Structure of a novel monocyclic carotenoid, 3''-hydroxy-2'-isopentenylsaproxanthin ((3*R*,2'*S*)-2'-(3-hydroxy-3-methylbutyl)-3', 4'-didehydro-1', 2'-dihydro- β , ψ -carotene-3, 1'-diol), from a flavobacterium *Gillisia limnaea* strain DSM 15749

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ABSTRACT

Gillisia limnaea strain DSM 15749 (=R-8282^T, =LMG21470, =CIP108418) is a Gram-negative, strictly aerobic, psychrophilic, and non-photosynthetic bacterium that belongs to the family *Flavobacteriaceae*, which was isolated from a microbial mat in Lake Fryxell, Antarctica (Van Trappen et al., 2004). The bacterium shows yellow colored colony, but the carotenoid composition has never been determined yet. In this study, we elucidated the structure of carotenoids produced by the strain DSM 15749. *G. limnaea* strain DSM 15749 synthesized a novel monocyclic-type carotenoid, 3''-hydroxy-2'-isopentenylsaproxanthin ((3*R*, 2'*S*)-2'-(3-hydroxy-3-methylbutyl)-3', 4'-didehydro-1', 2'-dihydro- β , ψ -carotene-3, 1'-diol), as well as (3*R*, 3'*R*)-zeaxanthin. Furthermore, another monocyclic carotenoid, 2'-isopentenylsaproxanthin, was also detected in the strain DSM 15749. 3''-Hydroxy-2'-isopentenylsaproxanthin is "Chimera"-like unique structures, in that one end group forms the same structure of zeaxanthin, and the other end group is the same as bacterioruberin which is produced by several extremophilic bacteria and archaea. This structure involved the hydroxy derivative of 2'-isopentenylsaproxanthin. The present study is the first report about the carotenoids produced by the genus *Gillisia*, of the family *Flavobacteriaceae*.

Keyword: *Gillisia limnaea* strain DSM 15749; 3''-hydroxy-2'-isopentenylsaproxanthin; 2'-isopentenylsaproxanthin; monocyclic carotenoid; *Flavobacteriaceae*.

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1. Introduction

Carotenoids are naturally occurring yellow, orange, and red pigments synthesized by photosynthetic organisms, such as higher plants, algae, and photosynthetic bacteria. In addition, several species of non-photosynthetic bacteria, yeasts, and fungi also produce carotenoids. To date, more than 750 carotenoids have been isolated and identified from natural sources (Jackson et al., 2008).

Certain species of marine bacteria have been reported to synthesize carotenoids with unique molecular structures that are different from higher plants containing β -carotene and lutein (Maoka, 2011; Misawa, 2011). We recently reported that strain 11shimoA1 (JCM 19538), which was isolated from seaweed collected at Nabeta Bay (Shizuoka, Japan), synthesized a novel monocyclic carotenoid, 2'-isopentenylsaproxanthin (Takatani et al., 2014a). The strain 11shimoA1 belongs to the genus *Jejuia* in the family *Flavobacteriaceae* and its draft genome sequence was recently reported (Takatani et al., 2014b). Further, we also isolated five strains of the marine flavobacteria *Nonlabens* spp., (NR17, NR24, NR27, NR32, and NR33), which produce another monocyclic carotenoid, myxol (3',4'-didehydro-1',2'-dihydro- β , ψ -carotene-3,1',2'-triol), and draft genome sequences were analyzed (Nakanishi et al., 2014).

Myxol and saproxanthin, which have similar structures as 2'-isopentenylsaproxanthin, have been reported to possess stronger antioxidative activities than those of β -carotene and zeaxanthin. In addition, those monocyclic carotenoids were observed to have a neuroprotective effect (Shindo et al., 2007). For this reason, monocyclic carotenoids and their synthetic pathways in the family *Flavobacteriaceae* are of great interest.

Several new genera of the *Flavobacteriaceae* were systematically collected from Antarctic maritime lakes in the MICROMAT project (Van Trappen et al., 2002). The Gram-negative, non-photosynthetic bacterium *Gillisia limnaea* strain DSM 15749, belongs to the family *Flavobacteriaceae*, and was isolated from a microbial mat in Lake Fryxell, Antarctica (Van Trappen et al., 2004). Further, the draft genome of strain DSM 15749 was sequenced for the first time in the genus *Gillisia* (Riedel et al., 2012). According to previous studies (Van Trappen et al., 2004; Bowman and Nichols, 2005), the strain DSM 15749 produced orange colonies, although the chemical structures of pigment were not identified.

