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Structures and Antioxidant and Intestinal Disaccharidase Inhibitory Activities of A-Type Proanthocyanidins from Peanut Skin

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Supporting Information

ABSTRACT: Nine compounds including a new A-type proanthocyanidin trimer, epicatechin-(2β→O→7,4β→8)-[catechin-(6→4β)]-epicatechin (**8**), and a known trimer, epicatechin-(4β→8)-epicatechin-(2β→O→7,4β→8)-catechin (**9**), being reported for peanut skin for the first time, were isolated and purified. Their structures were determined by spectroscopic methods and by degradation reactions with L-cysteine in acidic conditions. The DPPH radical scavenging activity and the inhibitory activity on maltase and sucrase of the isolated compounds were investigated. All compounds showed strong DPPH scavenging activities (EC_{50} < 20 μg/mL). Compound **8** showed the strongest inhibitory activity on maltase with an IC_{50} value of 0.088 mg/mL, while compound **9** exhibited the strongest inhibition on sucrase with an IC_{50} value of 0.091 mg/mL.

KEYWORDS: *peanut skin, condensed tannin, proanthocyanidin trimer, inhibition on intestinal disaccharidases*

INTRODUCTION

Peanut (*Arachis hypogaea* L. Fabaceae), the fourth oleaginous plant in the world, is known to be one of the most important economical crops owing to its wide distribution, nutritional characteristics, and its great application in the food industry for centuries. China, being the largest producer and exporter of peanuts, has a production of 14,385,000 tons every year, and almost 60% of the peanuts is consumed as feedstock for oil, which is one of the main vegetal oils used in Chinese daily life. However, over the years, the skin of the peanut has been generally neglected and considered as a useless byproduct. Of the yearly generated peanut skin, only a small part of it is used in traditional Chinese medicine (TCM) to treat illnesses such as chronic hemorrhage and bronchitis, etc.[1] Early investigations have shown that the most important chemical constituents of peanut skin are proanthocyanidins, particularly the A-type, in which the subunits are connected by a 4→8 or 4→6 carbon bond and a 2→O→7 ether bond.[2,3]

Proanthocyanidins in general have shown notable potentials of health benefits, e.g., antioxidant[4,5] and insulin action potentiation[6] activities. Moreover, the consumption of plant extracts containing A-type proanthocyanidins has been shown to increase sugar tolerance in type II diabetes patients.[7] Disaccharidase (or α-glucosidase) inhibitors could control postprandial hyperglycemia by delaying the absorption of intestinal carbohydrates as does the prototype clinically used as an antidiabetic α-glucosidase inhibitor drug, acarbose.[8]

In our further systematic search for proanthocyanidins, 9 compounds were isolated from the water-soluble fraction of peanut skin, including a novel trimer (compound **8**) and another trimer (compound **9**), which is being reported from peanut skin for the first time. Bioassays were carried out to test the antioxidant and intestinal disaccharidase inhibitory activities of the 9 compounds from peanut skin. This article describes the isolation, structure elucidation, antioxidant activity, and intestinal disaccharidase inhibitory activity of these proanthocyanidins to provide scientifically valuable information for better utilization of the peanut skin.

MATERIALS AND METHODS

Materials and Apparatus. Solvents used for the extraction and isolation were of analytical grade. L-Cysteine hydrochloride was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). High performance liquid chromatography (HPLC) grade solvents used for UPLC were purchased from Fisher Scientific Company (New Jersey, USA). The sources of the other materials are octadecylsilane (ODS, 38−63 μm, Wako Pure Chemcial Industries, Ltd., Osaka, Japan), Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and high porous polymer from Mitsubishi Chemical Corporation (MCI CHP 20, Mitsubishi Chemical Corporation, Tokyo, Japan). TLC was performed on silica gel plates (GF254, 0.2 mm, Rushan Taiyang Desiccant Co., Ltd., China). NMR spectra were obtained from a Bruker Avance III 500 spectrometer with tetramethylsilane as internal standard. UPLC-DAD-ESI-MS experiments were carried out on an Agilent 1290 infinity UPLC-DAD system [Agilent Technologies Singapore (International) Pte. Ltd., Singapore] with an autosampler and a photodiode array detector (DAD) coupled with an Agilent 6340 triple quad MS. High resolution electrospray ionization mass spectrometry (HRESI-MS) was performed with a Xevo G2 Q-TOF mass spectrometer (Waters). Circular dichroism (CD) was recorded with a JASCO-J-18 spectro-polarimeter (Waters). UV and IR spectra were taken using a Shimadzu UV 240 spectrophotometer and a Shimadzu IR spectrophotometer, respectively.

