Chemical constituents of the leaves of rabbiteye blueberry (Vaccinium ashei) and characterization of polymeric proanthocyanidins containing phenylpropanoid units and A-type linkages

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**Abstract:** Chemical constituents of the leaves of rabbiteye blueberry (*Vaccinium ashei* Reade) were investigated in detail. The major phenolic components were caffeoyl quinic acids, flavonol glycosides, flavan-3-ols and proanthocyanidins. Catechins and proanthocyanidins having additional phenylpropanoid units, such as cinchonains, kandelins and mururins, characterized the polyphenols of this plant. Among them, vaccinin A, an isomer of mururin A, was found to be a new compound, and the structure was characterized by spectroscopic methods. The most abundant polyphenols (11.3% of freeze dried leaves) were oligomeric proanthocyanidins. Thiol degradation with mercaptoethanol indicated that the polymer was constituted of (+)-catechin and (-)-epicatechin as the terminal units and (-)-epicatechin, procyanidin A-2, and cinchonains Ia and Ib as the extension units. Mass spectral analysis suggested the presence of at least dodecamers with A-type linkages and phenylpropanoid moieties.

**Keywords:** blueberry; *Vaccinium ashei*; polyphenol; proanthocyanidin; catechin; cinchonain
1. Introduction

Many reports have suggested that blueberry fruits have various biological activities, including prevention of urinary tract infections (Jepson & Craig, 2007), antioxidative (Dulebohn, Yi, Srivastava, Akoh, Krewer & Fischer, 2008; Castrejón, Eichholz, Rohn, Kroh & Huyskens-Keil, 2008) and anticancer activities (Seeram, 2008; Neto, 2007). The fruit anthocyanins and proanthocyanidins are believed to be responsible for the activities (Gu, Kelm, Hammerstone, Beecher, Cunningham, Vannozzi, & Prior, 2002; Prior, Lazarus, Cao, Muccitelli, & Hammerstone, 2001). The leaves have been known to be used in a tea for diabetics among the alpine peasantry (Allen, 1927; Watson, 1928). More recently, strong oxygen radical absorbance capacity (Ehlenfeldt & Prior, 2001), hypotensive effects (Sakaida, Nagao, Higa, Shirouchi, Inoue, Hidaka, Kai, & Yanagita, 2007), hypolipidemic effects (Nagao, Higa, Shirouchi, Nomura, Inoue, Inafuku, & Yanagita, 2008), and antileukemic activity (Skupień, Oszmiański, Kostrzewa-Nowak, & Tarasiuk, 2006) of the leaves have been reported. However, the detail of the chemical constituents of the leaves has not yet been clarified. Only the presence of a large amount of polyphenols and tannins has been identified (Ehlenfeldt & Prior, 2001; Naczk, Grant, Zadernowski & Barre, 2006). In this study we investigated the chemical constituents in the leaves of rabbiteye blueberry (*Vaccinium ashei* READE, Ericaceae), which is tolerant
to heat and drought and thus is cultivated in the southern part of Japan. The structures of polyphenols, including oligomeric proanthocyanidins, were clarified in detail using spectroscopic and chemical methods.

2. Materials and methods

2.1. Materials

The fresh leaves of rabbiteye blueberry were cultivated and collected at Unkai Shuzo Co., Ltd., Miyazaki, Japan. The leaves were freeze-dried, pulverized and stored at -20°C until use.

