Food Chemistry (Research paper)

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

Yosuke Matsuo[a], Yusuke Fujita[a], Sachiko Ohnishi[a], Takashi Tanaka[a,*], Hideaki Hirabaru[b], Takanori Kai[b], Hiroshi Sakaida[b], Shoko Nishizono[c], Isao Kouno[a]

[a] *Department of Natural Product Chemistry, Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo Machi, Nagasaki 852-8521, Japan*

[b] *Research Institute, Unkai Shuzo Co., Ltd., 1800-5 Minamimata, Miyazaki 880-1303, Japan*

[c] *Cooperative Research Center, University of Miyazaki, 1-1 Gakuen Kibanadai-nishi, Miyazaki 889-2192, Japan*

[*] Corresponding author. Fax: +81 95 8192433, E-mail address: t-tanaka@nagasaki-u.ac.jp (T. Tanaka)

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 -20ºC 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_2O (19:1, v/v) at 27ºC 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 δ (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 □μm, GE Healthcare Bio-Science AB, Uppsala), Diaion HP20SS (Mitsubishi Chemical, Japan), MCI gel CHP 20P (75–150 μm; 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 CH_3CN in 50 mM H_3PO_4, 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, Et_2O to give hexane (13.7 g) and Et_2O (11.3 g) soluble fractions. TLC analysis indicated that the hexane soluble fraction mainly contained chlorophyll and waxes and was not examined further. The Et_2O layer was repeatedly chromatographed over Silica gel ($CHCl_3$-MeOH-H_2O, 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 H_2O 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 (H_2O-MeOH) and eluted

with H_2O 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_2O (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_2O-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_2O-MeOH), Chromatorex ODS (H_2O-MeOH), and silica gel ($CHCl_3$-MeOH-H_2O).

The fraction Aq-2 was separated by Diaion HP20SS (H_2O-MeOH). Aq-3 was fractionated by Diaion HP20SS (H_2O-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_2O-MeOH), Chromatorex ODS (H_2O-MeOH), and silica gel ($CHCl_3$-MeOH-H_2O).

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_2O, 9:1); UV (MeOH) λ_{max} nm (log ε): 217 (4.58), 271 (4.02), 314 (4.08), 347 sh (3.98), 374 sh (4.02), 390 (4.07); CD (MeOH) Δε (nm): 0 (224), +133.9 (240), 0 (260), -5.36 (271), 0 (278), +23.0 (295), 0 (326), -11.8 (347); IR (dry film) ν_{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 ^1H 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_2O (320 ml) and EtOH (550 ml), and the mixture was heated at 70ºC 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_2O 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, $[\alpha]_D^{21}$ -85.1º (*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 ^{13}C-NMR data see Table 3.

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

A tan amorphous powder, $[\alpha]_D^{22}$ 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 ^{13}C-NMR data see Table 3.

*Cinchonain IIb 4''-(2-hydroxyethyl) thioether (**16a**)*

A tan amorphous powder, $[\alpha]_D^{28}$ +149.3º (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) ν_{max} cm^{-1}: 3389, 1739, 1613, 1519, 1443; UV (MeOH) λ_{max} nm (log ε): 206 (4.92), 223 sh (4.74), 282 (3.95)); for the ^1H 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 ^1H NMR data with those of authentic samples.

3. Results and discussion

First, the extract was partitioned with *n*-hexane and Et_2O. The hexane layer mainly contained chlorophylls and waxes. From the Et_2O 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 *Brosimum 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 ^1H 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 δ 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 δ 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 CH_2 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). The

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

Allen, F. M. (1927). Blueberry Leaf Extract, *Canadian Medical Association Journal*, *17*, 1539–1540.

Castrejón, A. D. R., Eichholz, I., Rohn, S., Kroh, L. W., & Huyskens-Keil, S. (2008). Phenolic profile and antioxidant activity of highbush blueberry (*Vaccinium corymbosum* L.) during fruit maturation and ripening, *Food Chemistry*, *109*, 564-572.

Chassagne, D., Crouzet, J., Bayonove, C. L., Brillouet, J., & Baumes, R. L. (1996). 6-*O*-α-L-arabinopyranosyl-β-D-glucopyranosides as aroma precursors from passion fruit. *Phytochemistry*, *41*, 1497-1500.

Chen, H. F., Tanaka, T., Nonaka, G., Fujioka, T., & Mihashi, K. (1993). Phenylpropanoid-substituted catechins from *Castanopsis hystrix* and structure revision of cinchonains. *Phytochemistry*, *33*, 183-187.