In the present study, we identified carotenoid structures produced by *G. limnaea* strain DSM 15749 belonging to the family *Flavobacteriaceae*. This strain DSM 15749 synthesized a novel monocyclic carotenoid, named

3''-hydroxy-2'-isopentenylsaproxanthin ((3*R*, 2'*S*)-2'-(3-hydroxy-3-methylbutyl)-3', 4'-didehydro-1', 2'-dihydro- β , ψ -carotene-3, 1'-diol), and 2'-isopentenylsaproxanthin as well as zeaxanthin. Our present study is the first to report the identification of carotenoid structures produced by the genus *Gillisia*.

2. Materials and Methods

2.1. Cultivation of bacterium

The *Gillisia limnaea* strain DSM 15749 (=R-8282^T, =LMG 21470, =CIP 108418) was obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). The strain was cultivated aerobically on Marine Agar 2216 (Difco) plate at 20°C.

2.2. Isolation of carotenoids

The strain DSM 15749 was incubated for several weeks on Marine Agar 2216 plates at 20°C, because the growth of this particular was very slow. After cultivation, the cells were collected by cell-scraper (Asahi glass Co., Ltd. Japan), and the total lipid fraction was extracted from the pellet with methanol. The methanol extract was then subjected to high performance liquid chromatography (HPLC) using Develosil ODS-UG-5 column (4.6 × 250 mm, Nomura Chemical Co., LTD., Japan), and elution with methanol/acetonitrile (7:3, v/v) at a flow rate of 1.0 mL/min. Carotenoids were detected with a UV-VIS detector (Hitachi L-2400) at 450 nm.

To isolate each carotenoid, the methanol extract was applied to a silica gel preparative thin-layer chromatography (TLC) plate (1 mm; Merck, Germany) and developed with n-hexane/ethyl acetate (4:6, v/v). Yellow, dark shade of orange, and pale orange fractions (R_f = 0.35 of yellow, 0.10 of deep orange, 0.46 of pale orange) were scraped, and pigments were eluted from the silica gel with acetone. Each carotenoid was purified from the pigmented fraction by HPLC with Develosil ODS-5 column (20 × 250 mm, Nomura Chemical Co., LTD.), eluting with methanol/acetonitrile (7:3, v/v) at a flow rate of 8.0 mL/min. Carotenoids were detected with UV-VIS detector at 450 nm.

2.3. Spectroscopic analysis of isolated carotenoids

The proton nuclear magnetic resonance (^1H -NMR) spectra of the carotenoids in CDCl_3 were measured at 500 MHz using a UNITY INOVA-500 NMR system (Varian, USA) with TMS as an internal control. The positive ion electro-spray ionization mass spectrometry (ESI MS) spectra of the carotenoids were measured by an Acquity LC Xevo G2-S Q time-of-flight (TOF) MS spectrometer (Waters, USA). The ESI TOF MS spectra were acquired by scanning from m/z 100 to 1,500 with a capillary voltage of 3.2 kV, cone voltage of 40 eV, and source temperature of 120 °C. Nitrogen was used as a nebulizing gas at a flow rate of 30 L/h. MS/MS spectra were measured with a quadrupole-TOF MS/MS instrument with argon as the collision gas at a collision energy of 30 V. The circular dichroism (CD) spectra were as recorded in ether at room temperature using a J-500C spectropolarimeter (Jasco, Japan).