Plant Materials. The peanut skin used in this research was purchased from Weikang Drug Store, Huhhot, Inner Mongolia, China, and identified by the authors to be the seed skin of *Arachis hypogaea* L. Fabaceae.

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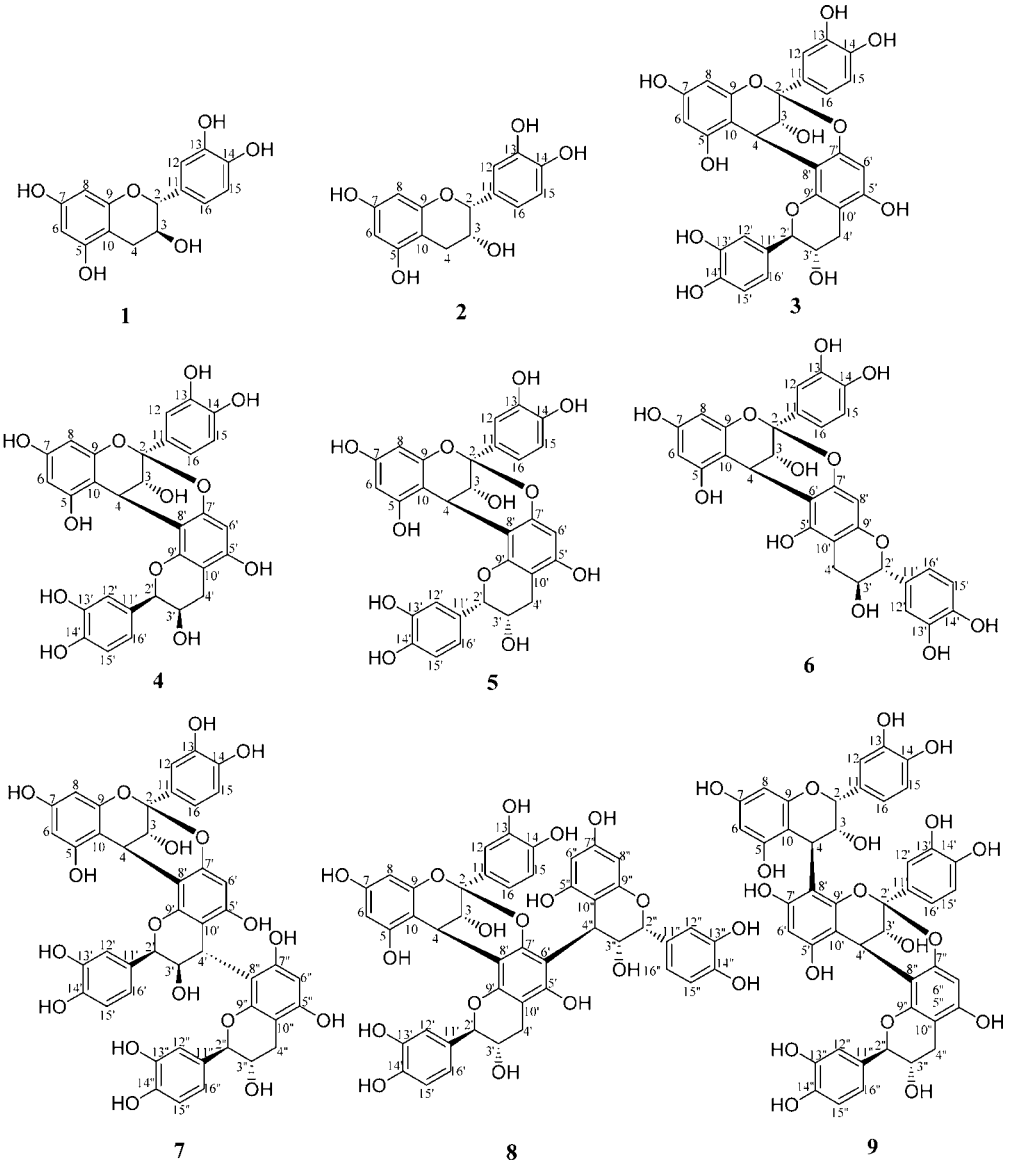

Figure 1. Structures of compounds **1**–**9** from peanut skin.