Dulebohn, R. V., Yi, W., Srivastava, A., Akoh, C., Krewer, G., & Fischer, J, G. (2008). Effects of Blueberry (*Vaccinium ashei*) on DNA Damage, Lipid Peroxidation, and Phase II Enzyme Activities in Rats. *Journal of Agricultural and Food Chemistry*, *56*, 11700-11706.

Ehlenfeldt, M. K., & Prior, R. L. (2001). Oxygen Radical Absorbance Capacity (ORAC) and Phenolic and Anthocyanin Concentrations in Fruit and Leaf Tissues of Highbush Blueberry. *Journal of Agricultural and Food Chemistry*, *49*, 2222-2227.

Ek, S., Kartimo, H., Mattila, S., & Tolonen, A. (2006). Characterization of Phenolic

Compounds from Lingonberry (Vaccinium vitis-idaea). *Journal of Agricultural and Food Chemistry*, *54*, 9834-9842.

Gu, L., Kelm, M., Hammerstone, J. F., Beecher, G., Cunningham, D., Vannozzi, S., & Prior, R. L. (2002). Fractionation of Polymeric Procyanidins from Lowbush Blueberry and Quantification of Procyanidins in Selected Foods with an Optimized Normal-Phase HPLC−MS Fluorescent Detection Method. *Journal of Agricultural and Food Chemistry*, *50*, 4852-4860.

Haslam, E., & Turner, M. J. (1971). Shikimate pathway. I. Conformational analysis of (-)-quinic acid and its derivatives by proton magnetic resonance spectroscopy. *Journal of the Chemical Society C*, 1496-500.

Hsu, F. L., Nonaka, G., & Nishioka, I. (1985). Tannins and related compounds. XXXI. Isolation and characterization of proanthocyanidins in *Kandelia candel* (L.) Druce *Chemical & Pharmaceutical Bulletin*, *33*, 3142-3152.

Jepson, R. G., & Craig, J, C. (2007). A systematic review of the evidence for cranberries and blueberries in UTI prevention. *Molecular Nutrition & Food Research*, *51*, 738-745.

Khan, M. L., Halson, E., & Williamson, M. P., Structure and conformation of the procyanidin B-2 dimer. *Magnetic Resonance in Chemistry*, *35*, 854-858 (1997)

Kitajima, J., Ishikawa, T., Tanaka, Y., Ono, M., Ito, Y., & Nohara, T. (1998). Water-Soluble Constituents of Fennel. V. Glycosides of Aromatic Compounds, *Chemical & Pharmaceutical Bulletin*, *46*, 1587-1590.

Li, H., Nakashima, T., Tanaka, T., Zhang, Y.-J., Yang, C.-R., & Kouno, I. (2008). Two new maltol glycosides and cyanogenic glycosides from *Elsholtzia rugulosa* Hemsl. *Journal of Natural Medicine*, *62*, 75-78.

López-Tamames, E., Carro-Mariño, N., Gunata, Y. Z., Sapis, C., Baumes, R., & Bayonove C. (1997). Potential Aroma in Several Varieties of Spanish Grapes. *Journal of Agricultural and Food Chemistry*, *45*, 1729-1735.

Miyase, T., Ueno, A., Takizawa, N., Kobayashi, H., & Karasawa, H. (1987). Studies on the glycosides of *Epimedium grandiflorum* Morr. var. *thunbergianum* (Miq.) Nakai. I. *Chemical & Pharmaceutical Bulletin*, *36*, 1109-1117.

Miyase, T., Ueno, A., Takizawa, N., Kobayashi, H., & Oguchi, H. (1988). Studies on the glycosides of *Epimedium grandiflorum* Morr. var. *thunbergianum* (Miq.) Nakai. III. *Chemical & Pharmaceutical Bulletin*, *36*, 2475-2484.

Morimoto S., Nonaka G., & Nishioka I., Tannins and related compounds. XXXV. Proanthocyanidins with a doubly linked unit from the root bark of *Cinnamomum sieboldii* Meisner. *Chemical & Pharmaceutical Bulletin*, *33*, 4338-4345 (1985)

Murakami, N., Morimoto, T., Imanuma, H., Ueda, T., Nagai, S., Sakakibara, J., & Yamada, N. (1991). Studies on Glycolipids. III. Glyceroglycolipids from an Axenically Cultured Cyanobacterium, *Phormidium tenue*, *Chemical & Pharmaceutical Bulletin*, *39*, 2277-2281.