2.2. Analytical Procedures

UV spectra were obtained with a JASCO V-560 UV/VIS spectrophotometer (JASCO Co., Tokyp, Japan). $^1$H and $^{13}$C NMR spectra were recorded in a mixture of acetone-$d_6$ and D$_2$O (19:1, v/v) at 27°C with a JEOL JNM-AL400 spectrometer (JEOL Ltd., Tokyo, Japan) operating at 400 MHz for $^1$H and 100 MHz for $^{13}$C. $^1$H-$^1$H COSY, NOESY, HSQC and HMBC spectra were recorded in a mixture of acetone-$d_6$ using a Varian Unity plus 500 spectrometer (Varian Inc., Palo Alto, CA, USA) operating at 500 MHz for $^1$H and 125 MHz for $^{13}$C. Coupling constants are expressed in Hz and chemical
shifts are given on a $\delta$ (ppm) scale. HMQC, HMBC and NOESY experiments were performed using standard Varian pulse sequences. The matrix-assisted laser desorption time-of-flight mass spectra (MALDI TOF MS) were recorded on a Voyager-DE Pro spectrometer (Applied Biosystems, USA), and 2, 5-dihydroxybenzoic acid (10 mg/ml in 50% acetone containing 0.05% trifluoroacetic acid) was used as the matrix. Fast atom bombardment (FAB) MS was recorded on a JMS 700N spectrometer (JEOL Ltd., Japan), and $m$-nitrobenzyl alcohol or glycerol was used as a matrix.

Column chromatography was performed using Sephadex LH-20 (25-100 $\mu$m, GE Healthcare Bio-Science AB, Uppsala), Diaion HP20SS (Mitsubishi Chemical, Japan), MCI gel CHP 20P (75–150 $\mu$m; Mitsubishi Chemical, Tokyo, Japan), Chromatorex ODS (100–200 mesh; Fuji Silysia Chemical, Kasugai, Japan), and Silica gel 60 N (Kanto Chemical Co., Inc., Tokyo, Japan). Thin layer chromatography was performed on precoated Kieselgel 60 F$_{254}$ plates (0.2 mm thick, Merck KGaA, Darmstadt, Germany) with toluene-ethyl formate-formic acid (1:7:1, v/v), CHCl$_3$-MeOH-water (14:6:1, v/v) and Cellulose F$_{254}$ (0.2-mm thick, Merck) with 2% AcOH. Spots were detected using ultraviolet (UV) illumination and by spraying with 2% ethanolic FeCl$_3$ or 10% sulfuric acid reagent followed by heating. Analytical HPLC was performed on a Cosmosil 5C$_{18}$-AR II (Nacalai Tesque Inc., Kyoto, Japan) column.
(4.6 mm i.d. × 250 mm) with gradient elution from 4–30% (39 min) and 30–75% (15 min) of CH₃CN in 50 mM H₃PO₄, flow rate, 0.8 ml/min; detection, JASCO photodiode array detector MD-910.

2.3. Extraction and separation

The freeze-dried leaves (500 g) were pulverized and extracted with methanol (3 l) three times and filtered. The plant debris was further extracted with 70% acetone (3 l) two times and filtered. The filtrate was combined, concentrated by rotary evaporator (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) and dried under vacuum to give the extract (304 g). The extract was suspended in water and successively partitioned with hexane, Et₂O to give hexane (13.7 g) and Et₂O (11.3 g) soluble fractions. TLC analysis indicated that the hexane soluble fraction mainly contained chlorophyll and waxes and was not examined further. The Et₂O layer was repeatedly chromatographed over Silica gel (CHCl₃-MeOH-H₂O, 95:5:0, 90:10:1, 40:10:1, 14:6:1, v/v).

The aqueous layer was separated by Sephadex LH-20 column chromatography with H₂O containing increasing proportions of MeOH to give three fractions: Aq-1, Aq-2 (42.7 g), and Aq-3 (51.9 g).

The Aq-1 was applied to a Diaion HP20SS column (H₂O-MeOH) and eluted
with H$_2$O containing increasing proportions of MeOH (10 % – 100 %) to give fractions Aq-1-1 (110 g), Aq-1-2 (29.5 g) and Aq-1-3 (28.5 g). A portion (11 g) of the Aq-1-1 was acetylated with Ac$_2$O (100 ml) and pyridine (100 ml) at room temperature for 10 h and then 80ºC for 1 h. After evaporation of the reagent by rotary evaporator the syrup was separated by Silica gel column chromatography (20 – 50 % acetone in hexane) to yield tetraacetyl quinic acid (5.77 g) and octaacetyl sucrose (3.28 g).