Extraction and Purification. Peanut skin (2 kg) was soaked with 70% acetone (32L) for 24 h and extracted with ultrasonication for 30 min. After filtration, the residue was extracted once again by repeating the above procedure. The solvent was evaporated under reduced pressure and the ensuing extract (276 g) fractionated by chromatography over Sephadex LH-20 with ethanol (7500 mL) to give 8 fractions (E1-E8). TLC and LC-MS analysis revealed that E2 contained proanthocyanidin monomers, while E3 and E5 contained dimeric and trimeric proanthocyanidins, respectively. E2 was subjected to further chromatography on ODS, eluted with a 0–100% methanol/water gradient to give compounds **1** (122 mg) and **2** (63 mg). Similarly, E3 (27 g) was subjected to further chromatography on ODS, eluted with a 0–100% methanol/water gradient. The 20%–30% gradient fraction contained polyphenols as indicated by TLC and was further purified by repeated chromatography on MCI gel (CHP20/P120) and Sephadex LH-20 with methanol/water gradients to afford compounds **3**–**6**. Compound **3** (780 mg) was recrystallized from water as colorless needles, while **4** (56 mg), **5** (25 mg), and **6** (68 mg) were obtained as amorphous powders. Furthermore, E5 (20 g) was purified in the same way as described for E3 to obtain compounds **7** (145 mg), **8** (54 mg), and **9** (72 mg) as amorphous powders.

Compounds **1**–**2**: amorphous powder, MS (ESI) m/z 289 ($[M - H]^-$, 100). Compounds **3**–**6**: colorless needles (**3**) or amorphous powder (**4**–**6**), MS (ESI) m/z 575 ($[M - H]^-$, 100). NMR, see Supporting Information. Compounds **7**–**9**: amorphous powder, MS (ESI) m/z 863 ($[M - H]^-$, 100). NMR, see Supporting Information.

Degradation of Proanthocyanidin Trimers. L-Cysteine-induced degradations of the trimeric proanthocyanidins were carried out according to the reported methods[9,10] with modification. The trimer and cysteine·HCl (1:2) were dissolved in methanol in a centrifuge tube. The tube was tightly sealed and heated at 65 °C for 24 h. The reaction mixture was filtered though a 0.22 μm microfilter and analyzed by UPLC-DAD-EIS-MS in comparison with standard **3** (procyanidin A1) and standards catechin–cystein and epicatechin–cystein, which were recently prepared in our earlier work.[11]

DPPH Radical Scavenging Assay. We examined the DPPH radical scavenging activity with the method described by Ma et al.[12] The antioxidant activities were examined at four different concentration (2.5, 5, 10, and 20 μg/mL) in 96-well plates. Ten microliters of a compound solution (in DMSO) and 190 μL of DPPH solution (1,1-diphenyl-2- picrylhydrazyl radical in ethanol, 0.1 mM) were added to each well. For the color control, 10 μL of compound solution and 190 μL of ethanol were mixed in each well. In the control wells, 10 μL of DMSO and 190 μL of DPPH were added. After 20 min at room temperature in the dark, the absorbance (A) was measured at 520 nm, and the activity was calculated as follows:

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Scheme 1. Degradation of Compound 7 with L-Cysteine HCl

