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Four analogues of spiroleptosphol isolated from *Leptosphaeria doliolum*

Takanori Murakami [a], Taro Tsushima [a], Noboru Takada [a], Kazuaki Tanaka [a], Ken-ichi Nihei [b], Tomisato Miura [c], Masaru Hashimoto [a,*]

[a] Faculty of Agriculture and Life Science, Hirosaki University, 3 Bunkyo-Cho, Hirosaki, Aomori 036-8561, Japan
[b] Faculty of Agriculture, Utsunomiya University, 350 Mine-Machi, Utsunomiya, Tochigi 321-8505, Japan
[c] Graduate School of Health Sciences, Hirosaki University, 66-1 Honcho, Hirosaki, Aomori 036-8564, Japan

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ABSTRACT

Spiroleptosphol B (**2**), spiroleptosphol C (**3**), norleptosphol C (**4**) and hydroleptosphol (**5**) were isolated from ascomycete *Leptosphaeria doliolum*. Detailed ^{1}H and ^{13}C NMR spectral analyses revealed these were structural analogues of spiroleptosphol (**1**) which we have recently isolated from the same fungi. Spiroleptosphol B (**2**) carried an unprecedent 5,3-dioxatricyclo[4.4.0.1^{1,4}]undecane framework in place of the spirobicyclo ring system of **1**. Spiroleptosphol C (**3**) was a 17-(*R*)-hydroxy derivative of **1**. Norleptosphol C (**4**) was deduced to be the monocyclic structure biosynthetically resulted by decarboxylation from **3**. Although **5** gave broaden ^{1}H NMR spectrum, it was gradually transformed to **2** which suggested being a hydrolysate of **1**.

1. Introduction

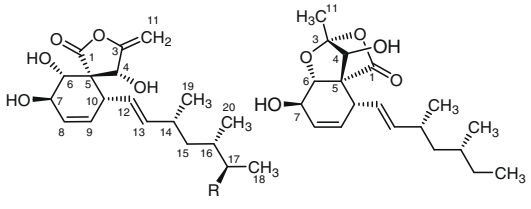
In the course of studies exploring novel metabolites from fungi with unique ecologies, we recently reported spiroleptosphol (**1**)[1] as a cytotoxic compound from *Leptosphaeria doliolum*.[2] Our structural studies revealed 3-methylidene-2-oxaspiro[4.5]decan-1-one framework[3–10] with a (*E*)-3,5-dimethyl-1-heptenyl side chain for **1**. Further investigation led us to isolate spiroleptosphol B (**2**), spiroleptosphol C (**3**), norleptosphol C (**4**) and hydroleptosphol (**5**) as novel metabolites. Structural analyses revealed that these were biosynthetically related compound to **1**. Here, we report isolations and structural elucidations of them.

2. Results and discussion

Ascomycete *L. doliolum* collected from mugwort stems[2] was cultured in potato-sucrose medium at 25 °C for 2 weeks on a rotary shaker (100 rpm). The crude ethyl acetate extracts were subjected to extensive column chromatography to obtain **2** (92 mg), **3** (65 mg), **5** (18 mg), and **4** (16 mg) along with known **1** (230 mg). These were analyzed mainly employing NMR spectroscopy. Summary of the ^{1}H and ^{13}C NMR spectral data are shown in Table 1.

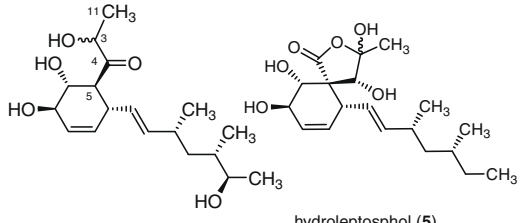
2.1. Spiroleptosphol B (2)

Spiroleptosphol B (**2**) showed the protonated molecular ion signal at *m/z* 337.2013 $[M+H]^{+}$ in the ESIMS, suggesting the same formula of **1**, $C_{19}H_{28}O_5$. The ^{1}H NMR and COSY spectra revealed that **2** possessed a C5–C10 cyclohexene ring with the same C12–C18 side chain (*spiroleptosphol numbering*) as those of **1**. The two methyl



spiroleptosphol (**1**) : R = H
spiroleptosphol C (**3**): R =OH

spiroleptosphol B (**2**) :



norleptosphol C (**4**)

hydroleptosphol (**5**)
(predominant acetal form)

* Corresponding author. Tel./fax: +81 172 39 3782.
E-mail address: hmasaru@cc.hirosaki-u.ac.jp (M. Hashimoto).