The Aq-1-2 and Aq-1-3 were separately chromatographed over Sephadex LH-20 (H$_2$O-MeOH) to give fractions Aq-1-2-1 (7.3 g), Aq-1-2-2 (19.2 g), Aq-1-3-1 (7.5 g) and Aq-1-3-2 (20.6 g). The fractions Aq-1-2-1 and Aq-1-3-1 were separately subjected to a combination of chromatography over Diaion HP20SS (H$_2$O-MeOH), Chromatorex ODS (H$_2$O-MeOH), and silica gel (CHCl$_3$-MeOH-H$_2$O).

The fraction Aq-2 was separated by Diaion HP20SS (H$_2$O-MeOH). Aq-3 was fractionated by Diaion HP20SS (H$_2$O-MeOH).into three fractions: Aq-3-1 (10.7 g), Aq-3-2 (9.7 g), and Aq-3-3 (16.9 g). The fractions Aq-3-1 and Aq-3-2 were separately subjected to a combination of chromatography over Diaion HP20SS (H$_2$O-MeOH), Chromatorex ODS (H$_2$O-MeOH), and silica gel (CHCl$_3$-MeOH-H$_2$O).

The fractions Aq-1-2-2, Aq-1-3-2 and Aq-3-3 were characterized to be polymeric proanthocyanidins by reddish coloration with vanillin-HCl reagent and
HPLC analysis of the thiol degradation products.

2.4. Vaccinin A (19)

A yellow amorphous powder; \([\alpha]^{20}_D\) -15.5° (c=0.07, acetone-H\(_2\)O, 9:1); UV (MeOH) 
\(\lambda_{\text{max}}\) nm (log \(\varepsilon\)): 217 (4.58), 271 (4.02), 314 (4.08), 347 sh (3.98), 374 sh (4.02), 390
(4.07); CD (MeOH) \(\Delta\varepsilon\) (nm): 0 (224), +133.9 (240), 0 (260), -5.36 (271), 0 (278), +23.0
(295), 0 (326), -11.8 (347); IR (dry film) \(\nu_{\text{max}}\) cm\(^{-1}\): 3250, 2922, 1674, 1615, 1562, 1519,
1469, 1432; FAB-MS \(m/z\): 449 [M+H]\(^+\); HR-FAB-MS \(m/z\): 449.0873 [M+H]\(^+\) (Calcd for
C\(_{24}\)H\(_{17}\)O\(_9\): 449.0873); for the \(^1\)H and \(^13\)C-NMR data see Table 2.

2.5. Thiol degradation

The fraction Aq-3-3 (10.0 g) was dissolved in a solution (1.0 l) consisting of
2-mercaptoethanol (50.0 ml), 0.5M HCl (80 ml), H\(_2\)O (320 ml) and EtOH (550 ml), and
the mixture was heated at 70°C for 10 h (Tanaka, Takahashi, Kouno, & Nonaka, 1994).
After concentration by rotary evaporator, the aqueous solution was fractionated by
Sephadex LH-20 column chromatography (5.5 cm i.d. × 30 cm) with H\(_2\)O containing
increasing proportions of MeOH to give three fractions: TD-1 (5.15 g), TD-2 (1.42 g)
and TD-3 (4.00 g). The TD-1 was separated by MCI-gel CHP20P, Chromatorex ODS,
and Sephadex LH-20 column chromatography to give 3 (99.6 mg), 4 (62.1 mg), and 
(-)-epicatechin 4-(2-hydroxyethyl)thioether (4a) (2.4 g) (Tanaka et al., 1994). Similar separation of TD-2 afforded cinchonain Ia 4-(2-hydroxyethyl)thioether (11a) (340.6 mg), cinchonain Ib 4-(2-hydroxyethyl)thioether (12a) (166.6 mg), cinchonain Iib 4''-(2-hydroxyethyl)thioether (16a) (38.2 mg). From TD-3, 8 (131.8 mg), 9 (30.1 mg), and procyanidin A-2 4'-(2-hydroxyethyl)thioether (20) (411.2 mg) (Tanaka, Kondou, & Kouno, 2000) were isolated.