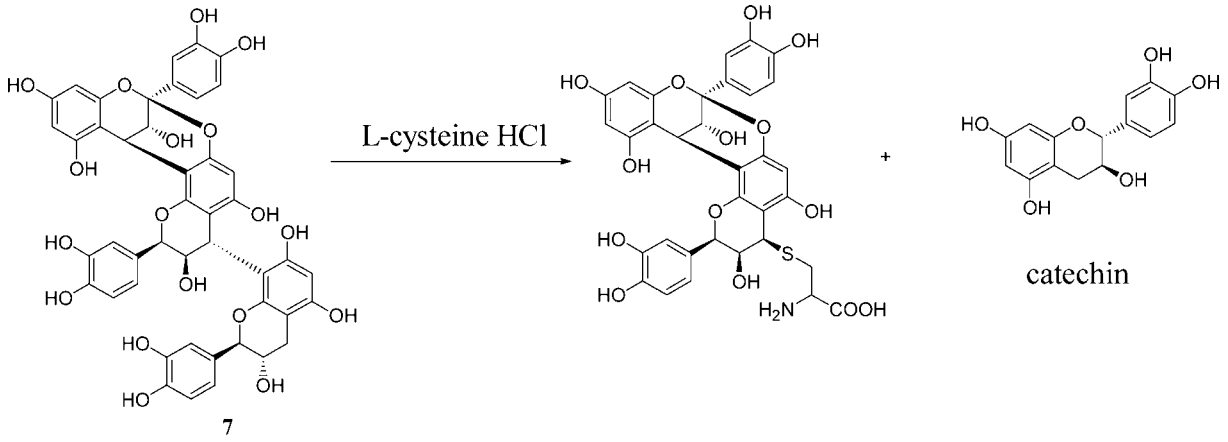


Table 1. NMR Spectral Data of Compound 8 in CD_3OD

ring	no.	^{13}C	^{1}H	ring	no.	^{13}C	^{1}H	ring	no.	^{13}C	^{1}H
C	2	100.88		F	2′	84.21	4.79, d (6.5)	I	2″	77.21	5.33, br.s
	3	67.49	4.20, br.s		3′	68.02	4.15, m		3″	73.34	4.09, m
	4	29.46	4.35, br.s		4′	28.58	2.76, dd (16.1, 7.5)		4″	37.06	4.63, br.s
							2.50, dd (16.1, 7.5)				
A	5	156.54		D	5′	149.29		G	5″	159.21	
	6	96.05	6.10, br.s		6′	109.35			6″	96.45	6.04, br.s
	7	157.82			7′	150.1			7″	154.55	
	8	98.35	5.96, br.s		8′	107.33			8″	99.18	5.93, br.s
	9	156.54			9′	154.44			9″	158.18	
	10	104.07			10′	103.93			10″	99.18	
B	11	131.86		E	11′	130.43		H	11″	132.93	
	12	116.14	7.18, br.s		12′	115.26	6.87, br.s		12″	115.7	6.94, br.s
	13	146.09			13′	145.91			13″	145.66	
	14	146.94			14′	146.85			14″	146.45	
	15	116.01	6.82, m		15′	120.59	6.10, br.s		15″	119.22	6.78, m
	16	120.19	7.06, d (7.8)		16′	120.08	6.77, m		16″	120.19	6.74, m

$$\text{effect } \% = 100 \times [A_{\text{control}} - (A_{\text{compound}} - A_{\text{color}})]/A_{\text{control}}$$

IC_{50}, the concentration of a sample that scavenged 50% of the DPPH radical, was obtained from a curve of effect % versus sample concentration.

Determination of Inhibitory Activity against Maltase and Sucrase. To determine the maltase and sucrase inhibitory activities of samples, we prepared the stocking intestinal disaccharidases as reported before.[11] The assay was carried out as follows: 3 μL of sample (dissolved by DMSO), 7 μL of disaccharidase [the stocking enzyme was diluted 10 times by phosphate buffer (pH 7)] and 20 μL of maltose solution (2 mg/mL) was added to each well of a 96-well plate to test the inhibition activity on maltase. Three microliters of DMSO was added instead of samples in the control wells. After 20 min of incubation at 37 °C, 10 μL of DMSO and the glucose detecting reagents of an assay kit from Nanjing Jiancheng Bio Company (Nan Jing, China) were added, and the absorbances (A) were measured at 520 nm with a plate reader. The activity was calculated as follows:

$$\text{inhibition } \% = 100 \times [(A_{\text{control}} - A_{\text{sample}})]/A_{\text{control}}$$

The inhibition activity on sucrase was tested using the same procedure described above, except that 20 μL of sucrose instead of maltose was used as the substrate, and the concentration of the working enzyme solution was twice of that for the maltase assay.

RESULTS AND DISCUSSION

Proanthocyanidins in Peanut Skin. Peanut skin was extracted with 70% (v/v) acetone, and the extract was fractionated with Sephadex LH-20 column chromatography. The fraction obtained from the 95% ethanol eluted part was then subjected to repeated chromatography on ODS and MCI eluted with aqueous methanol. Compounds **1**−**9** were obtained, and their structures were determined by interpretation of their spectral data and by L-cysteine-induced degradations (Figure 1).