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groups in the side chains were assumed to be (14*R*,16*R*)-stereochemistry because of the same producer with **1**. In the ^{1}H NMR spectrum, the C11 *exo*-methylene signals observed in **1** was disappeared and a methyl signal (3H, singlet) was newly detected at 1.60 ppm. This is consistent with the observation of its methyl carbon at 14.8 ppm in place of the C11 *exo*-methylene carbon (expected to be appeared around 90 ppm) in the ^{13}C NMR spectrum. This methyl proton signal exhibited a strong HMBC correlation with the C3 quaternary carbon at 111.5 ppm, which was assigned to an acetal carbon based on its chemical shift. Further analyses of the HMBC correlations, as shown in Figure 1, disclosed a unique 3,5-dioxatricyclo[4.4.0.1^{1,4}]undecane framework. The coupling constant J_{C6HC7H} (8.4 Hz) established *trans* relationship for C6C7 vicinal diol moiety. Irradiation of the signal due to C6H induced NOEs at C4H and C10H which established stereochemical relationship for the tricyclic moiety. Only the isomer shown in Figure 1 satisfied these NOEs.

2.2. Spiroleptosphol C (3)

Spiroleptosphol C (**3**) was found to carry one more oxygen as compared to **1** on the basis of the protonated molecular ion at 353.1959 [M+H]$^+$ (suggesting $C_{19}H_{28}O_6$ as the molecular formula) in the ESIMS. Comparison of the ^{1}H NMR spectra revealed that **3** was composed of the same spirobicyclic unit as **1**, but slightly different side chain. The large coupling constant (15.7 Hz) for the C11–C12 double bond (*spiroleptosphol numbering*) in the ^{1}H NMR spectrum confirmed the (*E*)-configuration of the side chain. The ^{1}H resonance for the C18H$_3$ of **3** was observed as a doublet (J = 6.3 Hz) at 1.06 ppm, while the corresponding signal in **1** was triplet (J = 7.2 Hz) at 0.85 ppm. The COSY spectrum of **3** indicated a coupling between the C18 H$_3$ doublet and a methyne proton appeared at 3.53 ppm. These suggest that the extra oxygen is attached to C17 in a form of alcohol also by taking account of index number of hydrogen deficiency. Fortunately, **3** gave nice single crystalline needles. The stereochemistry of the C17 position was established to be *R* configuration by X-ray crystallographic analysis as shown in Figure 2.[11] This also confirmed the structure of **1** that we have proposed in the preceding studies.

2.3. Norleptosphol C (4)

The ESIMS of norleptosphol C (**4**) (*m/z* 327.2164, [M+H]$^+$) suggested that the molecular formula was estimated to be $C_{18}H_{30}O_5$ which was one carbon less than **1**–**3**. The ^{1}H NMR spectrum of **4** was similar to those of other compounds. The COSY spectrum indicated the same C12–18 side chain as that of **3**. The C6H (*spiroleptosphol numbering*) was observed as doubled-doublet (J = 8.1 and 10.6 Hz) at 3.64 ppm in the ^{1}H NMR spectrum, while the corresponding signal appeared as doublet in the cases of **1**–**3**. A proton connected to C5 was newly detected at 3.03 ppm as a triplet (J = 10.6 Hz) and the COSY revealed the coupling C5H↔C6H, and C5H↔C10H. The large coupling constants suggested that all of C5H, C6H and C10H were arranged as quasi-axial configurations as shown in Figure 3. The HMBC indicated that a $CH_3CH(OH)CO$– group, corresponding to the C11C3C4 carbon unit of **1**, was attached to C5. These disclosed the cyclohexene ring moiety of **4** as shown. The stereochemistry of C3-alchohol has remained unknown. We assumed that **4** was biosynthetically caused by a decarboxylation of **3** accompanying a migration of the 1,2-ketoalchol (C3C4) moiety.