2.4. Detection of 2'-isopentenylsaproxanthin

In the present study, 2'-isopentenylsaproxanthin in *G. limnaea* strain DSM 15749 was detected and identified by HPLC and LC-MS/MS. In HPLC analysis, each carotenoid in the methanol extract from the strain DSM 15749 was separated by Develosil ODS-UG-5 column, isocratically eluting with methanol/acetonitrile (7:3, v/v) at a flow rate of 1.0 mL/min and detected by UV-VIS diode array detector (Hitachi L-7455) at 200-650 nm. Further, the pale orange pigmented fraction (Fig. 1, peak 3) was purified by HPLC and subjected to LC-MS/MS using LCMS-8040 (Shimadzu, Japan) to characterize the structure. A binary solvent system of water (A)/methanol(B) with the following gradient elution was developed: 100% B in the beginning, changed to 70% B at 4 min, 100% B at 20 min again. The column (Develosil HB ODS-UG-3, 2.0×100 mm, Nomura Chemical) temperature was 25 °C, flow rate was 0.2 mL/min, the elute was detected using UV-VIS detector at 470 nm. A triple quadrupole mass spectrometer with ESI, positive ion mode, was used for analyzing the total ion scanning range being m/z 50-1000, under nebulizer nitrogen gas flow rate 3.0 L/min, drying nitrogen gas flow rate 15.0 L/min, DL temperature of 250 °C, and heat block temperature of 400 °C. 2'-Isopentenylsaproxanthin, used as an authentic standard, was prepared from *J. pallidilutea* strain 11shimoA1 according to the previous method described by Takatani et al. (2014a).

3. Results

3.1. Identification of pigments produced by *Gillisia limnaea* strain DSM 15749

Strain DSM 15749 produced yellow pigmented colonies on the agar plates. After culturing the bacterium on Marine Agar 2216, two major and several minor peaks in the methanol extract were detected by HPLC analysis (Fig. 1).

Peak 1 was the main peak at a retention time of 6.0 min. It was the same retention time of the zeaxanthin authentic standard. Analyses using ESI TOF MS and ^1H NMR (data not shown) showed that peak 1 was zeaxanthin (Fig. 2). The CD spectrum was comparable to that of (3*R*, 3'*R*)-zeaxanthin as described in a previous report by Buchecker and Noack (1995).

3.2. Structure of new carotenoid, 3''-hydroxy-2'-isopentenylsaproxanthin

Peak 2 in the HPLC analysis was detected at a retention time of 5.4 min (Fig. 1). This compound showed absorption maxima at 456, 481, and 512 nm, indicating the presence of saproxanthin type double bond system (Shindo et al., 2007). The molecular formula was determined to be $\text{C}_{45}\text{H}_{66}\text{O}_3$ by HR-ESI MS (m/z 655.5071 MH^+ , $\text{C}_{45}\text{H}_{67}\text{O}_3$ calc. for 655.5090). ESI TOF MS/MS showed characteristic product ions of $[\text{MH}-\text{H}_2\text{O}]^+$ (m/z 637.4981), $[\text{MH}-2\text{H}_2\text{O}]^+$ (m/z 619.4856), $[\text{MH}-92]^+$ (m/z 563.4345), and $[\text{MH}-160]^+$ (m/z 495.3614) (Fig. 3). These MS spectral data suggested that an isoprene unit was attached to the C40 carotenoid skeleton (Enzell and Bach, 1995). The NMR spectral data of this carotenoid are shown in Table 1. Because of the small amount of available sample (below 500 μg), complete ^{13}C -NMR measurement of this carotenoid could not be performed. Thus the part of the ^{13}C -chemical shift values were estimated from hetero-nuclear single quantum coherence (HSQC) and hetero-nuclear multiple-bond connectivity (HMBC) spectrum. Namely, ^{13}C chemical shift values of direct proton bonded carbons and quaternary carbons were estimated by HSQC and HMBC spectral data, respectively. The ^1H -NMR spectral data of this peak 2 carotenoid was almost similar to those of saproxanthin (Englert, 1995, Shindo et al., 2007) and 2'-isopentenylsaproxanthin (Takatani et al., 2014a) except for the signals of an isoprene moiety. The saproxanthin structural moiety was confirmed by correlated spectroscopy (COSY), nuclear overhauser enhancement spectroscopy (NOESY), HSQC, and HMBC experiments. The key NOESY correlations (H19/H-7 and H-11, H-20/H-11 and H-15, H-20'/H-15' and H-11', H-19'/H-11' and H-7', H-18'/H-3' and H-7') revealed the all *trans* geometry of polyene chain. The chemical shift values of the isoprene moiety of