Compounds **1** and **2** were identified as catechin and epicatechin by comparison with authentic standards in UPLC-DAD-ESI-MS. Compounds **3**−**6** were identified as procyanidins A1, A2, epicatechin-(2β→O→7,4β→8)-ent-epicatechin, and epicatechin-(2β→O→7,4β→6)-catechin by comparison of their spectral data with those reported in literature.[3]

Structural Elucidation of Compound 7. Compound **7** [circular dichroism (CD): $\Delta\varepsilon_{272.6}$, −3.93; $\Delta\varepsilon_{231}$, +21.57] was obtained as a white amorphous powder. It showed a molecular formula of $C_{45}H_{36}O_{18}$ from the negative HRESI-MS (m/z [M − 1]$^-$ 863.1823, which revealed that **7** was a trimeric proanthocyanidin. Degradation of **7** (Scheme 1) produced catechin and another compound with a poseudo-molecular weight of 694 in negative ESIMS, suggesting that it is composed by A-type procyanidins and a catechin. By comparison of its ^{1}H and ^{13}C NMR spectral data with those previously reported,[13,14] compound **7** was confirmed as cinnamtannin D-1.[14]

Structural Elucidation of Compound 8. Compound **8** [UV (MeOH) λ_{max} (log ε): 280 (4.13) nm. IR (ν, KBr, cm^{-1}): 3350, 1610, 1520, 1450, 1290, 1150, 1100. CD: $\Delta\varepsilon_{273.8}$, −2.58; $\Delta\varepsilon_{239.4}$, +16.07.] was obtained as a white amorphous powder. It showed a molecular formula of $C_{45}H_{36}O_{18}$ from the negative HRESI-MS (m/z [M − 1]$^-$ 863.1823), which revealed that **8** was a trimeric proanthocyanidin. The presence of three flavanyl units was also indicated by ^{13}C resonances at δ 100.88 (C-2), 67.49 (C-3), 29.46 (C-4), 84.21 (C-2′), 68.02 (C-3′), 28.58 (C-4′), 77.21 (C-2″), 73.34 (C-3″), and 37.06 (C-4″) (Table 1), arising from the heterocyclic rings (rings C, I, and F). The existence of an A-type proanthocyanidin unit was supported by the downfield C-2 signal at δ 100.88 (Table 1) and an isolated AB coupling system at δ 4.20 (br.s, H-3) and 4.35 (br.s, H-4) (C-ring) in the ^1H NMR spectrum. The two flavan-3-ol units of the A-type entity in **8** were deduced to be linked through C4 (C ring) and C-8′ (D ring) based on the long-range correlations of H-4 (C-ring) at δ 4.35 with C-7′ at δ 150.1, C-8′ at δ 107.33, and C-9′ at δ 154.44 in the heteronuclear multiple-bond correlation (HMBC) spectrum. The long-range correlations of H-4″ (I-ring) at δ 4.63 with C-5′ at δ 149.29, C-6′ at δ 109.35, and C-7′ at δ 150.1 in the HMBC indicated the C-4″→C-6′ linkage (Figure 2). The C-4″→C-6′ linkage was further confirmed by its complex NMR spectra from conformational isomers due to the steric hindrance for the free rotation of the epicatechin unit.

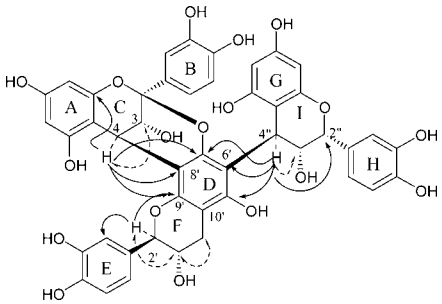


Figure 2. Key correlations observed in the HMBC and $^1H-^1H$ COSY spectra of **8**. HMBC, ↶; $^1H-^1H$ COSY, dashed arrow.

The terminal flavanol unit was determined to be 2,3-cis (I ring) from the singlet at δ 5.33 (1H, s, H-2″) and the corresponding carbon at δ 77.21(C-2″), [4] while the middle unit (the doubly substituted terminal unit) was deduced to have a 2,3-trans configuration (F ring) from the proton signal at δ 4.79 (1H, d, J = 6.5, H-2′) and the corresponding carbon at δ 84.21 (C-2′) in ^{13}C NMR.[4]

Treatment of **8** with cysteine·HCl in methanol (Scheme 2) yielded compound **3**, which was identified to be procyanidin A1 by direct comparison of its ^1H and ^{13}C NMR spectral data with those previously reported.[4] The cysteine conjugate derived from the terminal flavan-3-ol unit in the degradation of **8** was identified as epicatechin−cysteine by direct comparison by UHPLC-MS with a standard compound, which had been prepared and reported in our laboratory recently[11] (Figure 3).