Naczk, M., Grant, S., Zadernowski, R., & Barre, E. (2006). Protein precipitating capacity of phenolics of wild blueberry leaves and fruits, *Food Chemistry*, *96*, 640-647.

Nagao, K., Higa, K., Shirouchi, B., Nomura, S., Inoue, N., Inafuku, M., & Yanagita, T. (2008). Effect of Vaccinium ashei reade leaves on lipid metabolism in Otsuka Long-Evans Tokushima Fatty rats. *Bioscience, Biotechnology, and Biochemistry*, *72*, 1619-1622.

Neto, C. C. (2007). Cranberry and blueberry: Evidence for protective effects against cancer and vascular diseases. *Molecular Nutrition & Food Research*, *51*, 652-664.

Nonaka G., Nishioka I., Nagasawa T., & Oura H. (1981). Tannins and related compounds. I. Rhubarb (1). *Chemcal & Pharmaceutical Bulletin*, *29*, 2862-2870.

Nonaka G., Kawahara O., & Nishioka I. (1982). Tannins and related compounds. VIII. A new type of proanthocyanidin, cinchonains IIa and IIb from *Cinchona succirubra*. (2). *Chemcal & Pharmaceutical Bulletin*, *30*, 4277-4282.

Nonaka G., Morimoto S., & Nishioka I. (1983). Tannins and related compounds. Part 13. Isolation and structures of trimeric, tetrameric, and pentameric proanthocyanidins from cinnamon. *Journal of the Chemical Society, Perkin Transactions 1*, 2139-2145

Price, K. R., Colquhoun, I. J., Barnes, K. A., & Rhodes, M. J. C. (1998). Composition and Content of Flavonol Glycosides in Green Beans and Their Fate during Processing. *Journal of Agricultural and Food Chemistry*, *46*, 4898-4903.

Prior, R. L., Lazarus, S. A., Cao, G., Muccitelli, H., & Hammerstone, J. F. (2001). Identification of procyanidins and anthocyanins in blueberries and cranberries (Vaccinium Spp.) using high-performance liquid chromatography/mass spectrometry. *Journal of Agricultural and Food Chemistry*, *49*, 1270-1276.

Reyes, A., Muñoz, M., Garcia, H., & Cox, C. (1986). Chemistry of Myzodendraceae, I. Myzodendrone, a new phenylbutanone of *Myzodendron punctulatum*. *Journal of Natural Products*, *49*, 318-320, 1986

Sakaida, H., Nagao, K., Higa, K., Shirouchi, B., Inoue, N., Hidaka, F., Kai, T., & Yanagita, T. (2007). Effect of Vaccinium ashei reade leaves on angiotensin converting enzyme activity in vitro and on systolic blood pressure of spontaneously hypertensive rats in vivo. *Bioscience, Biotechnology, and Biochemistry*, *71*, 2335-2337.

Seeram, N. P. (2008) Berry Fruits for Cancer Prevention: Current Status and Future Prospects. *Journal of Agricultural and Food Chemistry*, *56*, 630-635.

Sekine, T., Arita, J., Yamaguchi, A., Saito, K., Okonogi, S., Morisaki, N., Iwasaki, S., & Murakoshi, I. (1991). Two flavonol glycosides from seeds of *Camellia sinensis*. *Phytochemistry*, *30*, 991-995.

Skupień, K., Oszmiański, J., Kostrzewa-Nowak, D., & Tarasiuk, J. (2006). In vitro antileukaemic activity of extracts from berry plant leaves against sensitive and multidrug resistant HL60 cells, *Cancer Letters*, *236*, 282-291.

Sugiyama, M., & Kikuchi, M. (1992). Studies on the Constituents of Osmanthus Species. X. Structures of Phenolic Glucosides from the Leaves of *Osmanthus asiaticus* Nakai. *Chemical & Pharmaceutical Bulletin*, *40*, 325-326.

Takashima, J., Asano, S., & Ohsaki, A. (2002). Mururins A-C, three new lignoids from Brosimum acutifolium and their protein kinase inhibitory activity. *Planta Medica*, *68*, 621-625.

Tanaka, T., Takahashi, R., Kouno, I., & Nonaka, G. (1994). Chemical evidence for the de-astringency (insolubilization of tannins) of persimmon fruit. *Journal of the Chemical Society, Perkin Transactions. 1*, 3013-3022.