Cinchonain Ia 4-(2-hydroxyethyl) thioether (11a)

A tan amorphous powder, [α]_D^{21} -85.1° (c=0.1, MeOH), FAB-MS m/z 529 [M+H]^+ HR-FAB-MS m/z 529.1174 (Calcd for C_{26}H_{25}O_{10}S: 529.1168); IR (dry film) ν_max cm⁻¹: 3388, 1746, 1615, 1521, 1446; UV (MeOH) λ_max nm (log ϵ): 282 (3.94); for the ^1H and ^13C-NMR data see Table 3.

Cinchonain Ib 4-(2-hydroxyethyl) thioether (12a)

A tan amorphous powder, [α]_D^{22} 76.2 (c=0.1, MeOH), FAB-MS m/z: 529 [M+H]^+, HR-FAB-MS m/z: 529.1163 (Calcd for C_{26}H_{25}O_{10}S: 529.1168); IR (dry film) ν_max cm⁻¹: 3389, 1745, 1613, 1520, 1443; UV (MeOH) λ_max nm (log ϵ): 282 (3.95); for the ^1H and ^13C-NMR data see Table 3.

Cinchonain Iib 4''-(2-hydroxyethyl) thioether (16a)
A tan amorphous powder, \([\alpha]_D^{28} +149.3^\circ \) (c=0.1, MeOH), FAB-MS \(m/z\) 817 [M+H]\(^+\); HR-FAB-MS \(m/z\) 817.1813 (Calcd for C\(_{41}\)H\(_{37}\)O\(_{16}\)S: 817.1802); IR (dry film) \(\nu_{\text{max}}\) cm\(^{-1}\): 3389, 1739, 1613, 1519, 1443; UV (MeOH) \(\lambda_{\text{max}}\) nm (log \(\varepsilon\)): 206 (4.92), 223 sh (4.74), 282 (3.95); for the \(^1\)H and \(^{13}\)C-NMR data see Table 3.

2.6. Desulfurization of thioethers.

The structures of thioethers 11a, 12a, 16a, and 20 were confirmed by desulfurization (Nonaka, et al., 1982). A thioether (10-15 mg) in EtOH (3 ml) was treated with Raney-nickel (W-4) at room temperature for 30 min. After filtration, the filtrate was concentrated and the residue was purified by Sephadex LH-20 (EtOH). The products were identified by comparison of physicochemical and \(^1\)H NMR data with those of authentic samples.