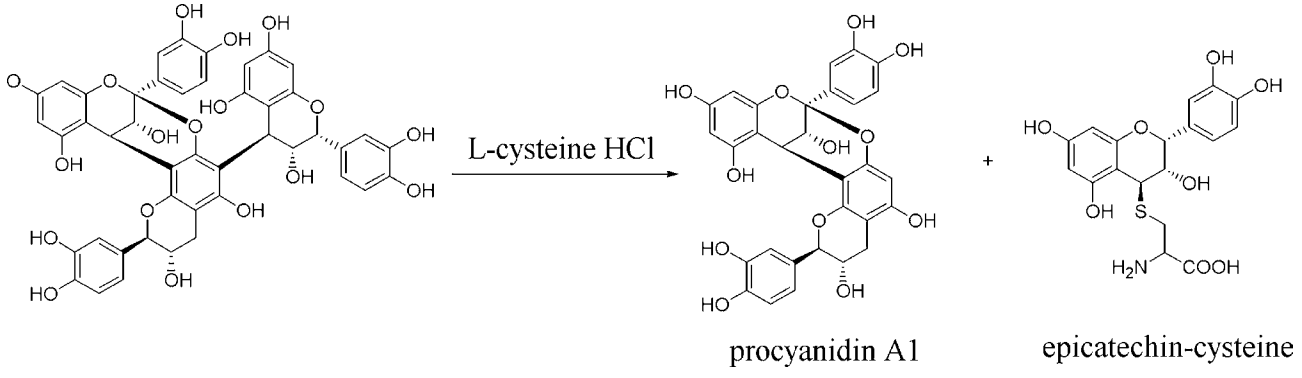
Of the three trimeric proanthocyanidins (**7**−**9**) with the same molecular weight (864), the ^1H NMR spectrum of **8** was observed to be relatively complicated compared to those of **7** and **9**. This relative complication alludes to the presence of conformers in **8**, due to hindered free rotation across its 4″→6′ linkage, which the structures of **7** and **9** conspicuously lack.

The diagnostic high amplitude positive Cotton effect at 239 nm wavelength of the CD spectrum of **8** informed the β-orientation assignment of the interflavonoid bonds.[15] Consequently, the structure of **8** was determined to be epicatechin-(2β→O→7,4β→8)-[catechin-(6→4β)]-epicatechin. This is a branched A-type proanthocyanidin with C4−C6, C4−C8 linkages to the same A ring of a flavanyl unit.

Structural Elucidation of Compound 9. Compound **9** (CD: $\Delta\varepsilon_{277.8}$, −4.67; $\Delta\varepsilon_{240}$, +5.39; $\Delta\varepsilon_{228.2}$, +2.86) was obtained as a white amorphous powder. It showed a molecular formula of $C_{45}H_{36}O_{18}$ from the negative HRESI-MS (m/z [M − 1]$^-$ 863.1823, which revealed that **9** was a trimeric proanthocyanidin. Treatment of **9** with cysteine·HCl in methanol yielded procyanidin A1 and epicatechin−cysteine (Scheme 3 and Figure 3), which were the same results as those for the degradation of compound **8**. The result indicated that compound **9** is also composed of procyanidin A1 and epicatechin with epicatechin being its upper flavan-3-ol unit. It was thus deduced that compounds **8** and **9** possess different linking positions between procyanidin A1 and epicatechin. The epicatechin was determined to be linked through C4→C8 with procyanidin A1, by the direct comparison of the ^1H and ^{13}C NMR spectral data of **9** with references.[16,17] The high amplitude positive Cotton effect at a wavelength of 240 nm in the CD spectrum of **9** confirmed its β-orientated interflavonoid bond. Compound **9** was thus identified as epicatechin-(4β→8)-epicatechin-(2β→O→7,4β→8)-catechin, which is reported from peanut skin for the first time.