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

epigallocatechin in banana fruits. *Phytochemistry, 53*, 311-316.

Watson, E. M. (1928). Some Observations on the Effect of Blueberry Leaf Extract in Diabetes Mellitus, *Canadian Medical Association Journal*, *19*, 166–171.

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
Compunds isolated from the Et_2O layer and aqueous layer of the leaves of Rabbiteye blueberry

fractions	compounds	Isolation yeld (%)[a]	references
Et_2O layer			
	p-hydroxybenzoic acid	0.002	[d]
	1,2-dilinolenyl-3-*O*-(α-D-galactopyranosyl-(1→6)-*O*-β-D-galactopyranosyl)-glycerol	0.006	Murakami, Morimoto, Imanuma, Ueda, Nagai, Sakakibara, & Yamada, 1991
	2-linolenyl-1-stearyl-3-*O*-(α-D-galactopyranosyl-(1→6)-*O*-β-D- galactopyranosyl)-glycerol	0.041	Murakami, et al., 1991
Aqueous layer			
Aq. 1-1	quinic acid[b]	6.16	Haslam, & Turner, 1971
	sucrose[c]	3.30	[d]
Aq. 1-2-1	maltol β–D-glucoside	0.003	Li, Nakashima, Tanaka, Zhang, Yang, Kouno, 2008
	benzylalcohol *O*-α-L-arabinofuranosyl-(1→6)-*O*- β-D-glucopyranoside	0.006	López-Tamames, Carro-Mariño, Gunata, Sapis, Baumes, & Bayonove, 1997
	benzylalcohol *O*-α-L-arabinopyranosyl-β-D-glucopyranoside	0.011	Chassagne, Crouzet, Bayonove, Brillouet, & Baumes, 1996
	2-(3',4'-dihydroxyphenyl)-ethanol 3'-*O*-β-D-glucopyranoside	0.009	Sugiyama, & Kikuchi, 1992
	4-hydroxy-3,5-dimethoxybenzyl alcohol 4-*O*-β-D-glucopyranoside	0.004	Kitajima, Ishikawa, Tanaka, Ono, Ito, Nohara, 1998
	4-(3',4'-dihydroxyphenyl)-butan-2-one 3'-*O*-β-D-glucoside	0.006	Reyes, Muñoz, Garcia, & Cox, 1986
	blumenol B β-D-glucopyranoside	0.003	Miyase, Ueno, Takizawa, Kobayashi, & Oguchi, 1988
	icariside B1	0.012	Miyase, Ueno, Takizawa, Kobayashi, & Karasawa, 1987
Aq. 1-3	icariside B6	0.017	Miyase et al., 1988
	quercetin 3-*O*-β-D-xylosyl-(1→2)-α-L-rhamnosyl-(1→6)-β-D-glucoside	0.004	Price, Colquhoun, Barnes, & Rhodes, 1998
	camelliaside B (kaempferol 3-*O*-β-D-xylosyl-(1→2)-α-L-rhamnosyl-(1→6)-β-D-glucoside)	0.007	Sekine, Arita, Yamaguchi, Saito, Okonogi, Morisaki, Iwasaki, & Murakoshi, 19
Aq. 2	rutin (quercetin 3-*O*-α-L-rhamnosyl-(1→6)-β-D-glucoside) (**2**)	0.92	[d]
	chlorogenic acid (**1**)	2.28	[d]
Aq. 3-1	quercetin 3-*O*-α-L-rhamnoside	0.022	Price et al., 1998
	quercetin 3-*O*-β-D-glucuronide	0.060	Price et al., 1998
	quercetin 3-*O*-β-D-glucoside	0.020	[d]
	quercetin 3-*O*-[4''-(3-hydroxy-3-methylglutaroyl)]-α-L-rhamnoside	0.005	Ek, Kartimo, Mattila, & Tolonen, 2006
	kaempferol 3-*O*-α-L-rhamnosyl-(1→6)-β-D-glucoside	0.001	[d]
	3,5-di-*O*-caffeoyl quinic acid	0.107	[d]
	4,5-di-*O*-caffeoyl quinic acid	0.013	[d]
	3,5-di-*O*-caffeoyl quinic acid methyl ester	0.001	
	(+)-catechin (**3**)	0.015	[d]
	(-)-epicatechin (**4**)	0.005	[d]
	procyanidin B-1 (**5**)	0.008	Nonaka, Nishioka, Nagasawa, & Oura, 1981
	procyanidin B-2 (**6**)	0.006	Khan, Halson, Williamson, 1997
	procyanidin C-1 (**7**)	0.002	Nonaka G., Kawahara O., & Nishioka I., 1982
	epicatechin-(4β→8, 2β→7)-epicatechin- (4α→8)-catechin (**8**)	0.008	Morimoto, Nonaka, Nishioka, 1985
	epicatechin-(4β→8, 2β→7)-epicatechin- (4α→8)-epicatechin (**9**)	0.010	Nonaka, Morimoto, & Nishioka, 1983
	[2*R*-(2α,3β,10β)]-2,10-bis(3,4-dihydroxyphenyl)-3,4,9,10-tetrahydro-3,5-dihydroxy-2H,8H-benzo[1,2-b:3,4-b']dipyran-8-one (**10**)	0.003	Chen, Tanaka, Nonaka, Fujioka, & Mihashi, 1993
	cinchonain Ia (**11**)	0.022	Chen, et al., 1993
	cinchonain Ib (**12**)	0.011	Chen, et al., 1993
	kandelin A-1 (**13**)	0.003	Hsu, Nonaka, & Nishioka, 1985; Chen et al., 1993
	kandelin A-2 (**14**)	0.004	Hsu, et al., 1985; Chen et al., 1993
	cinchonain IIa (**15**)	0.015	Nonaka et al., 1982; Chen et al., 1993
	cinchonain IIb (**16**)	0.021	Nonaka et al., 1982; Chen et al., 1993
	mururin A (**17**)	0.017	Takashima, Asano, & Ohsaki, 2002
	mururin B (**18**)	0.001	Takashima, et al., 2002
	vaccinin A (**19**)	0.001	
Aq. 1-2-2, Aq. 1-3-2, Aq. 3-3	oligomeric proanthocyanidins	11.34	