3. Results and discussion

First, the extract was partitioned with \(n\)-hexane and Et\(_2\)O. The hexane layer mainly contained chlorophylls and waxes. From the Et\(_2\)O layer \(p\)-hydroxybenzoic acid and two digalactosyl glycerolipids were isolated. A major part of the constituents remained in the aqueous layer, and chromatographic separation yielded compounds.
listed in Table 1. The presence of a large amount of quinic acid was confirmed by isolation of their acetate derivatives. From the yield of the acetate, the quinic acid content was estimated to be about 6% of the freeze dried leaves. On HPLC analysis, chlorogenic acid (1) and rutin (2) were found to be major phenolic constituents of the leaves, and their concentration in the freeze dried leaves was estimated to be 2.0% and 0.48%, respectively, by HPLC analysis. The presence of a large amount of proanthocyanidins was shown using a vanillin-HCl test on thin-layer chromatography. Although most proanthocyanidins are astringent and bitter, two A-type proanthocyanidin trimers 8 and 9 isolated in this experiment (Fig. 1) are known to have a sweet taste (Morimoto et al., 1985). In addition, the presence of phenylpropanoid substituted catechins (10 – 12, 17 – 19) and procyanidins (13 – 16) is characteristic of the leaves. The phenylpropanoid units are structurally related to the caffeic acid, and this is probably related to the coexistence of a large amount of caffeoyl quinic acids. *In vitro* biomimetic synthesis of the cinchonains from catechin and caffeic acid has been achieved (Chen et al., 1993). Mururins A (17) and B (18) are oxidative metabolites of cinchonain isomers having phenylpropanoid units at the C-6 position, and these compounds were only found in *Brothum acutifolium* before this study (Takashima et al., 2002).
A new compound named vaccinin A (19) was obtained as a yellow amorphous powder and showed UV absorption bands at 271, 314, and 390 nm. The high-resolution FAB-MS (m/z 449.0873 [M+H]+, C_{24}H_{16}O_{9}) suggested that this compound is an isomer of mururins. The $^1$H and $^{13}$C NMR spectroscopic data (Table 2) were closely related to those of mururin A (17), showing signals attributable to tri-, tetra- and penta-substituted aromatic rings, one enone system, two oxygenated methines and a benzylic methylene. The oxygenated methine proton at $\delta$ 4.89 was assignable to the C-ring H-2, and its relatively large coupling constant (6.9 Hz) indicated 2,3-trans configuration of the C-ring. The HMBC correlations of the C-ring H-2 and H-4 and A-ring aromatic proton at $\delta$ 6.51 illustrated in Fig. 2 confirmed the presence of a catechin moiety in the molecule. The remaining 9 $sp^2$ carbon signals arising from the caffeoyl moiety (C-1" – C-9") were almost the same as those of 17. The degree of unsaturation (17) calculated from the molecular formula as well as the spectroscopic similarity between these two compounds indicated that the C-7" of the caffeoyl moiety was attached to the C-6 of the A-ring, and both oxygen atoms at the C-5 and C-7 form ether or lactone rings. This was supported by the appearance of a correlation peak between H-8" and the A-ring of C-6. The difference between 19 and 17 was the chemical shifts of the oxygen-bearing A-ring carbons. The C-7 and C-5 signals were distinguishable by observation of HMBC
correlation with H-4. The C-5 signal at δ 149.0 was observed at higher field compared with that of 17 (δ 153.0), and inversely, the C-7 resonated at lower field δ 153.6 compared with that of 17 (δ 150.0). This observation strongly suggested that the carboxyl group of the caffeoyl moiety was connected to the C-7 oxygen and the C-5 oxygen forms a pyran ring by oxidative coupling with the C-6" of the catechol ring. Thus, 19 and 17 differ in the arrangement of the caffeoyl moiety. The absolute configuration was determined to be the same as that of 17 by comparison of CD spectral data. It is noteworthy that in this study we did not identify cinchonain isomers having phenylpropanoid units at the C-6 position of the catechin A-ring. The catechol ring of the caffeoyl moiety in the C-6 isomers may be oxidized to form pyran rings with a C-5 or C-7 hydroxyl group.

Besides these compounds with relatively low molecular weights, the extract contained a large amount of oligomeric and polymeric proanthocyanidins. The isolation yield of the oligomers was 11.3 % of freeze dried leaves (Table 1). The oligomers were observed as a broad hump on the base line on HPLC analysis and remained at the origin on silica gel TLC analysis. To determine the structural components, the proanthocyanidins underwent thiol degradation with mercaptoethanol under acidic conditions. The products indicated that terminal units of the oligomers (the terminal
units having CH₂ structure at the C-4 position) were (+)-catechin (3) and (-)-epicatechin (4) (the ratio is about 3:2). Epicatechin-(4β→8, 2β→7)-epicatechin-(4α→8)-catechin (8) and epicatechin-(4β→8, 2β→7)-epicatechin-(4α→8)-epicatechin (9) were also isolated as the terminal units. The extension units were obtained as 4-(2-hydroxyethyl)-thioethers (4a, 11a, 12a, and 20) in this thiol degradation (Fig. 3), and the structures were confirmed by reductive desulfurization with Raney Ni. The most dominant extension unit was (-)-epicatechin (about 71% of the extension units), followed by procyanidins A-2 (17.7%), cinchonain Ia (7.5%), and cinchonain Ib (3.4%). A small amount of cinchonain IIb thioether (16a) (0.5%) was also isolated, and this may suggest that the caffeic acid reacts with the stereochemically unhindered upper terminal units of the proanthocyanidin chains with free C-8 and C-6 positions. A rough estimate based on yield of the thiol degradation products suggested the degree of the oligomerization is about 12. To avoid excess complexity of the products by side reactions such as cleavage of C-rings, the thiol degradation was terminated before complete degradation; therefore, the yield of the products may not reflect the actual composition. However, obviously the presence of A-type linkage and cinchonain units characterizes the blueberry leaf proanthocyanidin oligomers. This was also demonstrated by the MALDI-TOF MS analysis of the oligomer fractions (Fig. 4).
spectrum exhibited two series of peaks: one is assignable to proanthocyanidins composed of usual flavan-3-ol units with one or two A-type linkages, and the other is proanthocyanidins with one additional caffeic acid moiety and A-type linkages. The spectrum indicated the presence of at least dodecamers. Due to the low sensitivity to proanthocyanidins with higher molecular weights, probably proanthocyanidins larger than dodecamers are present.

