JOURNAL OF NATURAL PRODUCTS

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α-Amylase and Lipase Inhibitory Activity and Structural Characterization of Acacia Bark Proanthocyanidins

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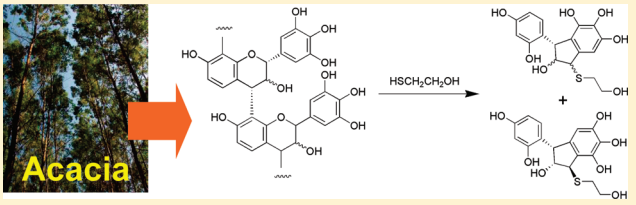
Supporting Information

ABSTRACT: The bark extract of *Acacia mearnsii* showed strong lipase and α-amylase inhibition activities. Fractionation of the extract by column chromatography and subsequent ^{13}C NMR and MALDI-TOF-MS analysis revealed that the active substances are proanthocyanidin oligomers mainly composed of 5-deoxyflavan-3-ol units. In addition, 4′-*O*-methylrobinetinidol 3′-*O*-β-D-glucopyranoside, fisetinidol-(4α,6)-gallocatechin, and epirobinetinidol-(4β,8)-catechin were isolated as new compounds, and their structures were determined from spectroscopic data. Furthermore, a modified thiol degradation method using strongly acidic conditions was applied to the extract to yield three thiol degradation products derived from robinetinidol units. This method is useful for characterizing acacia proanthocyanidins (wattle tannins).



Acacia mearnsii of the family Fabaceae, commonly called "black wattle", is a tree native to Australia. The bark is rich in condensed tannins (syn. proanthocyanidins) known as "wattle tannins",[1−4] which are industrially used for particleboard adhesives and leather tanning. Proanthocyanidins are known to have a variety of health benefits, including antitumor effects,[5] hairgrowth promotion,[6] antihypertension,[7] and antiallergic properties.[8] Antioxidative activity[9] and tyrosinase inhibitory activity[10] have been reported for acacia proanthocyanidins. Recently, antidiabetes and antiobesity activities of acacia bark extract have been demonstrated in mouse models.[11] Since proanthocyanidins constitute 68 wt % of bark extract, acacia proanthocyanidins are a readily available and promising food additive with health benefits. Gastrointestinal absorption of monomeric flavan-3-ols and proanthocyanidin dimers, such as catechin and procyanidin B-2, has also been reported;[12] however, the absorption of proanthocyanidin oligomers is known to be very low.[13] Therefore, the *in vivo* biological activities of proanthocyanidin oligomers, such as suppression of sugar and lipid uptake, are believed to be mainly attributable to the inhibition of digestive enzymes in the gastrointestinal tract.[14−16] In this study, we evaluated the effect of acacia proanthocyanidins on two digestive enzymes, α-amylase and lipase, and then characterized the active fractions of the bark extract using spectroscopic and chemical methods. It is known that the acacia proanthocyanidin oligomers are mainly composed of 5-deoxyflavan-3-ol units, such as robinetinidol (**1**) and fisetinidol (**2**) (Figure 1),[17] and their chemical properties are different from those of many other proanthocyanidins. The interflavan linkages of proanthocyanidins composed of 5-hydroxyflavan-3-ol units are readily cleaved by acid-catalyzed thiolysis.[18,19] This reaction is commonly used for their structural characterization. In contrast, the acacia proanthocyanidins are resistant to thiolysis, making the chemical characterization of these oligomers difficult. This paper presents a modified thiolysis method that can discriminate acacia proanthocyanidin oligomers from other proanthocyanidins.

RESULTS AND DISCUSSION

Measurement of α-Amylase and Lipase Inhibitory Effects. Acacia bark extract showed strong α-amylase inhibition activity (73.7 ± 3.8% inhibition at 250 μg/mL), which is comparable to that of black tea (68.5 ± 4.6%) and much stronger than that of green tea (21.0 ± 3.7%), oolong tea (10.9 ± 2.7%), or guava leaf (32.4 ± 9.5%) extracts. To identify the active substance, the acacia bark extract was separated into three major fractions by column chromatography over Diaion HP20SS resin, and inhibition activities (IC_{50} values) of the fractions were compared (Table 1). Adjusting for yields, the second fraction (Fr. 2) had the highest activity (86.0% at 250 μg/mL). Fraction 2 was further separated into four fractions by size-exclusion column chromatography using Sephadex LH-20 eluted with aqueous acetone containing a high concentration of urea.[20] Although three fractions (212, 221, and 222) showed strong activities (92.8, 89.2, and 65.8% inhibition, respectively, at 250 μg/mL), fraction 221 was obtained with the highest yield and was therefore further fractionated by Sephadex LH-20 column chromatography to afford the most active fraction, 2217. The α-amylase inhibition activity of fraction 2217 was stronger than that of robinetinidol

Received: June 3, 2010
Published: December 30, 2010

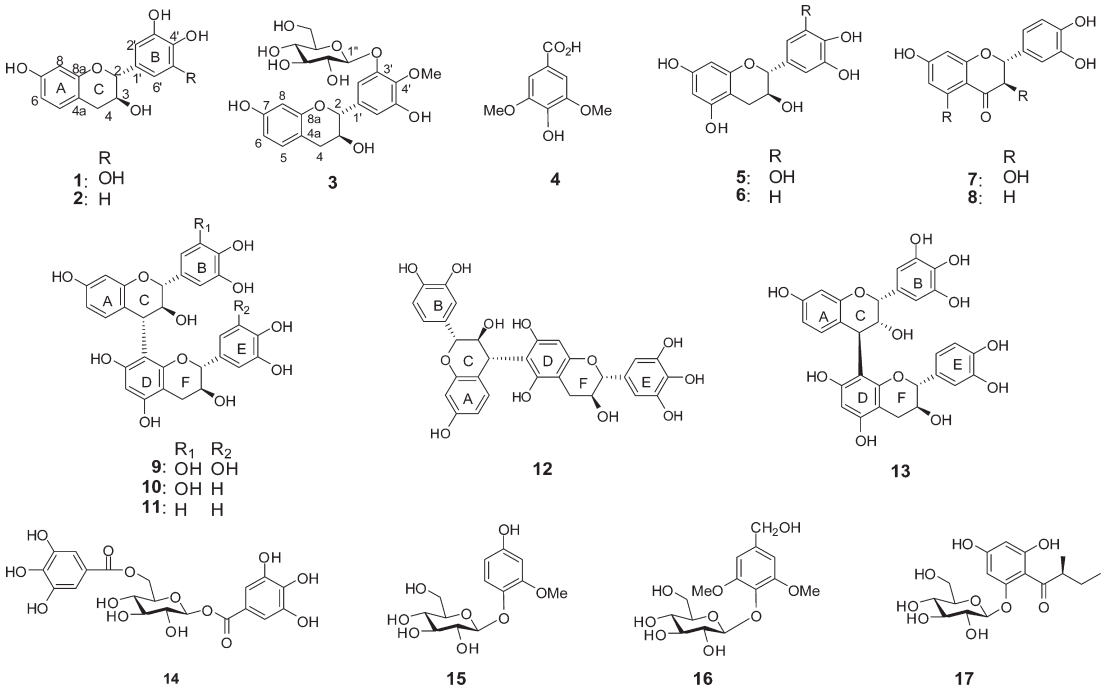


Figure 1. Structures of compounds **1**−**17**.

Table 1. α-Amylase Inhibitory Activity and Yield of Fractions

Fr. no.	IC_{50} (μg/mL)	yield (%)[a]
1	>500	20.6
2	68.4	70.4
3	80.1	1.8
211	>500	0.6
212	60.9	16.1
221	60.8	44.4
222	83.7	7.5
2211	>500	0.6
2212	>500	0.9
2213	>500	1.8
2214	335.0	4.1
2215	58.8	7.1
2216	52.6	8.8
2217	38.0	15.9
robinetinidol (**1**)	137.5	
robinetinidol-(4α,8)-catechin (**10**)	368.5	

[a] Yield calculated from the original extract.