[a] From freeze dried leaves (500g).
[b] Isolated as tetraacetate after acetylation with Ac_2O and pyridine.
[b] Isolated as octaacetate after acetylation with Ac_2O and pyridine.
[d] Identified by comparison of the ^1H and ^{13}C NMR data with those of authentic samples.

2

Table 2

^{1}H and ^{13}C NMR data for vaccinin A (**19**)[a] and mururin A(**17**)[b] in acetone-d_6+D_2O (δ in ppm, *J* in Hz)

Position	**19**		**17**	
	^{1}H	^{13}C	^{1}H	^{13}C
2	4.89 (d, 6.9)	83.0	4.87 (d, 6.8)	83.0
3	4.21 (m)	66.5	4.18 (m)	66.5
4	2.81 (dd, 16.3, 7.8)	26.9	2.75 (dd, 16.4, 7.8)	26.9
	3.03 (dd, 16.3, 5.2)		2.96 (dd, 16.4, 4.9)	
4a		104.2		104.6 [c]
5		149.0		153.0
6		100.7		100.9
7		153.6		150.0
8	6.51 (s)	98.3	6.48 (s)	98.2
8a		158.9		159.2
1'		130.7		130.7
2'	6.89 (d, 1.6)	114.7	6.87 (d, 2.0)	115.0 [d]
3'		145.7		145.9 [e]
4'		145.9		145.8 [e]
5'	6.80 (d, 8.0)	115.7	6.78 (d, 8.3)	116.0 [d]
6'	6.73 (dd, 8.0, 1.6)	119.3	6.71 (dd, 8.3, 2.0)	119.5
1"		107.5		107.5
2"		152.7		152.7
3"	6.86 (s)	104.2	6.72 (s)	104.1 [c]
4"		147.9		148.1
5"		144.5		144.4 [f]
6"	7.35 (s)	108.9	7.27 (s)	109.1
7"		142.6		143.2 [f]
8"	6.03 (s)	93.1	6.01 (s)	92.7
9"		162.7		163.5

[a] Measured at 500 MHz for ^{1}H and 125 MHz for ^{13}C.

[b] Measured at 400 MHz for ^{1}H and 100 MHz for ^{13}C.

[c – f] Assignments may be interchanged in each column.

