Human immunodeficiency virus (HIV) is the etiologic agent of acquired immuno-deficiency syndrome (AIDS). HIV protease being a member of the aspartic protease family plays an important role in maturation of this virus, and is regarded as one of the most promising targets for development of anti-HIV agents.[1] Inhibitors of this enzyme have been successfully developed as effective drugs, *e.g.*, Saquinavir and Indinavir. A combination of these drugs with HIV reverse transcriptase inhibitors can reduce the blood virus to an undetectable level, however, a few viruses are still lurking on and hiding inside immune cells in a resting state.[2,3] One of the major obstacles to long-term treatment of the disease is the remarkable mutation of HIV to, if not all, most of the clinically used chemotherapeutic agents. Therefore, there is an ongoing need for new, structurally diverse anti-HIV

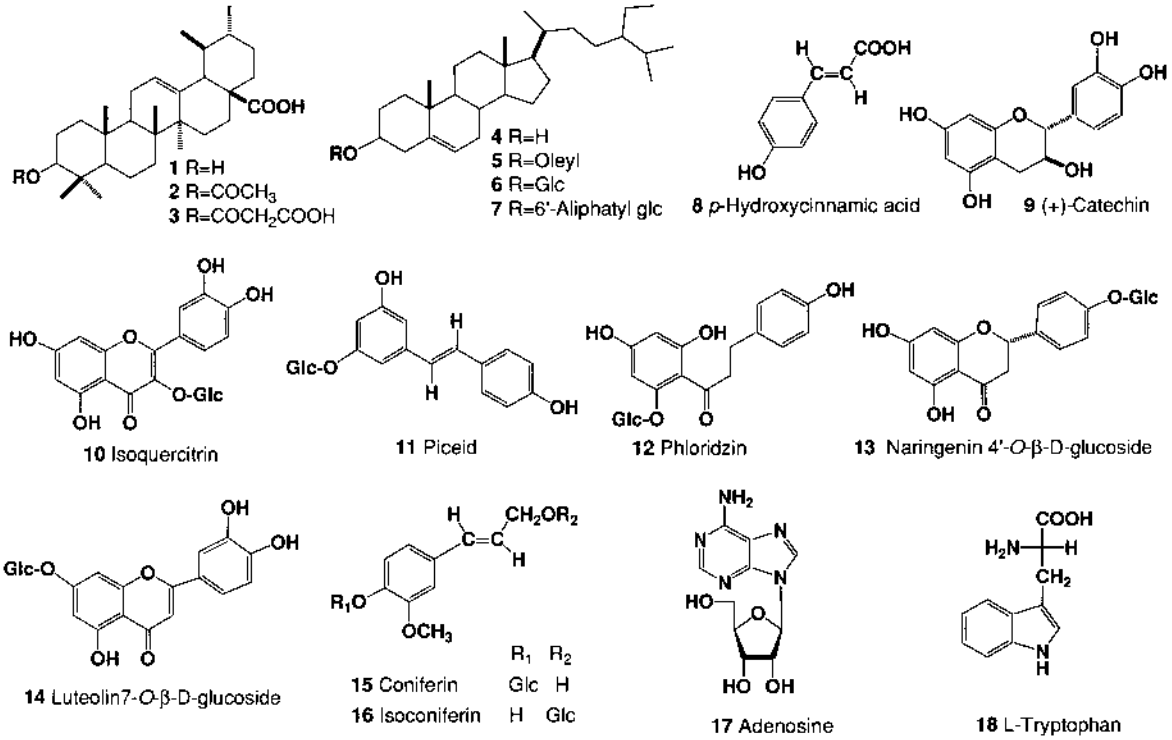

Chart 1

Table 1. Spectral Data of Triterpene Derivatives

Compd.	mp (°C)	Yield (%)	IR (KBr) cm^{-1}	^{1}H-NMR		MS (*m/z*) (A: FAB; B: API; C: EI)
			C=O	H-3	Acyl or methoxyl residure	
20[a]	106—112	97	1725	3.20	3.6 (3H, s)	C: 470 $[M]^+$
21	219—223	quant.	1740 br, 1690	4.66		B: 551 $[M+Na]^+$
22	240—243	39	1735, 1715, 1695	4.52	2.64 (4H, s)	A: 557 $[MH]^+$, 579 $[M+Na]^+$
23	220—223	49	1735, 1705 br	4.51	1.96[b] (2H, m), 2.41 (4H, t, J=7.2 Hz)	A: 571 $[MH]^+$, 593 $[M+Na]^+$
24[a]	40—46	quant.	1730 br	4.50	1.96[b] (2H, m), 2.37 (4H, m), 3.60 (3H, s), 3.68 (3H, s)	C: 598 $[M]^+$
27[a]	190—192	quant.	1725	3.20	3.62 (3H, s)	C: 470 $[M]^+$
28	264—266	quant.	1730, 1680	4.49	2.05 (3H, s)	B: 499 $[MH]^+$, 521 $[M+Na]^+$