181 this peak 2 carotenoid resembled to those of bacterioruberin (Englert, 1995). The
182 structural moiety of this peak 2 carotenoid was confirmed by COSY, HSQC, and
183 HMBC experiments. The HMBC correlation was observed from the terminal methyl
184 groups in isoprene unit at 1.21 and 1.22 ppm (H4" and H5") to quaternary carbon at
185 71.2 ppm (C-3") (Fig. 2, Table 1). These data clearly indicate that the hydroxy group is
186 attached at C3" position in isoprene unit. The COSY spectrum showed the correlation
187 between methin proton at 2.01 ppm (H-2') in sproxanthin moiety and methylene
188 proton at 1.30 ppm (H-1") in the isoprene moiety. This indicated that the isoprene unit
189 was attached at C-2' position of sproxanthin. Furthermore, characteristic product ions
190 of ESI MS/MS at m/z 495.3614 [MH-160]⁺ (attributed to cleavage between C-2' and
191 C-3') was in agreement with this structure (Fig. 3). This compound has two asymmetric
192 carbons at C-3 and C-2' (Fig. 2). The CD spectrum of this compound showed [(EPA)
193 nm ($\Delta\epsilon$) 235 (+1.5), 245 (0), 270 (-2.5), 305 (-3.0), 320 (0), 340 (+3.0), 360 (+3.0)] and
194 is the same shape as that of myxol (Yokoyama and Miki, 1995, Takaichi et al., 2001),
195 having 3*R*,2'*S* chirality with the same chromophore system. Therefore the structure of
196 peak 2 was elucidated to be
197 (3*R*,2'*S*)-2'-(3-hydroxy-3-methylbutyl)-3',4'-didehydro-1',2'-dihydro- β,ψ -carotene-3,1'-d
198 iol. This structure involved the hydroxy derivative of 2'-isopentenylsaproxanthin. So, it
199 is proposed to call the compound as 3"-hydroxy-2'-isopentenylsaproxanthin.

3.3. Detection of 2'-isopentenylsaproxanthin

204 Peak 3 was observed in the HPLC analysis at a retention time of 7.8 min as minor
205 peak (Fig. 1). This peak had the same retention time as 2'-isopentenylsaproxanthin (Fig.
206 2) from *J. pallidilutea* strain 11shimoA1 (Takatani et al., 2014a). The absorbance
207 spectrum for peak 3 also showed a maximum at 471.9 nm, which is the same as that of
208 2'-isopentenylsaproxanthin. Furthermore, the pale orange pigment was purified from
209 peak 3 by HPLC and then subjected to LC-MS/MS. As shown in Fig. 4, MS
210 chromatogram at m/z 636 [M], as well as UV-VIS chromatogram detected at 470 nm
211 showed the same pattern as 2'-isopentenylsaproxanthin standard. In addition, in MS/MS
212 fragmentation pattern of m/z 636 [M], several characteristic peaks (m/z 578 [M-58], 544
213 [M-92], and 486 [M-58-92]) were observed in both samples. These data indicated that
214 the pale orange pigment isolated from peak 3 in the strain DSM 15749 was
215 2'-isopentenylsaproxanthin (Fig. 2).

4. Discussion

G. limnaea strain DSM 15749 is a yellow-colored bacterium, belonging to the family *Flavobacteriaceae*, isolated from Antarctica lakes (Van Trappen et al., 2004). In the present study, carotenoids synthesized by strain DSM 15749 were identified (Fig. 2). The major carotenoids are zeaxanthin and a novel monocyclic carotenoid, 3''-hydroxy-2'-isopentenylsaproxanthin. Further, 2'-isopentenylsaproxanthin was also detected in the strain DSM 15749.

According to the genome information of the strain DSM 15749 (Riedel et al., 2012), this strain possesses a carotenoid biosynthesis gene cluster (phytoene desaturase: Gilli_2873, phytoene synthase: Gilli_2872 and carotene hydroxylase: Gilli_2871) that is similar to other *Flavobacteriaceae* bacteria. Lycopene cyclase (Gilli_2870) also locates downstream of the gene cluster. The genome information suggests that these genes are responsible for the production of zeaxanthin in the strain DSM 15749. Zeaxanthin has a dicyclic structure and is synthesized by some species of bacteria including the *Flavobacteriaceae* bacteria (McDermott et al., 1974). Zeaxanthin and lutein are normally found in the human eye and prevent age-related macular degeneration and light-induced photoreceptor death (Beatty et al., 2001; Thomson et al., 2002). Therefore zeaxanthin and lutein have been applied as a nutraceutical worldwide.