3

4

Table 3
^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for thioethers **11a**, **12a** and **16a** in acetone-d_6+D_2O (δ in ppm, *J* in Hz)

Position	**11a**		**12a**		**16a**					
	^{1}H	^{13}C	^{1}H	^{13}C	Position	^{1}H	^{13}C	Position	^{1}H	^{13}C
2	5.22 (br s)	75.3	5.3 (br s)	75.6	2	5.57 (s)	75.4 [a]	2"	4.76 (s)	76.5 [a]
3	4.12 (br s)	71.1	4.11 (br s)	71.2	3	3.99 (br s)	71.7 [b]	3"	3.68 (br s)	72.1 [b]
4	4.14 (d, 2.0)	43.2	4.14 (d, 2.0)	43.2	4	4.64 (br s)	36.6 [c]	4"	4.02 (d, 2.0)	44.1
4a		104.9		104.9	4a		105.1	4a"		99.9
5		157.5		157.3	5		156.4 [d]	5"		156.4 [d]
6	6.26 (s)	96.6	6.25 (s)	96.7	6	6.21 (s)	94.9	6"	5.98 (s)	96.7
7		152.8 [a]		152.7 [a]	7		155.7 [d]	7"		155.3 [d]
8		105.5		105.7	8		108.1 [e]	8"		108.2 [e]
8a		152.6 [a]		152.6 [a]	8a		153.2 [d]	8a"		150.5 [d]
1'		131.3		131.1	1'		132.3 [f]	1'''		131.8 [f]
2'	7.06 (d, 2.0)	114.8	6.89 (d, 2.0)	114.7 [b]	2'	6.62-6.89 (m)	115.8 [h]	2'''	6.62-6.89 (m)	115.5 [h]
3'		145.5 [b]		144.6 [c]	3'		145.5 [g]	3'''		145.1 [g]
4'		145.2 [b]		145.2 [c]	4'		145.2 [g]	4'''		145.0 [g]
5'	6.79 (d, 7.9)	115.6	6.68-6.74 (m)	115.5 [d]	5'	6.62-6.89 (m)	115.2 [h]	5'''	6.62-6.89 (m)	115.2 [h]
6'	6.82 (dd, 2.0, 7.9)	118.7	6.68-6.74 (m)	118.9 [e]	6'	6.62-6.89 (m)	118.9 [i]	6'''	6.62-6.89 (m)	118.9 [i]
1"		134.8		134.7	1''''		135.3			
2"	6.57 (d, 2.0)	114.6	6.67 (d. 2.0)	114.9 [b]	2''''	6.62-6.89 (m)	115.1 [h]			
3"		145.8		145.4 [c]	3''''		144.8 [g]			
4"		144.6		145.7 [c]	4''''		144.3 [g]			
5"	6.63 (d, 7.8)	116.0	6.68-6.74 (m)	116.0 [d]	5''''	6.62-6.89 (m)	114.9 [h]			
6"	6.41 (dd, 2.0, 7.8)	118.7	6.51 (dd, 2.0, 7.8)	119.1 [e]	6''''	6.48 (dd, 2.0, 7.8)	119.2 [i]			
α	2.85 (dd, 2.0, 15.6)	38.0	2.87 (dd, 2.0, 15.6)	37.6	α	1.95 (dd, 6.6, 15.6)	36.9 [c]			
	3.09 (dd, 6.8, 15.6)		3.07 (dd, 6.8, 15.6)			2.58 (br d, 15.6)				
β	4.56 (br d, 6.8)	34.6	4.43 (br d, 6.8)	34.5	β	4.18 (br d, 6.6)	34.3			
COO		168.5		168.4	COO		169.0			
CH_2S-	2.80 (m)	35.4	2.81 (m)	35.5	CH_2S-	2.63 (m)	34.8			
	2.97 (m)		2.97 (m)			2.82 (m)				
CH_2OH	3.76 (m)	62.7	3.79 (m)	62.7	CH_2OH	3.73 (m)	62.5			
	3.89 (m)		3.93 (m)			3.93 (m)				

[a - e] Assignments may be interchanged in each compound.