4. Conclusion

This study revealed that the polyphenols of blueberry leaves are mainly composed of proanthocyanidins, followed by caffeoyl quinic acids and flavonol glycosides. The proanthocyanidins was characterized by the presence of A-type linkages and cinchonain units. It was deduced from the concentration in the leaves that the polyphenols, especially the oligomeric proanthocyanidins, contribute to the biological activities of the blueberry leaves.

References


Tanaka, T., Kondou, K., & Kouno, I. (2000). Oxidation and epimerization of

Figure Captions

Fig. 1 Structures of major polyphenols isolated from rabbiteye blueberry leaves

Fig. 2 Selected HMBC correlations observed for vaccinin A (19)

Fig. 3 Thioethers obtained by thiol degradation of oligomeric proanthocyanidins

Fig. 4 MALDI-TOF-MS of the oligomeric proanthocyanidin fraction of rabbiteye blueberry leaves and a possible structure. CA: caffeic acid moiety
Table 1
Compounds isolated from the EtO layer and aqueous layer of the leaves of Ribbiteye blueberry

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Compounds</th>
<th>Isolation yield (%)</th>
<th>References</th>
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<tr>
<td>EtO layer</td>
<td>2,3-dihydroxybenzonic acid</td>
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<td>1,2-dihydroxy-3-O-β-D-galactopyranosyl-(1→5)-O-β-D-galactopyranosyl)-glycerol</td>
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<td>2,3-dihydroxy-3-O-β-D-galactopyranosyl-(1→5)-β-D-galactopyranosyl)-glycerol</td>
<td>0.041</td>
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<td>Aqueous layer</td>
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<td>Quinic acid&lt;sup&gt;a&lt;/sup&gt;</td>
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<td></td>
<td>Sucrose&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Benzylalcohol O-α-L-arabinofuranosyl-(1→5)-O-β-D-glucopyranoside</td>
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<td>Quercitin 3-O-β-D-xyllosyl-(1→2)-α-L-rhamnosyl-(1→6)-β-D-glucoside</td>
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<td>Camelliaside (β-kamphoterin 3-O-β-D-xyllosyl-(1→2)-α-L-rhamnosyl-(1→6)-β-D-glucoside)</td>
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<td>Rutin (quercitin 3-O-α-L-rhamnosyl-(1→6)-β-D-glucoside) (2)</td>
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<td>(+)-catechin (3)</td>
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<td>(−)-epicatechin (4)</td>
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<td>Aq. 3-3</td>
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<sup>a</sup> From freeze dried leaves (500g).
<sup>b</sup> Isolated as tetraacetate after acetylation with Ac<sub>2</sub>O and pyridine.
<sup>c</sup> Isolated as octaacetate after acetylation with Ac<sub>2</sub>O and pyridine.
<sup>d</sup> Identified by comparison of the 1H and 13C NMR data with those of authentic samples.
Table 2
1H and 13C NMR data for vaccinin A (19)a and mururin A(b) in acetone-d6+D2O (δ in ppm, J in Hz)

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<thead>
<tr>
<th>Position</th>
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<th>1H</th>
<th>13C</th>
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<td>4.87 (d, 6.8)</td>
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<tr>
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a Measured at 500 MHz for 1H and 125 MHz for 13C.
b Measured at 400 MHz for 1H and 100 MHz for 13C.
c–f Assignments may be interchanged in each column.
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<th>1H (400 MHz)</th>
<th>13C (100 MHz)</th>
<th>1H (400 MHz)</th>
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*a-e Assignments may be interchanged in each compound.
Fig. 1
Fig. 2
Fig. 3
Fig. 4