Table 2. Lipase Inhibitory Activity

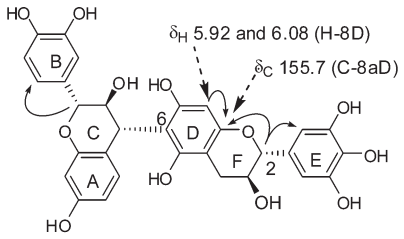
Fr. no.	inhibition (%)[a]
1	0.3 ± 0.7
2	79.6 ± 11.5
3	69.3 ± 8.7
211	22.7 ± 7.3
212	71.3 ± 8.9
221	87.6 ± 7.9
222	86.4 ± 9.1
2211	28.4 ± 0.8
2212	64.1 ± 4.0
2213	58.7 ± 8.2
2214	69.1 ± 6.4
2215	75.1 ± 6.0
2216	87.9 ± 6.4
2217	82.8 ± 1.8

[a] At 80 μg/mL.

and robinetinidol-(4β,8)-catechin,[1−4] which are known acacia polyphenols (Table 1). The activity was also compared to the activities of other α-amylase inhibitors. The activity of fraction 2217 (92.4 ± 1.9% inhibition at a concentration of 250 μg/mL) was comparable to that of epigallocatechin 3-*O*-gallate (77.7 ± 6.5%) from green tea[15] and theaflavin 3,3′-di-*O*-gallate (88.7 ± 2.5%) from black tea[15] and stronger than the activities of sanguiin H-6 (65.3 ± 5.4%) from Chinese sweet tea[21] and raspberry.[14] α-Amylase inhibition activities of the acacia proanthocyanidin fractions roughly paralleled their inhibition effects on lipase (Table 2). Fractions 212, 221, and 223 showed high lipase inhibition activity (71.3, 87.6, and 86.4% inhibition, respectively, at 80 μg/mL), and subfractions 2215, 2216, and 2217 obtained from fraction 221 exhibited strong inhibitory effects (75.1, 87.9, and 82.8%, respectively, at 80 μg/mL). Adjusting for yield, fraction 2217 exhibited the highest lipase inhibition activity. Fraction 2217 was qualitatively characterized as containing proanthocyanidins by its characteristic coloration following the addition of $FeCl_3$ (dark blue) and vanillin-HCl (red). HPLC analysis of the fraction provided two broad humps; there were no sharp peaks arising from flavan-3-ols or proanthocyanidin dimers (see Supporting Information), suggesting that this fraction is a complex mixture of proanthocyanidin oligomers with relatively high molecular weights.

Identification of Low Molecular Weight Constituents. The extract was chromatographically separated on several different

dx.doi.org/10.1021/np100372t |*J. Nat. Prod.* 2011, 74, 119–128



12

Figure 2. Selected HMBC correlations for compound **12**.

columns to identify the components. Sixteen compounds, including a new flavan-3-ol glycoside (**3**) (Figure 2) and two new proanthocyanidin dimers (**12** and **13**), were isolated. The known compounds were identified as robinetinidol (**1**),[22] syringic acid (**4**),[23] gallocatechin (**5**),[24] catechin (**6**),[24] taxifolin (**7**),[25] butin (**8**),[26] robinetinidol-(4α,8)-gallocatechin (**9**),[27] robinetinidol-(4α,8)-catechin (**10**),[27] fisetinidol-(4α,8)-catechin (**11**),[27] 1,6-di-*O*-galloyl-β-D-glucose (**14**),[28] 4-hydroxy-2-methoxyphenyl 1-*O*-β-D-glucopyranoside (**15**),[29] 3,5-dimethoxy-4-hydroxybenzyl alcohol 4-*O*-β-D-glucopyranoside (**16**),[30] and multifidol glucoside (**17**)[31,32] (Figure 1). The identifications were made by comparing the ^{1}H and ^{13}C NMR spectra of the isolated compounds with those of authentic samples or with literature data. Fisetinidol (**2**), a constituent unit of profisetinidins, was not isolated in this experiment.

Compound **3** showed an $[M]^+$ peak at *m/z* 466.1471 in HRFABMS analysis, confirming its molecular formula to be $C_{22}H_{26}O_{11}$. The ^{1}H NMR spectrum was related to that of robinetinidol (**1**) and showed ABX-type signals at δ 6.29 (1H, d, *J* = 2.4 Hz), 6.35 (1H, dd, *J* = 2.4, 8.3 Hz), and 6.86 (1H, d, *J* = 8.3 Hz), attributable to the aromatic protons of a resorcinol-type A-ring. In addition, signals of methylene protons at δ 2.67 (1H, d, *J* = 7.8, 15.6 Hz) and 2.84 (1H, d, *J* = 4.9, 15.6 Hz) and two oxygenated methine protons at δ 4.03 (1H, m) and 4.69 (1H, d, *J* = 6.6 Hz) indicated that **3** is a 5-deoxyflavan-3-ol with a 2,3-*trans* configuration. The appearance of mutually *m*-coupled aromatic protons at δ 6.59 and 6.68 (each d, *J* = 1.8 Hz) suggested that the B-ring is an asymmetric pyrogallol ring. In addition to these signals, one methoxy proton signal at δ 3.83 (3H, s) and a sugar anomeric proton signal at δ 4.91 (1H, d, *J* = 7.5 Hz), accompanied by oxygenated methine proton signals in the range δ 3.35−3.75, indicated the presence of a methoxy group and a sugar moiety. The sugar was determined to be β-D-glucose on the basis of its ^{13}C NMR chemical shifts[33] and the results of acid hydrolysis.[34] The glucose and methoxy groups were located at C-3′ and C-4′ of the B-ring, respectively, on the basis of the HMBC correlations of the methoxy protons with C-4′ (δ 137.3), and the anomeric proton with C-3′ (δ 151.4) (see Supporting Information). As for absolute configuration, the circular dichroism (CD) spectrum of compound **3** showed a negative Cotton effect (CE) at 284 nm, indicating a 2*R* configuration.[35] Thus, compound **3** was established to be 4′-*O*-methylrobinetinidol 3′-*O*-β-D-glucopyranoside (Figure 1).

HRFABMS analysis of compound **12** provided an $[M + Na]^+$ peak at *m/z* 601, consistent with the molecular formula $C_{30}H_{26}O_{12}$ and indicating that **12** is an isomer of **10**. The ^{1}H and ^{13}C NMR spectra of **12** showed duplicated signals arising from two conformational isomers of a proanthocyanidin dimer created by rotational hindrance at the interflavan linkage. The two rotamers exist in equilibrium, as evidenced by strong NOESY exchange peaks between equivalent protons of each rotameric pair.[36] The large coupling constants ($J_{2C,3C} = J_{3C,4C}$ = 9.5 Hz, $J_{2F,3F}$ = 6.8 Hz) of the C and F rings indicated 2,3- and 3,4-*trans* configuration of the C-ring and a 2,3-*trans* configuration of the F-ring; this was supported by the ^{13}C NMR chemical shifts.[27] The HMBC correlations of H-2 (C) with C-6 (B) and H-2 (F) with C-2 (E) and C-6 (E) indicated that this compound is composed of fisetinidol and gallocatechin units (see Supporting Information). In the HMBC spectrum, the carbon at δ 155.7 [C-8a (D)] was correlated with aromatic singlet signals of the D-ring (δ 5.92 and 6.08) and H-2 (F); therefore, the carbon signal was assigned to C-8a (D). This observation indicated that the aromatic proton is located at C-8 (D), and thus the gallocatechin unit is attached at C-6 of the fisetinidol unit (Figure 2). In addition, compound **12** showed a negative CE at 236 nm, indicating an α-orientation of the interflavan linkage.[37] Therefore, **12** was determined to be fisetinidol-(4α,6)-gallocatechin.

HRFABMS analysis showed the molecular formula of **13** to be $C_{30}H_{26}O_{12}$, indicating that **13** is an isomer of **12**. The ^{1}H NMR spectrum measured at room temperature exhibited broad peaks due to rotational hindrance, as observed for **12**. However, the spectrum measured at −20 °C showed sharp peaks arising from two flavan-3-ol units (see Supporting Information). The terminal unit was shown to be catechin, based on the C-ring coupling constant of H-2 (F) (5.1 Hz) and the appearance of a long-range ^{1}H−^{1}H correlation between H-2 (F) and H-2 (E) in the ^{1}H−^{1}H COSY spectrum. Long-range ^{1}H−^{1}H coupling was also observed between H-5 (A) and H-4 (C) protons. The HMBC spectrum showed correlations of C-8a (D) (δ 155.4) with H-4 (C) of the extension unit, as well as correlations with H-2 (F) and H-4 (F). These observations revealed the C-4−C-8 linkage between two flavan units in **13**. The configuration of the C-ring was deduced to be 2,3-*cis* and 3,4-*trans* on the basis of comparison of the coupling constants of the C-ring protons ($J_{2,3} = J_{3,4}$ = 2.5 Hz) with those of epifisetinidol-(4β,8)-catechin ($J_{2,3}$ = 2.5 Hz, $J_{3,4}$ = 3.5 Hz).[37] This was confirmed by the NOESY correlations between H-2 (C) and H-3 (C) and between H-4 (C) and B-ring H-2, H-6. In addition, the CD spectrum of compound **13** showed a positive CE at 239 nm, being consistent with the β-orientation of the interflavan linkage.[37] Therefore, **13** was concluded to be epirobinetinidol-(4β,8)-catechin (**13**).