2.4. Hydroleptosphol (5)

Hydroleptosphol (**5**) gave very poor ^{1}H NMR spectra in all solvents we examined [$CDCl_3$, CD_3OD, acetone-d_6, and $CD_3OD/CDCl_3$ (1:1)]. Among them, deuterio-chloroform gave the ^{1}H NMR spectrum in the best quality, even though the signal pattern of each resonance was still not assignable. However, the obtained spectrum suggested that **5** consisted of two predominant components in ca 1:1 ratio. Although recrystallization of **5** from chloroform-hexane gave fine needles,[12] that did not exhibit distinct melting point. Further recrystallization did not sharpen the melting point, nor improve the ratio of the components in $CDCl_3$ when the resulting needle was dissolved. The HPLC gave a symmetrical peak in any conditions. The mass profile of **5** was constant during the elution period in the LC-ESIMS. These results led us an assumption that the sample **5** was chemically pure in the crystalline lattice under HPLC, and ESIMS conditions but dissolution in the NMR solvents allowed to cause mixture. Interestingly, standing **5** in $CDCl_3$ at room temperature resulted in slow generation of **2**. Heating conditions

Table 1
The ^{13}C and ^{1}H NMR chemical shifts (ppm) and the coupling pattern and coupling constants (parenthesized, Hz) in the ^{1}H NMR for **1**–**4**

Position	**1** ($CDCl_3$)		**2** ($CDCl_3$)		**3** (CD_3OD)		**4** ($CDCl_3$)	
	^{13}C	^{1}H (J in Hz)	^{13}C	^{1}H (J in Hz)	^{13}C	^{1}H (J in Hz)	^{13}C	^{1}H (J in Hz)
1	174.6	–	171.8	–	174.0	–	–	–
3	157.1	–	111.5	–	160.6	–	74.9	4.32 (bq, 7.1)
4	68.7	5.16 (dt, 6.5, 2.3)	82.3	4.23 (br s)	69.6	5.32 (t, 2.4)	215.0	–
5	58.7	–	58.8	–	60.0	–	53.3	–
6	72.2	3.84 (br d, 6.8)	79.6	4.04 (d, 8.4)	74.3	3.57 (d, 7.9)	75.3	3.64 (dd, 8.1, 10.6)
7	70.0	4.71 (br)	70.1	4.17 (ddt, 8.4, 3.4, 1.8)	71.6	4.71 (ddt, 3.1, 7.9, 2.1)	73.1	4.15 (br s)
8	127.7	5.75 (dt, 10.2, 2.8)	127.7	5.50 (dt, 10.2, 1.8)	130.5	5.66 (dt, 10.3, 2.1)	128.0	5.53 (dt, 10.1, 2.2)
9	129.8	5.58 (ddd, 1.6, 2.8, 10.2)	131.6	5.56 (dt, 10.2, 1.9)	129.2	5.44 (dt, 10.3, 2.1)	131.5	5.45 (bt, 10.1, 2.2)
10	39.0	3.45 (dq, 8.4, 2.8)	40.9	3.22 (m)	40.1	3.53 (m)	43.2	3.30 (bt, 9.4)
11	89.1	4.68 (t, 2.3) 4.80 (t, 2.3)	14.8	1.60 (br s)	87.6	4.55 (t, 2.4) 4.63 (t, 2.4)	18.0	1.29 (bd, 7.1)
12	124.3	5.33 (dd, 8.4, 15.5)	125.1	5.81 (ddd, 15.4, 9.1, 0.7)	127.2	5.45 (dd, 7.1, 15.7)	128.6	5.05 (dd, 8.6, 14.8)
13	141.7	5.43 (dd, 7.6, 15.5)	141.4	5.50 (dd, 15.4, 7.9)	141.3	5.34 (dd, 7.9, 15.7)	139.3	5.21 (dd, 8.4, 14.8)
14	34.3	2.16 (m)	34.1	2.22 (m)	35.8	2.12 (m)	34.5	2.08 (m)
15	43.9	0.98 (ddd, 5.3, 8.0, 13.4) 1.24 (ddd, 5.1, 8.6, 13.4)	43.9	0.99 (m) 1.21 (m)	41.0	0.99 (m) 1.36 (m)	40.4	0.95 (m) 1.24 (m)
16	31.6	1.32 (m)	32.0	1.21 (m)	38.4	1.50 (m)	37.5	1.34 (m)
17	29.8	1.11 (m) 1.28 (m)	29.7	1.07 (m) 1.24 (m)	72.5	3.53 (m)	71.9	3.54 (m)
18	11.9	0.85 (t, 7.2)	11.2	0.80 (t, 7.2)	19.1	1.06 (d, 6.3)	18.3	1.03 (d, 6.3)
19	20.9	0.91 (d 6.7)	21.3	0.93 (d, 6.7)	21.9	0.92 (d, 7.6)	22.1	0.91 (d, 6.7)
20	19.0	0.80 (d, 6.3)	18.9	0.77 (d, 6.2)	14.7	0.81 (d, 6.6)	13.6	0.74 (d, 6.8)