Antioxidant Activity. As shown in Table 2, all of the isolated compounds, **1**−**9**, demonstrated strong antioxidant activities. They scavenged 50% of the DPPH radical at a

Scheme 2. Degradation of Compound 8 with L-Cysteine HCl



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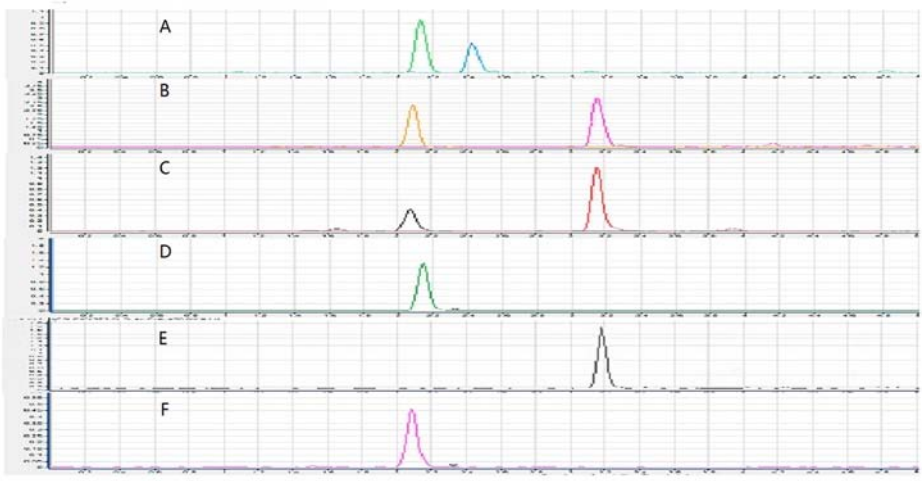

Figure 3. Extracted ion chromatograms of the LC-MS of cysteine-induced degradation of **7** (A), **8** (B), and **9** (C) in comparison with catechin (D), procyanidin A1 (E), and epicatechin−cysteine (F).

Scheme 3. Degradation of Compound 9 with L-Cysteine HCl

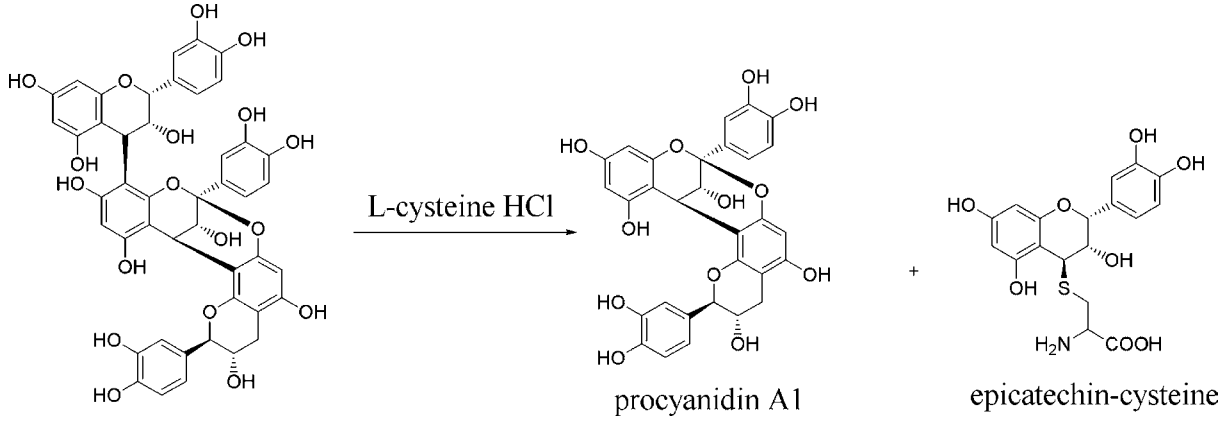

Table 2. DPPH Radical Scavenging Activities of Proanthocyanidins from Peanut Skin[a]

	effect %				
sample	20 (μg/mL)	10 (μg/mL)	5 (μg/mL)	2.5 (μg/mL)	EC_{50} (μg/mL)
1	83.01	61.69	37.42	31.88	8.21
2	94.52	74.33	42.07	34.59	6.12
3	94.02	63.14	29.93	26.28	8.55
4	86.91	58.93	28.80	20.00	9.71
5	79.43	47.29	30.06	15.28	11.36
6	93.39	67.29	47.10	38.61	5.68
7	93.33	54.21	26.79	19.43	9.74
8	84.21	53.01	37.61	34.02	8.61
9	89.37	85.66	48.49	20.56	6.32

[a]Catechin (**1**), a known anti-oxidant was used as a positive control.