Spectroscopic Analysis of the Active Fraction. Fraction 2217, which exhibited strong enzyme inhibition activities, was examined by ^{13}C NMR spectroscopy and MALDI-TOF-MS. The ^{13}C NMR spectrum of the fraction was compared with that of **10** (Figure 3).[27,38] The appearance of signals attributable to resorcinol-type A-ring, pyrogallol-type B-ring, catechol-type E-ring, and hydroxypyran C- and F- rings indicated that fraction 2217 contains a mixture of oligomers composed of robinetinidol (major) and fisetinidol (minor) units. The chemical shifts of C-2 (C), C-2 (F), C-3 (C), C-3 (F), and C-4 (C) were similar to those of **10** and **12**, indicating that the configuration of the C-ring is mainly 2,3-*trans* and 3,4-*trans*. No signals corresponding to D-6 and F-4a of the phloroglucinol-type aromatic ring of **10** were detected, suggesting that the terminal units of the oligomers are 5-deoxyflavan-3-ols.

The MALDI-TOF-MS spectrum of fraction 2217 showed a series of peaks arising from proanthocyanidin oligomers at *m/z* 1177, 1465, 1753, 2041, and 2329 (tetramer to octamer) (Figure 4). The distance between the major peaks was 288 Da, which coincides with the mass of a robinetinidol unit. The major

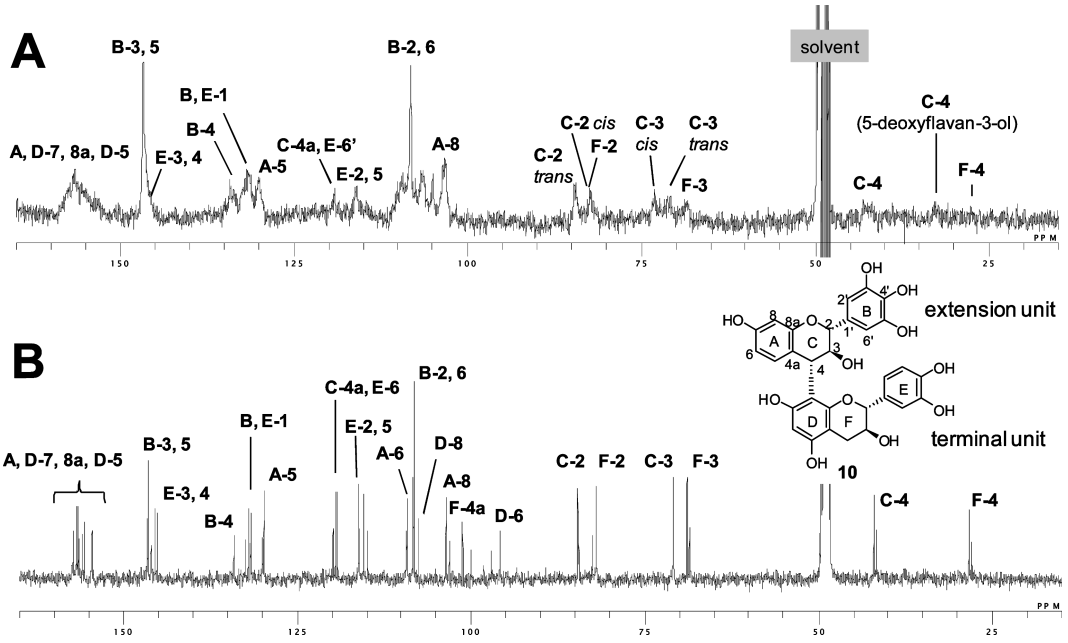


Figure 3. ^{13}C NMR spectra of the active fraction Fr. 2217 (A) and robinetinidol-(4α,8)-catechin (**10**) (B) measured at 100 MHz in methanol-d_4.

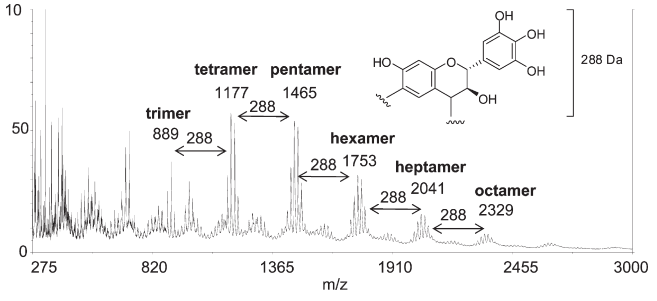


Figure 4. MALDI-TOF-MS spectrum of Fr. 2217.

peaks were accompanied by peaks 16 Da smaller or larger arising from molecules with fisetinidol or gallocatechin units. These results are similar to the mass spectra of the original extracts, except the peaks arising from trimers (m/z 905 corresponding to the $[M + K]^+$ of trimers) were much larger in the original extracts.

Size-Exclusion High-Performance Chromatography of the Active Fraction. Size-exclusion high-performance chromatography of fraction 2217 suggested that the number average molecular weight (M_n) and weight average molecular weight (M_w) are 1185 and 1556, respectively. The M_n value corresponded with the molecular weight of proanthocyanidin tetramer composed of robinetinidol and is consistent with the MALDI-TOF-MS data.

Thiolysis of the Extract. Thiol degradation is commonly used for characterization of proanthocyanidin oligomers composed of 5-oxygenated flavan-3-ols units.[19] Substitution reaction with thiol groups at the C-4 position under moderately acidic conditions cleaves interflavan linkages to produce catechins from the terminal units and 4-thioethers from the extension units. However, the method is not effective for proanthocyanidins with 5-deoxyflavan-3-ol units because the 4−8 (or 6) interflavan linkages are stable and resist the substitution reaction.[18] Accordingly, we attempted thiolysis reactions of fraction 2217 under strongly acidic conditions ($HSCH_2CH_2OH$ in EtOH containing

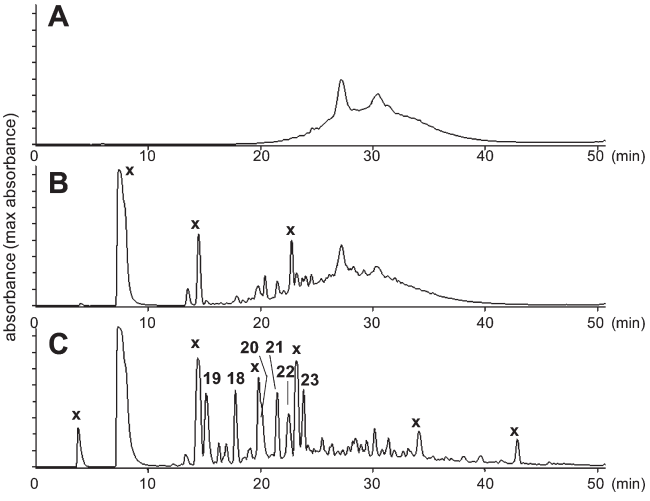


Figure 5. HPLC of the active fraction 2217 (A), the thiol degradation products of the active fraction under common reaction conditions (B), and the thiol degradation products under modified reaction conditions (C) (**18**−**23**: see Figure 6, × indicates peaks also observed in the blank).

0.75 mol/L of HCl, 80 °C for 7 h) (Figure 5). Although not quantitative, the degradation reaction proceeded gradually, and three characteristic products, **18**−**20**, derived from a 5-deoxyflavan-3-ol were obtained, as well as three from catechin and epigallocatechin (Figure 6). Acid thiolysis of the active fraction and the original extract provided similar results; therefore, due to the limited quantity of the active fraction, characterization of the products was conducted using the extract.

Product **18** exhibited an $[M + Na]^+$ peak at m/z 389, indicating its molecular weight is 366. On the basis of HRFABMS data, the molecular formula was determined to be $C_{17}H_{18}O_7S$, which is consistent with a structure composed of the $-SCH_2$-CH_2OH group and robinetinidol. The ^1H NMR spectrum (Table 3) showed signals of the SCH_2CH_2OH moiety and a

dx.doi.org/10.1021/np100372t |J. Nat. Prod. 2011, 74, 119–128

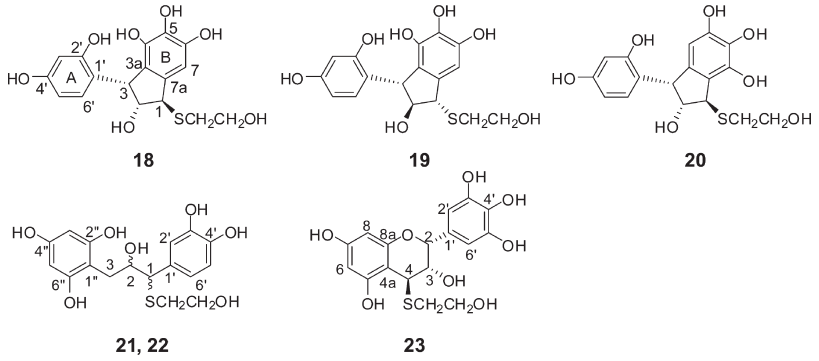


Figure 6. Structures of the thiol degradation products.