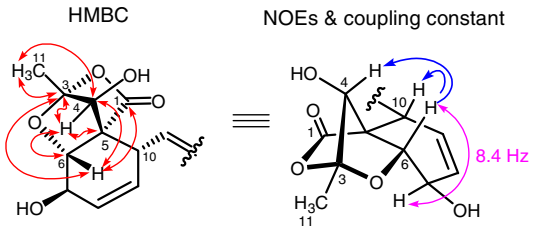


Figure 1. Characteristic HMBC correlations (red arrows), NOEs (blue arrows), and coupling constant (purple arrow) observed in **2**.

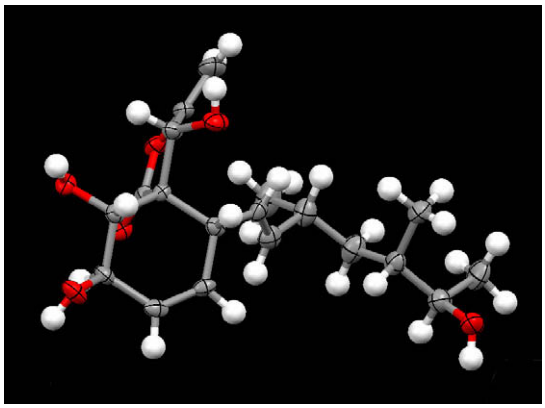


Figure 2. Structure of **3** suggested by the X-ray crystallographic analysis.

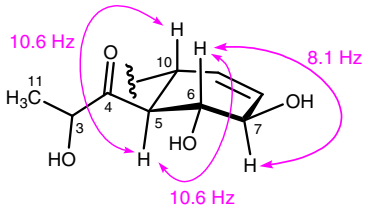
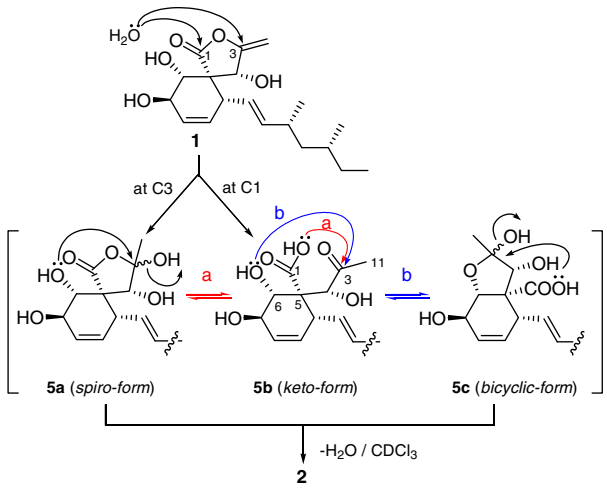


Figure 3. Characteristic coupling constant of the cyclic moiety of **4** in the ^{1}H NMR.



Scheme 1. Formation of **5** and its tautomerization.

accelerated its formation. Accordingly, **5** was estimated to be structurally related to **2.**