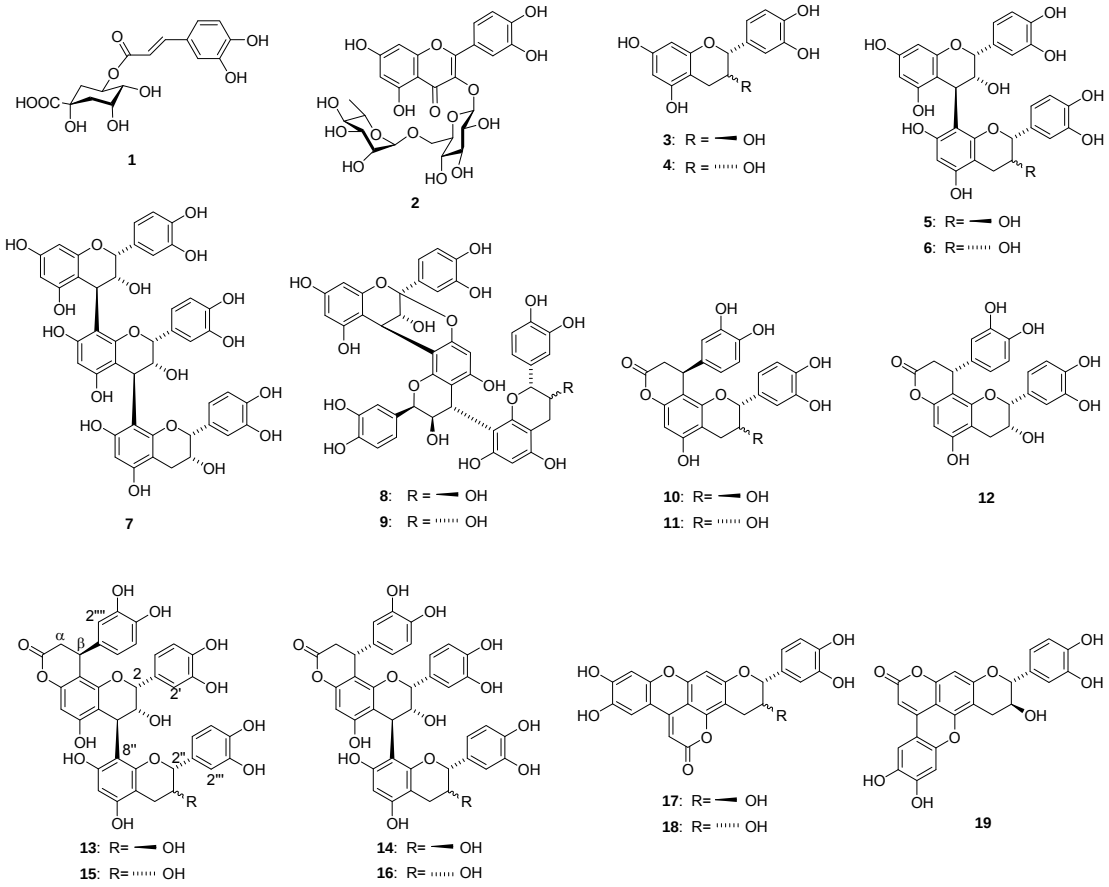

1
2
3: R = OH
4: R = OH
5: R= OH
6: R= OH
7
8: R = OH
9: R = OH
10: R= OH
11: R= OH
12
13: R= OH
15: R= OH
14: R= OH
16: R= OH
17: R= OH
18: R= OH
19