Table 3. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data of 18−20 in Methanol-d_4

	18		19		20	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	4.24, dd (1.1, 4.6)	56.1	3.96, d (3.4)	57.8	4.39, brs	54.7
2	4.30, dd (1.6, 4.6)	81.2	4.33, t (3.4)	88.5	4.43, d (4.7)	82.6
3	4.58, d (1.6)	50.0	4.42, d (3.4)	49.5	4.84, brd (4.7)	49.5[b]
3a		121.7		121.9		136.7
4		143.8		147.1	5.98, d (1.1)	104.8
5		133.9[a]		134.2		147.7
6		146.7		143.3		133.0
7	6.49, d (1.1)	104.7	6.46, s	104.8		144.2
7a		134.0[a]		133.4		120.0
1′		119.5		121.1		116.9
2′		157.1		156.7		158.1
3′	6.33, d (2.5)	103.4	6.32, d (2.5)	103.2	6.32, d (2.5)	103.7
4′		158.0		157.8		158.3
5′	6.10, dd (2.5, 8.6)	107.2	6.16, dd (2.5, 8.2)	107.5	6.26, dd (2.5, 8.2)	107.4
6′	6.27, d (8.6)	128.9	6.66, d (8.2)	130.6	6.92, d (8.2)	133.6
SCH_2	2.66−2.76, m	35.1	2.65−2.78, m	34.7	2.73−2.84, m	35.2
CH_2OH	3.67, t (6.6)	62.8	3.67, t (7.0)	62.7	3.72−3.81, m	62.9

[a] Assignments may be interchanged in the column. [b] Overlapped with solvent signal.

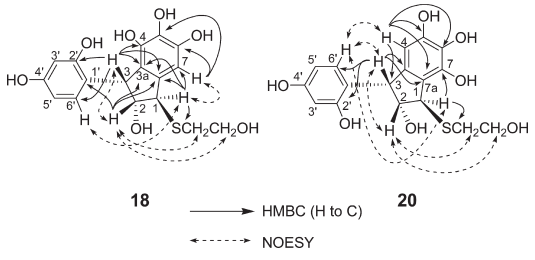


Figure 7. Selected HMBC and NOESY correlations for **18** and **20**.

set of ABX-type aromatic signals [δ 6.33 (d, J = 2.5 Hz, H-3′), 6.10 (dd, J = 2.5, 8.6 Hz, H-5′), and 6.27 (d, J = 8.6 Hz, H-6′)] attributable to a 1,2,4-trisubstituted benzene ring (A-ring). In addition, a singlet signal at δ 6.49 was assigned to a proton (H-7) attached to a pyrogallol ring (B-ring) based on the HMBC correlations with aromatic carbons (Figure 7). The ^{1}H−^{1}H COSY correlations of the remaining aliphatic methine protons resonated at δ 4.24 (dd, J = 1.1, 4.6 Hz, H-1), 4.30 (dd, J = 1.6, 4.6 Hz, H-2), and 4.58 (d, J = 1.6 Hz, H-3), showing the presence of a −CH−CH−CH− partial structure. In addition, allylic ^{1}H−^{1}H couplings of the pyrogallol-ring proton H-7 with the methine proton H-1, and the resorcinol H-6′ with the methine proton H-3, were observed. The HSQC spectrum revealed that H-2 was attached to an oxygenated carbon (δ 81.2). In the HMBC spectrum, H-1 was correlated to C-7, C-7a, and C-3a, confirming that C-1 was attached to the pyrogallol C-7a. Correlation of H-1 with a methylene carbon of the mercaptoethanol moiety was also observed. Furthermore, the HMBC correlations of H-3 with C-7a, C-3a, C-4, C-2′, and C-6′ indicated that the methine carbon C-3 was connected to the pyrogallol C-3a and to the resorcinol C-1′ (Figure 7). Accordingly, the structure of **18** was determined to be as shown in Figure 6. The relative configuration was determined by a NOESY experiment (Figure 7). The NOE correlations of H-1 with H-7 and H-6′, and H-2 with the methylene protons of the mercaptoethanol moiety, revealed 1,2-*trans* and 2,3-*cis* configurations. The absolute configuration was established by CD spectroscopic analysis,[39] which showed a positive CE at 278 nm and a negative CE at 244 nm. Positive CE at lower energy (longer wavelength) indicated two aromatic rings oriented with positive helicity,

dx.doi.org/10.1021/np100372t |J. Nat. Prod. 2011, 74, 119–128

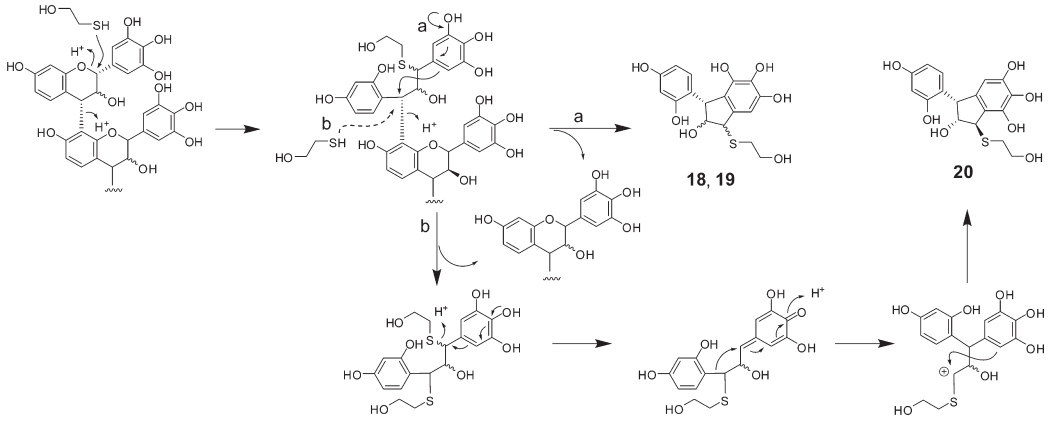


Figure 8. Possible mechanism for the production of **18**−**20**.

corresponding to the *R* configuration at C-3 (see Supporting Information). Therefore, the structure of product **18** was determined to be as shown in Figure 6.

HRFABMS indicated that the molecular formula of product **19** was the same as that of **18**, and the ^{1}H and ^{13}C NMR spectra were also closely related to those of **18** (Table 3). The ^{1}H−^{1}H COSY, HSQC, and HMBC spectra indicated that the planar structure of **19** was identical to that of **18**. However, differences were observed in the NOESY spectrum, which showed cross-peaks between the resorcinol H-6′ and methylene protons of the mercaptoethanol moiety, indicating that these two groups are located on the same side of the molecule. Furthermore, H-6′ also showed an NOE correlation with H-2, confirming the 1,2-*trans* and 2,3-*trans* configuration. The CD spectrum resembled that of **18** and showed a positive CE at 269 nm and a negative CE at 243 nm. Therefore, the configuration of C-3 was concluded to be *R*, and the structure of **19** was established to be as illustrated in Figure 6.

Product **20** was shown to be an isomer of **18** and **19** by its HRFABMS and ^{1}H and ^{13}C NMR spectra (Table 3). An important difference in the ^{1}H−^{1}H COSY spectrum of **20** compared to that of **19** was the appearance of an allylic coupling (J = 1.1 Hz) between the benzylic H-3 and a pyrogallol aromatic proton (δ 5.98, H-4). In addition, the HMBC spectrum showed a long-range coupling between H-4 and C-3 (Figure 7). Moreover, the NOESY correlations of the pyrogallol methine proton H-4 with H-3 and the resorcinol H-6′ were observed. These observations indicated that C-4 of the pyrogallol ring is a methine carbon. Consistent with this, the HMBC spectrum showed another benzylic methine H-1 (δ 4.39) correlated both with the C-7 (δ 144.2) bearing a hydroxy group and the methylene carbon of the mercaptoethanol moiety. NOESY correlations of H-1 and the resorcinol ring proton, and H-2 with H-3 and the mercaptoethanol methylene protons, indicated that the relative configuration of the five-membered ring of **20** is the same as that of **18**. A positive CE at 288 nm and a negative CE at 246 nm in the CD spectrum indicated that the absolute configuration of **20** is the same as that of **18** and **19**. Therefore, the structure of compound **20** was concluded to be as shown in Figure 6.

