiments.

The other known compounds isolated, *i.e.*, (3*R*,6*R*,7*E*)-3-hydroxymegstigma-4,7-dien-9-one [24], α-tocopherylquinone [25], isofraxidin [26], 5-formylmellein [27], mellein-5-carboxylic acid [28], 5-hydroxymethylmellein [29], 3β-hydroxystigmast-5-en-7-one [30], *N-trans*-feruloyltyramine [31], *N-cis*-feruloyltyramine [32], vanillic acid [33], methyl paraben [33], and syringaldehyde [33] were identified by comparison of their spectral data (UV, IR, ^{1}H-NMR, MS) with those in the corresponding literature.

Conclusions. – In this study, we focused on the minor secondary metabolites in the BuOH-soluble fraction of the 95% EtOH extract of long-grain rice (*Oryza sativa*) fermented with the endophytic fungus *Annulohypoxylon boveri* var. *microspora* (BCRC 34012). The new metabolite **1**, described in this study, is the first, naturally occurring compound. It is worthy to mention that this is the first report of a azaphilone derivative, which contains a dihydrobenzofuran-2,4-dione backbone combined with a γ-lactone ring, isolated from *Annulohypoxylon* species. Further, twelve known compounds were isolated for the first time from *Annulohypoxylon* species. However, the chemical characteristics, as well as the biological activities, of other *Annulohypoxylon* species' constituents still remain unclear. Thus, diverse biological activities of *Annulohypoxylon* spp. and related diverse secondary metabolites deserve further investigations.

This investigation was supported by a grant from the *Ministry of Economic Affairs of Republic of China* (Grant No. 94-EC-17-A-17-R7-0563). The authors also thank Senior Technician Mrs. *Chyi-Jia Wang* of *Center for Resources, Research and Development* (CRRD) of Kaohsiung Medical University (KMU) for recording the 2D-NMR data.

Experimental Part

General. TLC: silica gel *60* F_{254} precoated plates (*Merck*). Column chromatography (CC): silica gel *60* (70–230 or 230–400 mesh; *Merck*) or *Spherical C18* (20–40 μm; *Silicycle*). HPLC: *Spherical C18* column (250 × 10 mm, 5 μm) (*Waters*); *LDC-Analytical-III* apparatus; UV/VIS detector (*SPD-10A*, *Shimadzu*); MeCN/H_2O 10:1 as mobile phase, flow rate 1.0 ml/min. M.p.: *Yanaco* micro-melting point apparatus; uncorrected. Optical rotation: *Jasco DIP-370* polarimeter; in $CHCl_3$. UV Spectra: *Jasco UV-240* spectrophotometer; λ_{max} (log ε) in nm. IR Spectra: *Perkin-Elmer-2000* FT-IR spectrophotometer; $\tilde{\nu}$ in cm^{-1}. ^1H-, ^{13}C-, and 2D-NMR spectra: *Varian-Mercury*-400, *Varian-Unity-Plus*-400, and *Varian 600 VNMRS*-600 spectrometers; δ in ppm rel. to Me_4Si, *J* in Hz. GC/MS: *Trace GC/POLARIS Q Thermo Finnigan*; in *m*/*z* (rel. %). EI-MS: *VG-Biotech Quatro-5022* mass spectrometer; in *m*/*z* (rel. %). ESI- and HR-ESI-MS: *Bruker APEX-II* mass spectrometer; in *m*/*z*.

Microorganism. The fungal strain *Annulohypoxylon boveri* var. *microspora* was isolated from the bark of medicinal plant *Cinnamomum* sp., which was collected from Fu-Shan Botanical Garden, I-lan County, Taiwan, during August of 2001. *A. boveri* var. *microspore* was used throughout this study, and specimens (BCRC 34012) deposited with the Bioresource Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (FIRDI).

Cultivation and Preparation of the Fungal Strain. A. boveri var. *microspora* BCRC 34012 was maintained on potato dextrose agar (PDA), and the strain was cultured on potato dextrose agar slants at 25° for 7 d, and then the spores were harvested by sterile H_2O. The spores (5×10^5) were seeded into 300-ml shake flasks containing 50 ml RGY medium (3% rice starch, 7% glycerol, 1.1% polypeptone, 3% soybean powder, 0.1% $MgSO_4$, 0.2% $NaNO_3$), and cultivated with shaking (150 rpm) at 25° for 3 d. After the mycelium enrichment step, an inoculum mixing 100 ml of mycelium broth and 100 ml of RGY medium was inoculated into plastic boxes (25 cm × 30 cm) containing 1 kg of sterile rice and cultivated at 25° for producing rice, and the above mentioned RGY medium was added for maintaining the growth. After 21 d of cultivation, the rice was harvested and used as a sample for further extraction.

Extraction and Isolation. The rice of the *A. boveri* var. *microspora* BCRC 34012 (2 kg) were extracted with cold 95% EtOH (3 × 10 l, 3 d each) at r.t. The EtOH extract was concentrated under reduced pressure, and was partitioned with BuOH/H_2O 1:1 (*v*/*v*) to afford BuOH-soluble fraction (2.8 g), H_2O-soluble fraction (3.0 g), and insoluble fraction (500 mg).