Since the ESIMS suggested the molecular formula to be $C_{19}H_{30}O_6$ (*m/z* 355.2121, $[M+H]^+$), **5** was expected to be a hydrolysate of **1** ($C_{19}H_{28}O_5$) taking a mixture of *spiro-form* **5a**, *keto-form* **5b**, or *bicyclic form* **5c**, as shown in Scheme 1, in solutions. Diastereomers of **5a** and **5c** must be also involved. An attack of H_2O to C3 of **1** would generate **5a**, while that at C1 gives **5b**. Acetal formation between the C1 carboxylic acid and the C3 carbonyl of **5b** produced **5a** (path a). Similarly, that between C6 alcohol and C3 forms **5c** (path b). These should be reversible to result the tautomeric mixture. When steric energies of **5a**, **5b**, and **5c** and their diastereomers were calculated with HF 6-31G* after conformational searches using MMFF,[13] estimated energy differences were within 2.0 kcal/mol. These results suggested that most of them could exist under equilibrium conditions, although those calculations did not consider the solvation energies. This might be the reason why **5** provided unassignable ^{1}H NMR spectra in many solvents. Probably, **5** in crystalline lattice might involve only one structure, the heating during melting point measurement allowed the tautomerization was well as dehydration into **2**, which resulted broadening of its melting points.

As mentioned above, two predominant isomers presented in $CDCl_3$. The major structures in $CDCl_3$ solution were then discussed. Keto form **5b** could be discarded from the candidates in this solution, because there were no signals around 2.0 ppm in the ^{1}H NMR, where C11 (*spiroleptosphol numbering*) methyl group must appear if we assumed that[14]. The ^{13}C NMR spectrum in $CDCl_3$ barely provided a pair of carbonyls (175.8 and 176.8 ppm). Since their chemical shifts empirically suggested lactone forms,[15] the predominant structures exist in $CDCl_3$ were tentatively assigned as **5a** and its C3 diastereomer.

2.5. Discussion

Our studies disclosed that **5a**–**c** were hydrolysate forms of **1**. We have also demonstrated that **2** could be formed from **5** in vitro. These suggested that some of them were the artificial compounds generated during isolation process. This possibility could evidently be denied because direct HPLC analysis of the culturing medium detected all **1**, **2**, and **5**. However, the question whether these were endogenous compounds has remained unclear. Since only one diastereomer due to the C17OH group was isolated, **3** and **4** would be formed enzymatically. Our biological assay disclosed **3** inhibited HepG2 cells (20% inhibitory) in 50 μM.

As described we succeeded in disclosing four novel analogues of spiroleptosphol (**1**) from ascomycete *L. doliolum*. Further biological assays of these compounds are under investigation.

3. Experimental

3.1. General methods

Melting points were determined with a Yanako MP-J3 micro melting point apparatus and not corrected. Optical rotations were measured on a HORIBA SEPA300 high-sensitivity polarimeter. ^{1}H NMR and ^{13}C NMR spectra were measured on a JEOL ALPHA 400 (^{1}H 400 MHz, ^{13}C 100 MHz) and a JEOL JNM-ECA 500 spectrometer (^{1}H 500 MHz, ^{13}C 125 MHz). Chemical shifts (δ) in ppm were determined relative to the solvent signal ($\delta_H(CHCl_3) = 7.24$ ppm, $\delta_H(CHD_2OD) = 3.30$ ppm $\delta_C(CDCl_3) = 77.0$ ppm, $\delta_C(CD_3OD) = 49.0$ ppm). IR spectra were obtained with a HORIBA FT-720 Fourier transform infrared spectrometer on KBr cells. Reverse phase column chromatography was performed with YAMAZEN ULTRA PACK (ODS-SM, 50 μm, 120 Å, 26 × 300 mm) employing FMI LAB PUMP RRP. Analytical HPLC were performed Merck LiChrospher® 100 RP-18e 5-μm column (125 × 4.0 mm I.D.) employing on a Waters 600 pump equipped with Waters 2487 Dual λ Absorbance detector.

3.2. Fermentation of *L. doliolum*

L. doliolum isolated from mugwort stems was cultured with potato-sucrose medium in 500 ml baffled flask × 20 (200 ml of medium in each flasks) at 25 °C for 2 weeks on a rotary shaker (100 rpm). After filtration, the culture broth was extracted with ethyl acetate (1.0 l × 2), and concentrated in vacuo to give a residue, which was loaded on silica gel column chromatography. Successive elution with 2:8 and 4:6 AcOEt/hexane gave pure **2** (92 mg) and a mixture of other compounds. The mixture was further chromatographed with $CHCl_3$/MeOH to afforded **1** (230 mg) and a mixture of **3**, **4** and **5**. After the mixture was passed through Sep-Pak (ODS) with H_2O/MeOH, followed by concentration, the residue was subjected to reverse phase column chromatography. Elution with CH_3CN/H_2O (32: 68) to afford **3** (65 mg), **5** (18 mg) and crude **4**. The crude **4** was further purified by HPLC (Waters SunFire™, Prep C18, 5 μm, 10 × 250 mm, MeCN/H_2O = 3:7, 4.0 ml/min flow) to give **4** (16 mg).