Compounds **21** and **22** showed signals arising from phloroglucinol, catechol, mercaptoethanol, one methylene, and two methine groups in the ^{1}H and ^{13}C NMR spectra. These spectroscopic features are closely related to those of 2-[3-(3,4-dihydroxyphenyl)-2-hydroxy-3-[(phenylmethyl)thio]propyl]-1,3,5-benzenetriol, obtained as a byproduct of thiol degradation with α-toluenethiol.[40] On the basis of the HMBC, HSQC, and ^{1}H−^{1}H COSY spectra, the structures of these two products were determined to be 2-hydroxyethylthio analogues, as shown in Figure 6. The configuration of the benzylic methine carbon of these products was not determined. The production of these compounds indicated the presence of catechin at the terminal units. Product **23** was identified as 4β-(2-hydroxyethylsulfanyl)epigallocatechin by comparing the sample's spectroscopic data with those of an authentic sample.[41]

Thiol degradation of **10** under similar conditions also yielded **18**, **19**, and **20**. On the basis of the results, a mechanism for the generation of **18** and **19** from the 5-deoxyflavan-3-ol extension units of proanthocyanidins is proposed as shown in route a of Figure 8. In contrast, the mechanism for the production of **20** is not clear. In a possible mechanism (route b in Figure 8), an intermediate with two thioether groups is produced, and subsequent elimination of one of the ethylthio groups accompanied by migration of a resorcinol moiety produce the product **20**. McGraw et al. reported that products with an indane skeleton related to **18**−**20** were produced from typical proanthocyanidins composed of 5-hydroxyflavan-3-ols upon extended thiol degradation (for 24 to 72 h).[42,43] They proposed a mechanism that begins with cleavage of the carbon−carbon bond between C-4 and C-4a, which is different from the mechanism for proanthocyanidins with 5-deoxyflavan-3-ol units.

In conclusion, proanthocyanidins from the bark of *A. mearnsii* exhibited strong inhibitory activities toward α-amylase and lipase. The most active fraction was characterized by spectroscopic and chemical methods and shown to contain tetrameric to octameric compounds mainly composed of robinetinidol units. The α-amylase and lipase inhibitory activities of these oligomeric proanthocyanidins suggest that they may be a promising functional food material for suppressing sugar and lipid uptake. In this study, we also developed a chemical method for characterization of proanthocyanidins with robinetinidol extension units.

EXPERIMENTAL SECTION

General Experimental Procedures. Ultraviolet (UV) spectra were obtained with a Jasco V-560 UV/vis spectrophotometer. The CD spectra were measured with a Jasco J-725N spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded in methanol-d_4 or acetone-d_6 with a Varian Unity Plus 500 spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C and with a JEOL JNM-AL 400 spectrometer operating

dx.doi.org/10.1021/np100372t |*J. Nat. Prod.* 2011, 74, 119–128

at 400 MHz for ^{1}H and 100 MHz for ^{13}C. MS were recorded on a Voyager-DE Pro MALDI-TOF spectrometer in positive linear ion mode, with 2,5-dihydroxybenzoic acid (10 mg/mL in 50% aqueous MeOH) used as the matrix. HRFABMS were recorded on a JMS 700N spectrometer (JEOL Ltd., Tokyo, Japan), with *m*-nitrobenzyl alcohol or glycerol used as the matrix. Column chromatography was performed using Sephadex LH-20 (25−100 mm, GE Healthcare UK Ltd., Little Chalfont, UK), MCI-gel CHP 20P (75−150 mm, Mitsubishi Chemical Co., Tokyo, Japan), Diaion HP20SS (Mitsubishi Chemical Co.), Bondapak C_{18} 125A (Waters Co., Ltd., Milford, U.S.A.), Cosmosil 75C_{18}-OPN (Nacalai Tesque, Inc., Kyoto, Japan), and Chromatorex ODS (Fuji Silysia Chemical Ltd., Kasugai, Japan). The flow rate for column chromatography was about 3 mL/min. TLC was performed on precoated Kieselgel 60 F_{254} plates (0.2 mm thick, Merck) with toluene−ethyl formate−formic acid (1:7:1, v/v) and $CHCl_3$−MeOH−H_2O (7:3:0.5, v/v). Spots were detected using UV illumination and by spraying with 2% ethanolic $FeCl_3$ or 5% H_2SO_4 reagent followed by heating. Analytical HPLC was performed on a Cosmosil 5C_{18}-AR II (Nacalai Tesque Inc.) column (4.6 × 250 mm i.d.) with gradient elution from 4 to 30% (39 min) and 30 to 75% (15 min) of CH_3CN in 50 mM H_3PO_4 at 35 °C (flow rate, 0.8 mL/min; detection, Jasco photodiode array detector MD-910). Preparative HPLC was performed on a Cosmosil 5C_{18}-PAQ (Nacalai Tesque, Inc.) column (20 × 250 mm) using gradient program A: elution with 4−10% (60 min) and 10−30% (240 min) of CH_3CN (flow rate, 2 mL/min), and with gradient program B: elution with 4−10% (60 min) and 10−20% (120 min) of CH_3CN (flow rate, 2 mL/min). Size-exclusion HPLC was performed on a TSK gel α-3000 (TOSOH) column (7.8 × 300 mm) with *N*,*N*-dimethylformamide containing 10 mM LiCl as the mobile phase. Absorbance measurements for the α-amylase and lipase inhibitory assays were performed using an Emax microplate reader (Molecular Devices Inc., Sunnyvale, CA, U.S.A.).

Dried Extract of the Bark of *Acacia mearnsii*. Spray-dried aqueous extract of *A. mearnsii* bark was prepared in 2007 according to the method reported by Cutting[1] and was provided by mimozax Co., Ltd. (Hiroshima, Japan). Briefly, the bark was chipped and extracted with hot H_2O (100 °C) for 30 min. After filtration, the filtrate was spray-dried.

Preparation of Extracts. Green tea, black tea, oolong tea, and guava leaf (each 2.0 g) were separately extracted with 100 mL of boiling H_2O for 5 min, and the filtrates were lyophilized. The polyphenol fraction of guava leaf was prepared as follows. The dried leaf (2.0 g) was extracted with 200 mL of boiling H_2O for 5 min, and the filtrate was subjected to Diaion HP20SS chromatography. After washing the column with H_2O, the polyphenols were eluted with 50% aqueous acetone.

Chemicals. Iodine solution (0.5 mol/L) and L-cysteine methyl ester hydrochloride were purchased from Nacalai Tesque, Inc. Pancreatic α-amylase, pancreatic lipase (type II), orlistat, glyceryl trioleate, and 2,5-dihydroxybenzoic acid were purchased from Sigma (St. Louis, MO, U.S.A.). NEFAC-test Wako, acarbose, *N*,*N*-dimethylformamide, sodium cholate, lecithin, and soluble starch were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). LiCl and TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] were purchased from Kishida Chemical Co., Ltd. (Osaka, Japan). Isothiocyanic acid *o*-tolyl ester was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). (+)-Catechin was isolated from *Uncaria gambir* extract. Sanguiins H-6 and H-11 were isolated from *Sanguisorba officinalis* and *Rubus suavissimus*,[21,44] respectively. Epicatechin-(4β,8,2β,7)-epicatechin-(4α,8)-catechin and procyanidin B-1 were isolated from *Vaccinium ashei*.[45] Epigallocatechin-3-*O*-gallate was isolated from commercial green tea and recrystallized from H_2O. Theaflavin-3,3′-di-*O*-gallate was synthesized by enzymatic oxidative coupling of epigallocatechin-3-*O*-gallate and epicatechin-3-*O*-gallate.[46]

Fractionation of the *Acacia mearnsii* Extract. Dried extract of *A. mearnsii* bark (50 g) was first fractionated by Diaion HP20SS column chromatography (5.5 cm i.d. × 25 cm) with H_2O, and the eluate was monitored by TLC. Elution of the column with H_2O gave Fr. 1, which contained sugars (10.3 g). Further elution of the column with 10−100% aqueous MeOH yielded Fr. 2, containing polyphenols (35.3 g), and Fr. 3, containing nonpolar compounds (0.9 g). Fr.2 was dissolved in a small amount of 6 M urea−acetone mixture (adjusted to pH 2 with HCl) (2:3, v/v), applied to a Sephadex LH-20 column (8 × 60 cm), and eluted with 6 M urea−acetone pH 2 solution to give two fractions: Fr. 21, which contained relatively high molecular weight polyphenols, and Fr. 22, which contained lower molecular weight polyphenols. After evaporation of the acetone, the fractions were separately applied to an MCI-gel CHP20P column (4.5 cm i.d. × 25 cm). The HCl and urea were eluted with H_2O, and subsequent elution with H_2O containing an increasing concentration of MeOH (0−100% MeOH, 10% stepwise elution) furnished Fr. 211 (0.3 g) and Fr. 212 (8.1 g) from Fr. 21, and Fr. 221 (22.2 g) and Fr. 222 (3.7 g) from Fr. 22. Fr. 221 was further fractionated by Sephadex LH-20 column chromatography (5 cm i.d. × 25 cm) with EtOH containing increasing proportions of water (0−40% H_2O, 20% stepwise elution), and then elution with 60% aqueous acetone, to give seven fractions: Fr. 2211 (0.31 g), Fr. 2212 (0.48 g), Fr. 2213 (0.95 g), Fr. 2214 (2.19 g), Fr. 2215 (3.83 g), Fr. 2216 (4.73 g), and Fr. 2217 (8.61 g) (the separation scheme is shown in the Supporting Information).