The BuOH fraction (2.8 g) was subjected to CC (1.5 kg of SiO_2, 230–400 mesh; CH_2Cl_2/MeOH gradient) to give eight fractions: *Frs. 1–8*. *Fr. 1* (170 mg) was applied to CC (10 g of SiO_2, 230–400 mesh; CH_2Cl_2/AcOEt gradient) to obtain four fractions: *Frs. 1.1–1.4*. *Fr. 1.2* (55 mg) was to CC (*RP-C18* (10 g); acetone/H_2O 10:1) to give five fractions: *Frs. 1.2.1–1.2.5*. *Fr. 1.2.2* (9.0 mg) was purified by prep. TLC (SiO_2; CH_2Cl_2/AcOEt 10:1): (3*R*,6*R*,7*E*)-3-hydroxymegastigma-4,7-dien-9-one (5.8 mg). *Fr. 1.2.4* (6.8 mg) was purified by prep. TLC (SiO_2; CH_2Cl_2/AcOEt 15:1): α-tocopheryl quinone (5.6 mg). *Fr. 3* (856 mg) was subjected to CC (30 g of SiO_2, 70–230 mesh; CH_2Cl_2/AcOEt 70:1): *Frs. 3.1–3.10*. *Fr. 3.5* (20 mg) was purified by prep. TLC (*RP-18*; MeOH/H_2O 5:1): *annulohypoxylin* (**1**; 5.5 mg). *Fr. 3.7* (80.9 mg) was subjected to CC (4 g of SiO_2, 70–230 mesh; hexane/AcOEt gradient): *Frs. 3.7.1–3.7.10*. *Fr. 3.7.5* (32.4 mg) was purified by prep. TLC (SiO_2; CH_2Cl_2/AcOEt 30:1): *isofraxidin* (5.2 mg) and *5-formylmellein* (7.1 mg). *Fr. 3.8* (85 mg) was submitted to CC (4 g of SiO_2, 70–230 mesh; hexane/Me_2CO gradient): *Frs. 3.8.1–3.8.5*. *Fr. 3.8.5* (48 mg) was submitted to CC (*RP-18*; Me_2CO/H_2O 2:1): *Frs. 3.8.5.1–3.8.5.7*. *Fr. 3.8.5.7* (15 mg) was purified by prep. TLC (SiO_2; CH_2Cl_2/AcOEt 65:1): *3β-hydroxystigmast-5-en-7-one* (1.6 mg), and *syringaldehyde* (1.8 mg). *Fr. 7* (1.2 g) was subjected to CC (50 g of SiO_2, 70–230 mesh; CH_2Cl_2/Me_2CO gradient): *Frs. 7.1–7.10*. *Fr. 7.2* (40 mg) was subjected to CC (*RP-18*; Me_2CO/H_2O 1:1): *Frs. 7.2.1–7.2.5*. *Mellein-5-carboxylic acid* (1.4 mg) was obtained from *Fr. 7.2.1*. *Fr. 7.2.4* (26.8 mg) was further purified by prep. TLC (SiO_2; hexane/Me_2CO 3:2): *5-hydroxymethylmellein* (1.5 mg). *Fr. 7.2.5* (11 mg) was further purified by prep. TLC (SiO_2; CH_2Cl_2/Me_2CO 1:1): N-trans-*feruloyltyramine* (2.3 mg). *Fr. 8* (520 mg) was subjected to CC (31 mg of SiO_2, 70–230 mesh; CH_2Cl_2/MeOH 10:1): *Frs. 8.1–8.6*. *Fr. 8.2* (15 mg) was purified by prep. TLC (*RP-18*; MeOH/H_2O 5:1): N-cis-*feruloyltyramine* (1.5 mg). *Fr. 8.3* (50.9 mg) was subjected to CC (600 mg of SiO_2, 70–230 mesh; CH_2Cl_2/MeOH gradient): *Frs. 8.3.1–8.3.4*. *Fr. 8.3.2* (10.4 mg) was purified by prep. TLC (SiO_2; CH_2Cl_2/AcOEt

30:1): *methylparaben* (2.5 mg). *Fr. 8.3.3* (10.8 mg) was subjected CC (*RP-C18* 5.0 g); acetone/H_2O 20:1) to afford *vanillic acid* (1.7 mg).

Annulohypoxylin (= rel-*(3*R*,3a*S*,8a*S*)-3a,8a-Dihydro-3-[(1*R*)-1-hydroxyoctyl]-8a-methylbenzo[1,2-b:4,5-c′]difuran-2,7,8(3*H*,4*H*,5*H*)-trione*; **1**). Yellowish oil. $[\alpha]_D^{25} = \pm 0$ ($c = 0.04$, $CHCl_3$). UV (MeOH): 235 (3.85), 352 (4.14). IR (neat): 3400 (OH), 1710 (conj. ketone C=O), 1652 (ester C=O). ^{1}H- and ^{13}C-NMR: see the *Table*. ESI-MS: 373 ($[M+Na]^+$). HR-ESI-MS: 373.1627 ($[M+Na]^+$, $C_{19}H_{26}NaO_6^+$; calc. 373.1629).

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Received September 28, 2010