29	172—180	quant.	1740 br, 1695	4.66		B: 551 $[M+Na]^+$
30	178—182	22	1735 br, 1695	4.56	3.38 (br)	B: 565 $[M+Na]^+$
31	260—262	66	1740, 1710 br	4.53	2.65 (4H, m)	A: 557 $[MH]^+$, 579 $[M+Na]^+$
32	206—209	67	1710 br	4.50	1.97[b] (2H, m), 2.40 (4H, t, J=7.0 Hz)	A: 571 $[MH]^+$
33[a]	170—172	quant.	1749, 1720, 1715	4.49	1.95[b] (2H, m), 2.37 (4H, m), 3.62 (3H, s), 3.67 (3H, s)	C: 598 $[M]^+$
35[a]	211—213	quant.	1720	3.18	3.65 (3H, s)	C: 470 $[M]^+$
36	202—205	quant.	1760, 1740, 1695	4.67		B: 529 $[MH]^+$, 551 $[M+Na]^+$
37	225—228	14	1710 br	4.54	3.38 (br)	B: 565 $[M+Na]^+$
38	283—286	82	1730, 1715, 1690	4.51	2.67 (4H, br)	B: 557 $[MH]^+$, 579 $[M+Na]^+$
39	265—269	84	1730, 1705 br	4.48	1.96[b] (2H, m), 2.41 (2H, t, J=7.7 Hz), 2.44 (2H, t, J=7.7 Hz)	B: 571 $[MH]^+$, 593 $[M+Na]^+$
40	205—208	60	1730, 1705, 1685	4.48	0.95 (6H, s), 2.21[b], 2.36 (1H, d, J=16.4 Hz), 2.51 (1H, d, J=16.4 Hz), 2.76 (1H, d, J=12.6 Hz)	B: 621 $[M+Na]^+$
41[a]	41—46	91	1730 br	4.48	2.60 (4H, s), 3.68 (3H, s), 3.69 (3H, s)	C: 584 $[M]^+$

NMR spectra were measured in $CDCl_3$–MeOH (8 : 1) except for *a*). *a*) In $CDCl_3$. *b*) Overlapped with other signals. quant.: quantitative. Notes: 1) By-products were formed during the preparation of malonyl hemiesters, so the yields of compounds **30** and **37** were very low. 2) The yields of compounds **22** and **23** were low because of sample-loss during chromatographic separation.

protease activity. It was found that introduction of methyl groups to the 3′ position of glutaryl part did not affect the anti-HIV protease activity (**39**, **40**, IC_{50}=4 μM).