Table 3. Maltase and Sucrase Inhibitory Activities of Compounds from Peanut Skin[a]

sample	inhibition% of maltase at 1 mg/mL	IC_{50} of maltase (mg/mL)	inhibition% of sucrase at 1 mg/mL	IC_{50} on sucrase (mg/mL)
1	44	>1	45	>1
2	60	0.088	54	0.57
3	47	>1	44	>1
4	44	>1	31	>1
5	47	>1	33	>1
6	67	0.61	42	>1
7	62	0.78	55	0.66
8	83	0.088	61	0.41
9	79	0.54	64	0.091
acarbose	nt	0.0054	nt	0.0074

[a]Acarbose, a known α-glucosidase inhibitor, was used as a positive control. nt: not tested, as this concentration is too high for the this very potent α-glucosidase inhibitor, acarbose.

concentration less than or close to 10 μg/mL. The dimeric and trimeric proanthocyanidins showed radical scavenging activity with the potencies similar to those of the monomers, catechin and epicatechin, which are well-known potent antioxidants.

Inhibitory Activity Against Maltase and Sucrase. As shown in Table 3, five compounds showed maltase inhibitory activity (>50% inhibition), and four compounds showed sucrase inhibitory activity at concentrations of 1 mg/mL. Compound **8**, epicatechin-(2β→O→7,4β→8)-[catechin-(6→4β)]-epicatechin, showed the strongest inhibition against maltase, with an IC_{50} value of 0.088 mg/mL. The lowest IC_{50} value (0.091 mg/mL) observed in the sucrase inhibition experiment was exhibited by **9**, epicatechin-(4β→8)-epicatechin-(2β→O→7,4β→8)-catechin, making it the strongest sucrase inhibitor of all the samples. It can be deduced from the results that **8** and **9** have notable degrees of specificity for

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maltase and sucrase, respectively. As the component units of **8** and **9** are essentially the same, the variation in their biological activities as observed by their differing enzyme specificities alludes to their different unit-connectivities, impacting chirality (though of the same configuration) at different positions. All of the 3 trimeric proanthocyanidins showed inhibitory activities on maltase and sucrase. Their inhibitory activities were greater than those of their component dimeric proanthocyanidins. Further investigations are required to elucidate the detailed structure−activity relationships of A-type proanthocyanidins on intestinal disaccharidases.

Degradation of the trimeric proanthocyanidins was carried out with a mild reagent, L-cysteine, which also is relatively safe compared to the conventionally used thiolytic degradation reagent, benzyl mercaptan, with a strong irritating smell. LC-MS analysis of the products of L-cysteine-induced degradation clearly indicated the component units and their linking order in the structures of pranthocyanidin polymers. Some L-cysteine conjugates of catechin/epicatechin and low-molecular-weight B-type proanthocyanidins have been reported to have antioxidant,[10] glucosidase inhibitory,[11] and neuroprotective activities.[18] However, no literature could be found for any A-type proanthocyanidin−cysteine conjugate. In the present research, we detected an A-type proanthocyanidin−cysteine conjugate for the first time from the degradation product of cinnamtannin D-1 (**7**). Preparation, purification, and bioactivity evaluation of novel A-type proanthocyanidin−cysteine conjugates are planned to be carried out in our group in the near future.

Proanthocyanidins are rich in hydrophobic aromatic rings and hydrophilic hydroxyl groups, which could interact with biological molecules, especially proteins. It is generally believed that the activity of proanthocyanidins becomes stronger as the number of flavanol units increases. The result of our study confirmed that proanthocyanidins have strong antioxidant activities, which could provide protection against oxidative stress and thereby prevent a variety of human diseases thus associated.[19−21]

In summary, we have isolated nine compounds, including one novel trimeric A-type proanthocyanidin (**8**) and a known trimeric A-type proanthocyanidin (**9**) that was reported for the first time in peanut skin. All of these compounds showed antioxidant activity, and the 2 trimeric A-type proanthocyanidins, **8** and **9**, showed considerable inhibitory activities on the intestinal disaccharidases, maltase and sucrase.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H and ^{13}C NMR spectra of compounds **3**−**9** and ^{1}H NMR and ^{13}C NMR data of compounds **3**−**7** and **9** (methanol-d_4). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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