Isolation of Compounds. Dried extract of *A. mearnsii* bark (100 g) was dissolved in 500 mL of H_2O and successively partitioned with Et_2O and EtOAc. The Et_2O layer (1.13 g) was subjected to Sephadex LH-20 (3 cm i.d. × 28 cm) column chromatography with 60−100% MeOH in H_2O (10% stepwise elution, each 200 mL) and then with 60% aqueous acetone to give seven fractions: E-1−E-7. Fr. E-1 was successively subjected to Sephadex LH-20 and Cosmosil 75C_{18}-OPN (2 cm i.d. × 20 cm) column chromatography with 0−40% aqueous MeOH (5% stepwise elution, each 100 mL) to afford syringic acid (**4**) (3.2 mg)[23] and multifidol glucoside (**17**) (18.5 mg).[31,32] Crystallization of Fr. E-3 and E-5 from H_2O yielded robinetinidol (**1**) (87.3 mg)[22] and catechin (**6**) (29.0 mg),[24] respectively. Fr. E-6 was subjected to Cosmosil 75C_{18}-OPN (2 cm i.d. × 20 cm) column chromatography with 0−40% aqueous MeOH (5% stepwise elution, each 100 mL) to afford butin (**8**)[26] (9.5 mg, eluted with 15% aqueous MeOH) and taxifolin (**7**)[25] (8.6 mg, eluted with 20% aqueous MeOH). The EtOAc layer (31.1 g) was separated by Sephadex LH-20 (5 cm i.d. × 35 cm) column chromatography (0−20% H_2O in EtOH, 10% stepwise elution, each 500 mL) into five fractions: EA-1−EA-5. Fr. EA-3, containing flavan-3-ols and proanthocyanidin dimers, was separated by Sephadex LH-20 (2 cm i.d. × 20 cm) column chromatography with 60−100% aqueous MeOH (10% stepwise elution, each 200 mL) to give eight fractions, EA-31−EA-38. Crystallization of EA-34 from H_2O afforded gallocatechin (**5**)[24] (53.1 mg). EA-36 was subjected to MCI-gel CHP20P (3 cm i.d. × 30 cm) column chromatography with 0−50% aqueous MeOH (10% stepwise elution, each 200 mL) to give a mixture of proanthocyanidin dimers. Separation of the mixture by Chromatorex ODS (3 cm i.d. × 25 cm) column chromatography with 0−30% aqueous MeOH (5% stepwise elution, each 200 mL) afforded fisetinidol-(4α,8)-catechin (**11**)[27] (54.6 mg) and a mixture of **12** and **13**. This mixture was separated by preparative HPLC (elution program A) to yield fisetinidol-(4α,6)-gallocatechin (**12**) (2.3 mg) and epirobinetinidol-(4β,8)-catechin (**13**) (10.4 mg). The aqueous layer was fractionated into five fractions, AQ-1−AQ-5, by Sephadex LH-20 (5 cm i.d. × 35 cm) column chromatography with H_2O containing increasing proportions of MeOH (0−100%, 20% stepwise, each 300 mL), and then the column was washed with 50% aqueous acetone. Fr. AQ-1 was subjected to MCI-gel CHP 20P (5 cm i.d. × 35 cm, 0−80% aqueous MeOH, 10% stepwise) and Chromatorex ODS column chromatography (2.5 cm i.d. × 20 cm,

0−60% aqueous MeOH, 10% stepwise) to give 4-hydroxy-2-methoxyphenyl 1-*O*-β-D-glucopyranoside (**15**)[29] (11.2 mg) and 3,5-dimethoxy-4-hydroxybenzyl alcohol 4-*O*-β-D-glucopyranoside (**16**)[30] (16.3 mg). Fr. AQ-3 was applied to Chromatorex ODS (3 cm i.d. × 22 cm) and Cosmosil 75C_{18}-OPN (3 cm i.d. × 23 cm) columns and eluted with 0−50% aqueous MeOH (5% stepwise, each 100 mL) to afford 4′-*O*-methylrobinetinidol 3′-*O*-β-D-glucopyranoside (**3**) (165.8 mg). Successive column chromatography of Fr. AQ-5 using Diaion HP20SS (5 cm i.d. × 22 cm), Chromatorex ODS (5 cm i.d. × 25 cm), Sephadex LH-20 (3 cm i.d. × 25 cm), and Chromatorex ODS (3 cm i.d. × 22 cm) furnished 1,6-di-*O*-galloyl-β-D-glucose (**14**)[28] (40.6 mg), robinetinidol-(4α,8)-gallocatechin (**9**)[27] (22.3 mg), and robinetinidol-(4α,8)-catechin (**10**)[27] (312.7 mg) (the separation scheme is shown in the Supporting Information).

4′-*O*-Methylrobinetinidol 3′-*O*-β-D-glucopyranoside (3): pale brown, amorphous powder; $[\alpha]^{18}_{D}$ −74.0 (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ε) 280 (3.62) nm; CD (MeOH) $\Delta\varepsilon_{242}$ +8.1, $\Delta\varepsilon_{255}$ −2.6, $\Delta\varepsilon_{259}$ +2.7, $\Delta\varepsilon_{284}$ −15.3; IR ν_{max} 3399, 2931, 1599, 1511, 1453, 1348, 1158, 1079 cm^{-1}; ^1H NMR (methanol-d_4, 400 MHz); δ 2.67 (1H, d, *J* = 7.8, 15.6 Hz, H-4), 2.84 (1H, d, *J* = 4.9, 15.6 Hz, H-4), 3.35−3.50 (4H, m, H-2″, 3″, 4″, 5″), 3.64 (1H, dd, *J* = 5.1, 12.2 Hz, H-6″), 3.73 (1H, dd, *J* = 2.1, 12.2 Hz, H-6″), 3.83 (3H, s, OMe), 4.03 (1H, m, H-3), 4.69 (1H, d, *J* = 6.6 Hz, H-2), 4.91 (1H, d, *J* = 7.5 Hz, H-1″), 6.29 (1H, d, *J* = 2.4 Hz, H-8), 6.35 (1H, dd, *J* = 2.4, 8.3 Hz, H-6), 6.59 (1H, d, *J* = 1.8 Hz, H-6′), 6.68 (1H, d, *J* = 1.8 Hz, H-2′), 6.86 (1H, d, *J* = 8.3 Hz, H-5); ^{13}C NMR (acetone-d_6+D_2O, 100 MHz) δ 33.1 (C-4), 61.1 (C-OMe), 62.1 (C-6″), 67.8 (C-3), 70.8 (C-4″), 74.3 (C-2″), 77.4, 77.5 (C-3″, 5″), 82.5 (C-2), 101.7 (C-1″), 103.2 (C-8), 107.3 (C-2′), 109.1 (C-6′), 109.7 (C-6), 112.1 (C-4a), 130.9 (C-5), 136.1 (C-1′), 137.3 (C-4′), 151.1 (C-5′), 151.4 (C-3′), 155.6 (C-7), 157.6 (C-8a); HRFABMS *m*/*z* 466.1471 $[M]^+$ (calcd for $C_{22}H_{26}O_{11}$, 466.1475).