3.3. Physical data for 2

$[\alpha]_D^{21}$ −86 (*c* 2.1, $CHCl_3$). IR (film): 3420, 3960, 3910, 2875, 1775, 1460, 1400, 1140, 1060, 980, 890, 720 cm^{-1}. ^1H Characteristic HMBC Correlations: C1↔C4H, C1↔C6H, C1↔C10H, C3↔C4H, C3↔C6H, C3↔C11H, C4↔C11H, C5↔C4H, C5↔C6H, C5↔C10H, C5↔C12H, C6↔C4H, C6↔C8H. ESIMS (rel. int.) *m/z*: 355.2130 (17, $[M+H+H_2O]^+$), 337.2013 (100, $[M+H]^+$, calcd for $C_{19}H_{29}O_5$: 337.2015), 319.1912 (12, $[M+H-H_2O]^+$), 301.1801 (8, $[M+H-2\times H_2O]^+$).

3.4. Physical data for 3

t_R = 5.9 min (1.0 ml/min flow, 20–100% H_2O/MeCN linear gradient for 15 min). mp 147 °C. $[\alpha]_D^{20}$ −154 (*c* 2.4, MeOH). IR (film): 3370, 2960, 2920, 1785, 1680, 1105, 1060 cm^{-1}. Characteristic HMBC correlations: C1↔C6H, C3↔C4H, C3↔C11H, C4C↔11H, C5↔C6H, C5↔C9H, C6↔C4H. ESIMS (rel. int.) *m/z*: 353.1959 (18, $[M+H]^+$, calcd for $C_{19}H_{27}O_5$: 353.1964), 335.1857(100, $[M+H-H_2O]^+$), 317.1753 (13, $[M+H-2\times H_2O]^+$).

3.5. Physical data for 4

t_R = 5.6 min. (1.0 ml/min flow, 20–100% H_2O/MeCN linear gradient for 15 min). $[\alpha]_D^{20}$ −135 (*c* 1.6, $CHCl_3$). IR (film): 3400, 3965, 3910, 2875, 1715, 1455, 1375, 1060, 970, 855 cm^{-1}. Characteristic HMBC correlations: C4↔C5H, C4↔C11H, C5↔C10H. ESIMS (rel. int.) *m/z*: 653.4080 (80, $[2M+H]^+$), 327.2164 (72, $[M+H]^+$, calcd for $C_{18}H_{31}O_5$: 327.2171), 309.2072 (48, $[M+H-H_2O]^+$), 291.1968 (100, $[M+H-2\times H_2O]^+$), 274.1903 (12), 273.1868 (68, $[M+H-3\times H_2O]^+$).

3.6. Physical data for 5

t_R = 8.9 min (1.0 ml/min flow, 20–100% H_2O/MeCN linear gradient for 15 min) The optical rotation of **5** was not measured because of a mixture of tautomers. ESIMS (rel. int.) *m/z*: 355.2131 (18, $[M+H]^+$, calcd for $C_{19}H_{31}O_6$: 355.2121), 337.2024 (100, $[M+H-H_2O]^+$), 319.1921(20, $[M+H-2\times H_2O]^+$).

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11. CCDC 703050 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.
12. The obtained needles were too thin to apply the X-ray diffraction studies.
13. SPARTAN 06 ver. 1.1.2 Wavefunction Inc. 18401 Von Karman Avenue, Suite 370 Irvine, CA 92612.
14. The IR spectrum of crystalline **5** on KBr showed a characteristic C=O adsorption at 1745 cm^{-1}. Carboxylic acids **5b** and **5C** should take dimeric structure in the crystalline lattice to show C=O adsorption at ca. 1710 cm^{-1}. However, typical g-lactone adsorb at 1770 cm^{-1}. Thus it was difficult to conclude the structure of **5** in the crystalline lattice based on IR spectrum.
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