Fig. 1

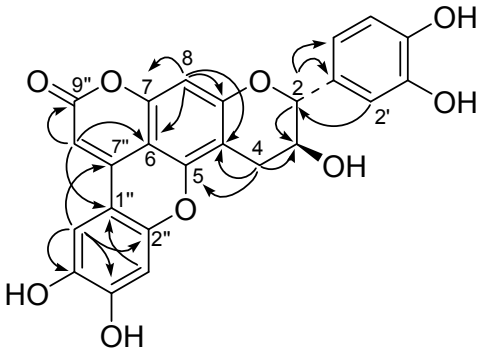


Fig. 2

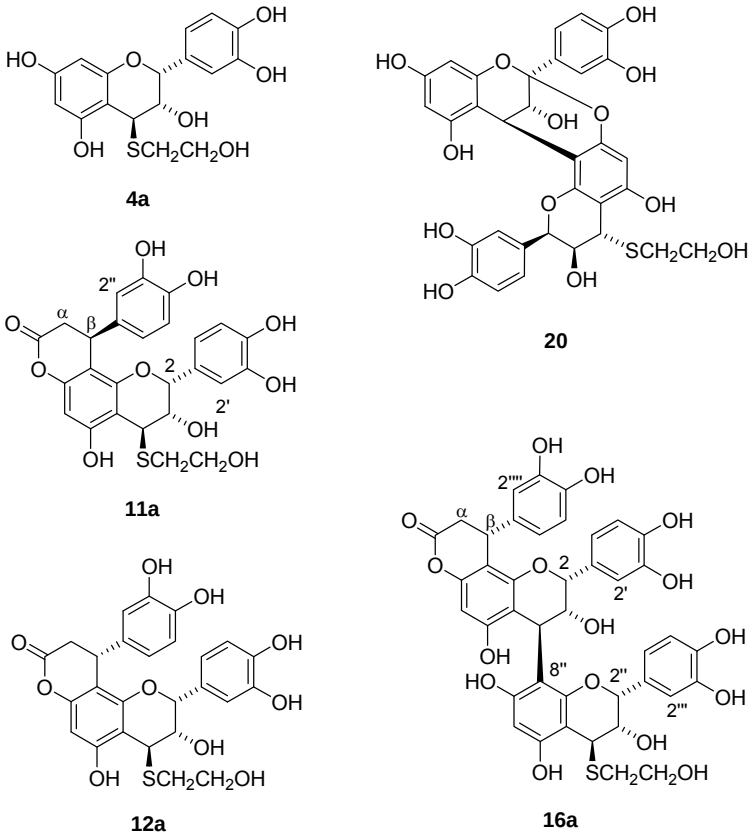
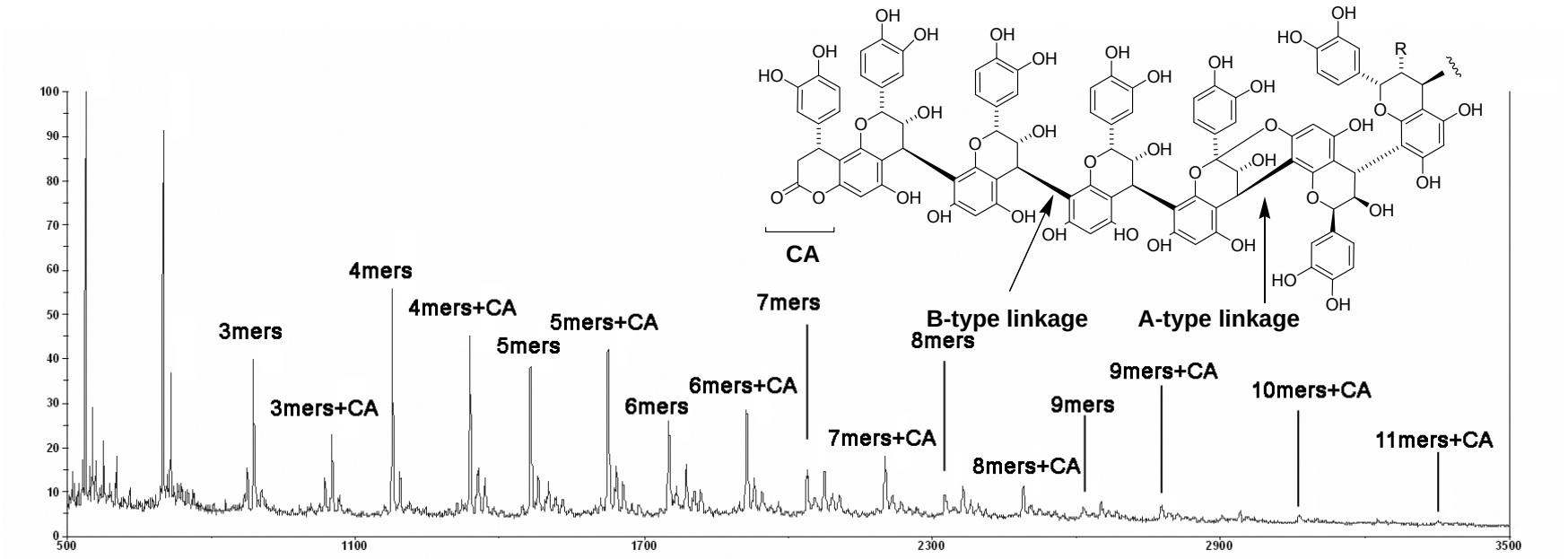


Fig. 3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4mers
4mers+CA
5mers
5mers+CA
6mers
6mers+CA
7mers
7mers+CA
8mers
8mers+CA
9mers
9mers+CA
10mers+CA
11mers+CA
CA
B-type linkage
A-type linkage
100
90
80
70
60
50
40
30
20
10
0
500
1100
1700
2300
2900
3500

Fig. 4