Fisetinidol-(4α,6)-gallocatechin (12): pale brown, amorphous powder; $[\alpha]_D$ −87.7 (*c* 0.06, MeOH); UV (MeOH) λ_{max} (log ε) 281 (3.92) nm; CD (MeOH) $\Delta\varepsilon_{216}$ −267.9, $\Delta\varepsilon_{236}$ −134.9, $\Delta\varepsilon_{274}$ +15.2, $\Delta\varepsilon_{287}$ −17.6; ^1H NMR (methanol-d_4, 500 MHz) δ of two rotational isomers 2.47, 2.61 (1H, dd, *J* = 7.5, 16.4 Hz, H-4F), 2.74, 2.77 (dd, *J* = 5.5, 16.4 Hz, H-4F), 3.73, 4.05 (1H, m, H-3F), 4.42, 4.76 (1H, d, *J* = 6.8 Hz, H-2F), 4.45 (2H, m, H-2C, H-3C), 4.53 (1H, d, *J* = 9.5 Hz, H-2C), 4.54, 4.62 (1H, br d, *J* = 9.5 Hz, H-4C), 4.65 (1H, t, *J* = 9.5 Hz, H-3C), 5.92, 6.08 (1H, s, H-8D), 6.03, 6.46 (2H, s, H-2E, 6E), 6.15, 6.18 (1H, d, *J* = 2 Hz, H-8A), 6.20, 6.27 (1H, dd, *J* = 2.0, 8.7 Hz, H-6A), 6.54, 6.83 (1H, dd, *J* = 2.0, 8.0 Hz, H-6B), 6.61, 6.64 (1H, dd, *J* = 1.5, 8.7 Hz, H-5A), 6.70, 6.76 (1H, d, *J* = 8 Hz, H-5B), 6.75, 6.96 (1H, d, *J* = 2.0 Hz, H-2B); ^{13}C NMR (methanol-d_4, 100 MHz) δ of two rotational isomers 27.5, 28.7 (C-4F), 41.8, 42.0 (C-4C), 68.6, 68.8 (C-3F), 71.1, 71.2 (C-3C), 82.5, 82.7 (C-2F), 84.3 (C-2C), 96.0, 97.1 (C-8D), 99.9, 101.7 (C-4aD), 103.2, 103.6 (C-8A), 106.8, 107.6 (C-2′E, 6′E), 108.1, 108.3 (C-6D), 109.4 (C-6A), 115.9, 116.1 (C-5B), 116.2, 116.5 (C-2B), 119.6 (C-4aA), 120.8, 121.1 (C-6B), 130.0, 130.1 (C-5A), 131.3, 131.1 (C-1E), 132.6, 132.8 (C-1B), 133.5 (C-4E), 145.6, 146.1 (C-3B, 4B), 146.4, 146.8 (C-3E, 5E), 155.1 (C-5D), 155.7, 157.2 (C-8aD, 7D), 156.4 (C-7A); HRFABMS *m*/*z* 601.1331 $[M + Na]^+$ (calcd for $C_{30}H_{26}O_{12}Na$, 601.1322).

Epirobinetinidol-(4β,8)-catechin (13): pale brown, amorphous powder; $[\alpha]_D$ −45.9 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 280 (3.85) nm; CD (MeOH) $\Delta\varepsilon_{212}$ −280.8, $\Delta\varepsilon_{239}$ +5.2, $\Delta\varepsilon_{278}$ −65.7; ^1H NMR (methanol-d_4, 400 MHz at −20 °C) δ 2.56 (2H, m, H-4F), 3.98 (1H, m, H-3F), 4.20 (1H, t, *J* = 2.5 Hz, H-3C), 4.68 (1H, d, *J* = 5.1 Hz, H-2F), 4.81 (1H, d, *J* = 2.5 Hz, H-4C), 5.15 (1H, d, *J* = 2.5 Hz, H-2C), 5.92 (1H, s, H-6D), 6.20 (2H, s, H-2B, 6B), 6.23 (1H, dd, *J* = 2.4, 8.5 Hz, H-6A), 6.38 (1H, d, *J* = 2.4 Hz, H-8A), 6.39 (1H, dd, *J* = 1.8, 8.3 Hz, H-6E), 6.53 (1H, d, *J* = 8.5 Hz, H-5A), 6.58 (1H, d, *J* = 1.8 Hz, H-2E), 6.60 (1H, d, *J* = 8.3 Hz, H-5E); ^{13}C NMR (methanol-d_4, 100 MHz at −20 °C) δ 26.6 (C-4F), 31.7 (C-4C), 67.8 (C-3F), 73.2 (C-3C), 81.1 (C-2F), 82.6 (C-2C), 97.4 (C-6D), 100.3 (C-4aD), 102.9 (C-8A), 104.6 (C-2, 6B), 105.8 (C-8D), 108.6 (C-6A), 113.8 (C-2E), 114.2 (C-4aA), 115.9 (C-5E), 118.2 (C-6E), 129.8 (C-5A), 131.8, 132.0 (C-1B, 1E), 132.9 (C-4B), 145.2, 145.6 (C-3E, 4E), 146.5 (C-3B, 5B), 154.5, 156.2, 156.4 (C-7A, 7D, 8aA), 155.4 (C-8aD), 157.5 (C-5D); HRFABMS *m*/*z* 601.1338 $[M + Na]^+$ (calcd. for $C_{30}H_{26}O_{12}Na$, 601.1322).

Hydrolysis of 3. Compound **3** (4 mg) was dissolved in 1 M H_2SO_4 (0.5 mL) and heated at 100 °C for 5 h. After neutralization with Amberlite IRA400 (OH form), the resin was removed by filtration and the filtrate was dried *in vacuo*. The residue was dissolved in pyridine (1 mL) containing L-cysteine methyl ester (10 mg) and heated at 60 °C for 1 h. The mixture was mixed with a solution (0.5 mL) of pyridine *o*-tolylisothiocyanate (10 mg) in pyridine and heated at 60 °C for 1 h. The final mixture was directly analyzed by HPLC [Cosmosil 5C_{18} AR II (250 × 4.6 mm i.d., Nacalai Tesque Inc.) with isocratic elution at 25% (40 min) and 25−90% gradient elution (5 min) with CH_3CN in 50 mM H_3PO_4]. The t_R of the peak at 16.9 min coincided with that of the thiocarbamoyl thiazolidine derivative of D-glucose (the t_R of the L-diastereomer was 15.4 min).

Measurement of α-Amylase Inhibitory Activity. The activity was measured using the method reported by Xiao et al.[47] and Yoshikawa et al.[48] with slight modifications. Acarbose was used as the positive control. Substrate solution was prepared as follows: soluble starch (500 mg) was dissolved in 25 mL of 0.4 M NaOH and heated for 5 min at 100 °C. After cooling in ice H_2O, the solution was adjusted to pH 7 with 2 M HCl, and H_2O was added to adjust the volume to 100 mL. Sample solutions were prepared by dissolving each sample in acetate buffer (pH 6.5) to make 2, 0.2, and 0.02 mg/mL solutions. The substrate (40 μL) and sample (20 μL) solutions were mixed in a microplate well, and the mixtures were preincubated at 37 °C for 3 min. Then 20 μL of α-amylase solution (50 μg/mL) was added to each well, and the plate was incubated for 15 min. The reaction was terminated by addition of 80 μL of 0.1 M HCl; then 200 μL of 1 mM iodine solution was added, and the absorbances were measured at 650 nm. Inhibitory activity (%) was calculated as follows:

$$\text{Inhibition } (\%) = \{1 - (\text{Abs } 2 - \text{Abs } 1)/(\text{Abs } 4 - \text{Abs } 3) \times 100\}$$

where Abs 1 is the absorbance of incubated solution containing sample, starch, and amylase, Abs 2 is the absorbance of incubated solution containing sample and starch, Abs 3 is the absorbance of incubated solution containing starch and amylase, and Abs 4 is the absorbance of incubated solution containing starch.

IC_{50} value was determined by curve-fitting using the graphing software DeltaGraph 5 for Windows (RockWare Inc., Golden, CO, U.S.A.).

Measurement of Pancreatic Lipase Inhibitory Activity. Lipase inhibitory activity was measured according to the method of Han et al.[49] with slight modifications. Orlistat was used as the positive control. Substrate solution was prepared by sonication (10 min in an ice bath) of a mixture of glyceryl trioleate (80 mg), lecithin (10 mg), and sodium cholate (5 mg) suspended in 9 mL of 0.1 M TES buffer (pH 7.0). Samples were separately dissolved in 0.1 M TES buffer to make 0.2 mg/mL solutions. The substrate (20 μL) and sample solutions (20 μL) in microplate wells were preincubated for 3 min; then 10 μL of lipase solution (20 μg/mL) was added to each reaction mixture and incubated for 30 min at 37 °C. The amount of released fatty acid was measured by a NEFAC-test Wako at 550 nm using a microplate reader. Inhibitory activity (%) was calculated as follows:

$$\text{Inhibition } (\%) = \{1 - (\text{Abs } 6 - \text{Abs } 5)/(\text{Abs } 8 - \text{Abs } 7) \times 100\}$$

where Abs 5 is the absorbance of incubated solution containing sample, substrate, and lipase, Abs 6 is the absorbance of incubated solution containing sample and substrate, Abs 7 is the absorbance of incubated

solution containing substrate and lipase, and Abs 8 is the absorbance of incubated solution containing substrate.