G. limnaea strain DSM 15749 also produces a novel monocyclic type carotenoid, 3''-hydroxy-2'-isopentenylsaproxanthin. One end group of this novel carotenoid has the same structure as zeaxanthin, and the other end group has the same structure as bacterioruberin, C₅₀ type long-chain carotenoid. Long-chain C₅₀ bacterioruberin-type carotenoids have been isolated from several extremophilic bacteria and archaea, including species of *Halobacterium*, *Haloferax* (D'Souza et al., 1997), and psychrotrophic bacteria, *Micrococcus roseus* (Jagannadham et al., 1991) and *Arthrobacter agilis* (Fong et al., 2001). In these organisms, bacterioruberin type carotenoids have been reported to play roles in adaptation and survival in extreme environments. Long-chain carotenoids may also reinforce the membrane bilayer to reduce O₂ diffusion in the cytoplasm (Wisniewska and Subczynski, 1998) and raise the hydrophobic barrier for polar molecules and ions. Since 3''-hydroxy-2'-isopentenylsaproxanthin has some structure similarity to bacterioruberin, it has been suggested that it may be carotenoid involved in adaptation to environmental stress.

3''-Hydroxy-2'-isopentenylsaproxanthin has a hydroxyl group at C-3'' position of the isopentenyl residue in 2'-isopentenylsaproxanthin, which is isolated from *J.*

pallidilutea strain 11shimoA1 (Takatani et al., 2014a). Identification of carotenoid structures suggests that the strain DSM 15749 produce 3''-hydroxy-2'-isopentenylsaproxanthin through hydroxylation of 2'-isopentenylsaproxanthin. Therefore, we attempted to detect 2'-isopentenylsaproxanthin in the methanol extract from the strain DSM 15749 by LC-MS/MS analysis. Fragmentation of minor peak 3 in the LC-MS/MS analysis revealed the same fragmentation pattern as authentic 2'-isopentenylsaproxanthin isolated from the strain 11shimoA1 (Fig. 4). These results strongly suggest that the strain DSM 15749 has the same biosynthetic pathways of 2'-isopentenylsaproxanthin as seen in the *J. pallidilutea* strain 11shimoA1, although these pathways have not been clearly defined.

Saproxanthin has been reported to be a stronger antioxidative activity than β -carotene and zeaxanthin against lipid peroxidation in rat brain homogenates (Shindo et al., 2007). In addition, another monocyclic carotenoid, myxol (Yokoyama and Miki, 1995; Teramoto et al., 2004), which has a similar structure to saproxanthin, was shown to have a neuroprotective effect against L-glutamate toxicity in the neuronal hybridoma cell line, N18-RE-105 (Shindo et al., 2007). Since the structure of 3''-hydroxy-2'-isopentenylsaproxanthin identified in this study is similar to that of saproxanthin and myxol, it is likely to have a strong antioxidant and a neuroprotective effects. Further, Yatsunami et al. (2014) evaluated DPPH radical scavenging activity of bacterioruberin and demonstrated that it possessed a greater antioxidant capacity than that of β -carotene. Considering one end group of 3''-hydroxy-2'-isopentenylsaproxanthin has the same structure as bacterioruberin, it is also expected that 3''-hydroxy-2'-isopentenylsaproxanthin has a strong antioxidative activity.

G. limnaea strain DSM 15749 produces both dicyclic and monocyclic carotenoids, zeaxanthin, 3''-hydroxy-2'-isopentenylsaproxanthin and 2'-isopentenylsaproxanthin. These results indicate that the strain DSM 15749 may have unique carotenoid synthetic pathways, which produces 3''-hydroxy-2'-isopentenylsaproxanthin via 2'-isopentenylsaproxanthin and γ -carotene. Both 3''-hydroxy-2'-isopentenylsaproxanthin and 2'-isopentenylsaproxanthin are novel monocyclic carotenoids found in the strain DSM 15749 and the strain 11shimoA1 in the family *Flavobacteriaceae*. Draft genome sequence of the strain DSM 15749 has already been reported (the GenBank accession number NZ_JH594605- NZ_JH594606) (Riedel et al., 2012). In addition, draft genome sequence of the strain 11shimoA1 was currently also reported (DDBJ/EMBL/GenBank under the accession number BBNY01000001-

BBNY01000098). Therefore, further study on potentially novel biosynthetic pathways of 3''-hydroxy-2'-isopentenylsaproxanthin and 2'-isopentenylsaproxanthin are expected.