MALDI-TOF-MS Spectrum of Active Fraction. Fr. 2217 was dissolved in 50% MeOH (1 mg/mL) and mixed 1:1 (v/v) with a 50% aqueous MeOH solution of 2,5-dihydroxybenzoic acid (10 mg/mL). The mixture (0.5 μL) was placed on a MALDI-TOF-MS target plate.

Size-Exclusion Chromatography of Active Fraction. High-performance size-exclusion chromatography was performed on a TSK gel α-3000 column (7.8 × 300 mm) with DMF containing 10 mM LiCl as the mobile phase at 40 °C.[50] Samples were dissolved in the elution solvent, and 5 μL was individually applied to the column and eluted using a flow rate of 0.5 mL/min; the eluate was monitored at 275 nm. Catechin (MW 290, t_R 15.9 min), procyanidin B-1 (MW 578, t_R 14.9 min), A-type trimer [epicatechin-(4β,8,2β,7)-epicatechin-(4α,8)-catechin, MW 864, t_R 14.3 min], and sanguiin H-11 (MW 3738, t_R 12.9 min) were used as standards for molecular weight estimation. The number average molecular weight (M_n) and weight average molecular weight (M_w) of Fr. 2217 were calculated from the SEC profile using a 807-IT integrator (Jasco).

Thiolysis of Dried Extract of *Acacia mearnsii* Bark. Bark extract (5.0 g) was dissolved in 50 mL of H_2O and mixed with 12 mL of concentrated HCl, 110 mL of EtOH, and 20 mL of 2-mercaptoethanol. After heating at 80 °C for 7 h, the solution was concentrated by rotary evaporation to remove the EtOH, the resulting aqueous solution was applied to a Sephadex LH-20 column (4 × 27 cm), and the HCl and mercaptoethanol were eluted with H_2O (Fr. 1). Subsequent elution of the column with 0, 50, and 100% aqueous MeOH afforded Fr. 2 (404 mg) and Fr. 3 (2.5 g). Fr. 2 was fractionated by Sephadex LH-20 column chromatography (2 × 30 cm) with 0−50% aqueous MeOH into four fractions. Fr. 23 thus obtained was further subjected to Bondapak C18 125A column chromatography (2 × 16 cm) with 0−15% aqueous MeOH to afford compounds **18** (24.2 mg) and **19** (22.0 mg). Fr. 3 was separated into five fractions by Sephadex LH-20 column chromatography (4 × 27 cm) with 100−80% aqueous EtOH and 50% aqueous acetone. Then Fr. 33 was further fractionated on a Diaion HP20SS column (3.5 × 22 cm) with 0−50% aqueous MeOH into five fractions. Fr. 334 was subjected to Cosmosil 5C_{18}-PAQ preparative HPLC (gradient elution program B) to afford **20** (4.1 mg), **21** (4.5 mg), **22** (3.3 mg), and **23** (8.0 mg).

Compound 18: pale brown, amorphous powder; $[\alpha]^{28}_D$ +76.1 (*c* 0.03, MeOH); UV (MeOH) λ_{max} (log ε) 282 (3.61) nm; CD (MeOH) $\Delta\varepsilon_{244}$ −34.2, $\Delta\varepsilon_{278}$ +30.5; IR ν_{max} 3351, 1605, 1510, 1463, 1304, 1210 cm^{-1}; HRFABMS *m*/*z* 389.0648 $[M + Na]^+$ (calcd for $C_{17}H_{18}O_7SNa$, 389.0671); ^1H and ^{13}C NMR data, see Table 3.

Compound 19: pale brown, amorphous powder; $[\alpha]^{18}_D$ −38.7 (*c* 0.17, MeOH); UV (MeOH) λ_{max} (log ε) 282 (3.60) nm; CD (MeOH) $\Delta\varepsilon_{243}$ −28.6, $\Delta\varepsilon_{269}$ +0.7; IR ν_{max} 3297, 1605, 1514, 1459, 1304, 1206 cm^{-1}; HRFABMS *m*/*z* 389.0705 $[M + Na]^+$ (calcd for $C_{17}H_{18}O_7SNa$, 389.0671); ^1H and ^{13}C NMR data, see Table 3.

Compound 20: pale brown, amorphous powder; $[\alpha]^{18}_D$ +67.7 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 282 (3.73) nm; CD (MeOH) $\Delta\varepsilon_{246}$ −28.6, $\Delta\varepsilon_{288}$ +23.4; IR ν_{max} 3358, 1604, 1505, 1457, 1300, 1210 cm^{-1}; HRFABMS *m*/*z* 389.0655 $[M + Na]^+$ (calcd for $C_{17}H_{18}O_7SNa$, 389.0671); ^1H and ^{13}C NMR data, see Table 3.

Compound 21: pale brown, amorphous powder; $[\alpha]^{18}_D$ +61.0 (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ε) 282 (3.57) nm; IR ν_{max} 3334, 1615, 1519, 1446, 1367, 1284 cm^{-1}; ^1H NMR (methanol-d_4, 500 MHz) δ 2.36 (1H, dd, *J* = 9.6, 14.4 Hz,, H-3a), 2.47 (2H, t, *J* = 7.0 Hz, SCH_2), 3.02 (1H, dd, *J* = 2.4, 14.4 Hz, H-3b), 3.55 (2H, t, *J* = 7.0 Hz, CH_2OH), 3.84 (1H, d, *J* = 5.3 Hz, H-1), 4.00 (1H, m, H-2), 5.85 (2H, s, H-3″, 6″), 6.70 (1H, d, *J* = 8.0 Hz, H-5′), 6.75 (1H, dd, *J* = 2.1, 8.0 Hz, H-6′), 6.98 (1H, d, *J* = 2.1 Hz, H-2′); ^{13}C NMR (methanol-d_4, 125 MHz) δ 30.1 (C-3), 34.3 (SCH_2), 56.9 (C-1), 62.3 (CH_2OH), 76.7 (C-2), 95.8 (C-3″, 5″), 105.8 (C-1″), 115.7 (C-5′), 117.6 (C-2′), 122.4 (C-6′), 132.3 (C-1′), 145.6 (C-3′, 4′), 157.5 (C-4″), 158.0 (C-2″, 6″); HRFABMS *m*/*z* 391.0849 $[M + Na]^+$ (calcd for $C_{17}H_{20}O_7SNa$, 391.0828).

Compound 22: pale brown, amorphous powder; $[\alpha]^{18}_D$ −22.6 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 281 (3.48) nm; IR ν_{max} 3309, 1614, 1518, 1455, 1358, 1287 cm^{-1}; ^1H NMR (methanol-d_4, 500 MHz) δ 2.50 (1H, dd, *J* = 9.5, 14.0 Hz,, H-3a), 2.48 (2H, m, SCH_2), 2.87 (1H, dd, *J* = 3.5, 14.0 Hz, H-3b), 3.53 (2H, t, *J* = 7.0 Hz, CH_2OH), 3.81 (1H, d, *J* = 7.0 Hz, H-1), 3.97 (1H, m, H-2), 5.83 (2H, s, H-3″, 6″), 6.69 (1H, d, *J* = 7.8 Hz, H-5′), 6.67 (1H, dd, *J* = 1.7, 7.8 Hz, H-6′), 6.87 (1H, d, *J* = 1.7 Hz, H-2′); ^{13}C NMR (methanol-d_4, 125 MHz) δ 30.0 (C-3), 34.3 (SCH_2), 57.9 (C-1), 62.3 (CH_2OH), 77.5 (C-2), 95.8 (C-3″, 5″), 105.6 (C-1″), 115.9 (C-5′), 116.9 (C-2′), 121.5 (C-6′), 133.7 (C-1′), 146.1 (C-3′), 145.5 (C-4′), 157.7 (C-4″), 158.2 (C-2″, 6″); HRFABMS *m*/*z* 369.0999 $[M + H]^+$ (calcd for $C_{17}H_{21}O_7S$, 369.1008).

Thiolysis of 10. Compound **10** (1 mg) was dissolved in 62% aqueous EtOH (2.6 mL) and mixed with concentrated HCl (0.12 mL) and 2-mercaptoethanol (0.2 mL). After heating at 80 °C for 7 h, the reaction mixture was analyzed by HPLC. Production of **18** (t_R 17.5 min), **19** (t_R 14.6 min), and **20** (t_R 19.5 min) was confirmed by comparisons of the retention times and UV absorptions.

■ ASSOCIATED CONTENT

Supporting Information. Separation schemes, HPLC chromatograms of the active fractions and the thiol degradation products, and selected NMR and CD spectra of the new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENT

The authors are grateful to Professor K. Fujita and Professor M. Tanaka (Nagasaki University) for use of the CD spectrometer and also thank Mr. K. Inada, Mr. N. Yamaguchi, and Mr. Y. Ohama (Nagasaki University) for NMR and MS measurements.

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