5. Conclusions

In the present study, we purified and identified the structure of pigments produced by strain *Gillisia limnaea* DSM 15749 belonging to the family *Flavobacteriaceae*. The strain DSM 15749 produced a novel monocyclic-type carotenoid, 3''-hydroxy-2'-isopentenylsaproxanthin, and 2'-isopentenylsaproxanthin as well as zeaxanthin.

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Figure Captions

Fig.1. HPLC chromatogram of pigments produced by *G. limnaea* strain DSM 15749. Strain DSM 15749 was cultivated in Marine Agar 2216 for several days at 20 °C. Pigments in the total lipid fraction extracted from the strain DSM 15749 were analyzed by HPLC equipped with ODS column. Mobile phase was methanol/acetonitrile (7:3, v/v) and flow rate was 1.0 mL/ min. Pigments were detected at 450 nm using UV detector.

Fig.2. Structure of carotenoids produced by strain DSM15749.

Fig.3. The characteristic product ions of peak 2 carotenoid detected by ESI TOF MS/MS.

Fig.4. Detection of 2'-isopentenylsaproxanthin in *G. limnaea* strain DSM 15749 using LC-MS/MS (LCMS-8040). (a), (b), and (c): 2'-isopentenylsaproxanthin authentic standard. (d), (e), and (f): a pale orange pigment (peak 3) isolated from the methanol extract of the strain DSM 15749 by HPLC. (a) and (d), LC chromatogram detected at 470 nm. (b) and (e), MS chromatogram at m/z 636 $[M]^+$. (c) and (f), MS/MS fragmentation pattern of m/z 636 $[M]^+$.

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Zeaxanthin

2'-Isopentenylsaproxanthin

3''-Hydroxy-2'-isopentenylsaproxanthin
((3*R*,2'*S*)-2'-(3-hydroxy-3-methylbutyl)-3', 4'-didehydro-
1', 2'-dihydro-β, ψ-carotene-3, 1'-diol)

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Fig. 3. Takatani et al.,

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Table 1 ^{13}C and ^1H -NMR data of 3''-hydroxy-2'-isopentenylsaproxanthin

position	$\delta^{13}\text{C}^*$	$\delta^1\text{H}$	mult. J (Hz)
1	37.2		
2	48.5	β 1.48 α 1.77	dd (12, 12) ddd (12, 5, 2)
3	65.1	4.00	m
4	42.5	β 2.05 α 2.39	dd (16, 10) ddd (16, 6, 2)
5	n.a.		
6	n.a.		
7	125.6	6.10	d (16)
8	138.7	6.15	d (16)
9	n.a.		
10	131.2	6.15	d (11)
11	125.0	6.62	dd (15, 11)
12	137.7	6.36	d (15)
13	n.a.		
14	~132.8	6.25	d (11)
15	~130.0	~6.64	m
16	28.8	1.08	s
17	30.3	1.08	s
18	21.9	1.74	s
19	~13.0	1.97	s
20	~13.0	1.97	s
1'	72.7		
2'	56.4	2.01	m
3'	129.2	5.45	dd (15, 9)
4'	138.7	6.20	d (15)
5'	n.a.		
6'	131.3	6.13	d (11)
7'	124.6	6.60	dd (15, 11)
8'	137.8	6.38	d (15)
9'	n.a.		
10'	131.5	6.25	d (11)
11'	125.2	6.64	dd (15, 11)
12'	137.5	6.39	d (15)
13'	n.a.		
14'	~132.8	6.28	d (12)
15'	~130.0	~6.64	m
16'	29.6	1.22	s
17'	28.0	1.18	s
18'	~13.0	1.93	s
19'	~13.0	1.97	s
20'	~13.0	1.99	s
1''	56.4	~1.30	m
2''	n.a.	n.a.	
3''	71.2	n.a.	
4''	29.5/27.3	1.21/1.22	s
5''	29.5/27.3	1.21/1.22	s

mated from HSQC and HMBC spectra

n.a. not assigned
d doublet
dd double doublet
m multiplet