Methylation of the carboxyls of the above triterpene derivatives significantly decreased the inhibitory activity against HIV-1 protease (**24**, **33**, **41**, IC_{50}=40, >50 μM). Methylation of the carboxyl group at C-17 in triterpenes **1**, **26** and **34** also appreciably decreased their inhibitory activities (**20**, **27**, **35**, IC_{50}=14, 20, >25 μM). In addition, replacement of the carboxyl group at C-17 with a methyl group led to a significant loss of activity [α-amyrin (**19**) (IC_{50}=80 μM) *vs*. ursolic acid (**1**) (IC_{50}=8 μM); β-amyrin (**25**) (IC_{50} >100 μM) *vs*. oleanolic

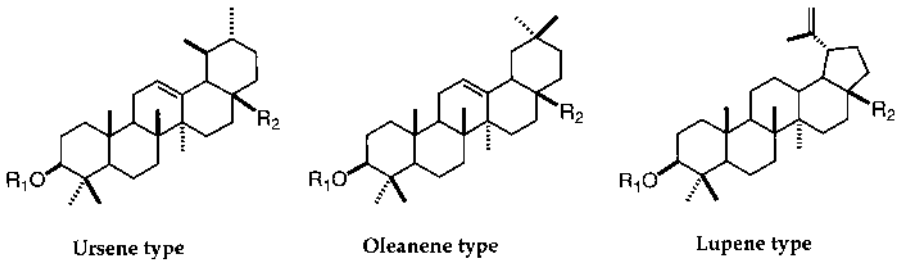

Fig. 1.

Table 2. Inhibitory Effects of Triterpene Derivatives on HIV-1 Protease

R_1	R_2	Ursene type IC_{50} (μM)		Oleanene type IC_{50} (μM)		Lupene type IC_{50} (μM)	
H	CH_3	α-Amyrin (**19**)	80	β-Amyrin (**25**)	>100		
H	COOH	Ursolic acid (**1**)	8	Oleanolic acid (**26**)	8	Betulinic acid (**34**)	9
H	$COOCH_3$	**20**	14	**27**	20	**35**	>25
$COCH_3$	COOH	**2**	13	**28**	9		
COCOOH	COOH	**21**	7	**29**	20	**36**	7
$COCH_2COOH$	COOH	**3**	6	**30**	8	**37**	6
$CO(CH_2)_2COOH$	COOH	**22**	6	**31**	4	**38**	6
$CO(CH_2)_3COOH$	COOH	**23**	4	**32**	4	**39**	4
$COCH_2C(CH_3)_2CH_2COOH$	COOH					**40**	4
$CO(CH_2)_2COOCH_3$	$COOCH_3$					**41**	40
$CO(CH_2)_3COOCH_3$	$COOCH_3$	**24**	>50	**33**	>50		

acid (**26**) (IC_{50}=8 μM)].

The above evidence suggests that the two polar substituents at C-3 and C-17 may be key functional groups which can interact with some amino acid residues of HIV-protease through hydrogen bonding or electrostatic interactions.

Recently, ursolic acid (**1**) was reported to have potent HIV-protease inhibitory activity[16] and various triterpenes were also reported to exhibit anti-HIV activity, and especially dicarboxylic acid hemiesters of betulinic acid demonstrated extremely potent activity against the virus.[15,17] Kashiwada *et al*.[15] proposed that a different mechanism of action other than inhibition of syncytia formation may be involved in the anti-HIV activity shown by these compounds. The present findings with ursolic acid (**1**), oleanolic acid (**26**), betulinic acid (**34**) and their hemiesters suggests a role for these compounds in the mechanism of their anti-HIV action. Since betulinic acid (**34**) and its 3′,3′-dimethylglutaryl hemiester (**40**) were reported not to inhibit HIV-reverse transcriptase,[15] these compounds tend to inhibit HIV-protease specifically.

Although these triterpenes were not as potent as acetyl pepstatin, used as a positive control (IC_{50}=0.09 μM), they are still interesting enough in that they are non-peptide HIV-protease inhibitors with low micromolar IC_{50}.

Inhibitory Substances from the Water Extract Since the H_2O extract of *C. songaricum* showed the most potent inhibitory activity against HIV-1 protease, the water extract was fractionated, whilst monitoring activity. The active substances obtained by passing through a porous polymer gel (Diaion HP-20) column were divided into EtOH-soluble and EtOH-insoluble parts. The EtOH-soluble part was repeatedly chromatographed on Sephadex LH-20 and MCI gel CHP20P to give procyanidin B1 (**42**),[18] B6 (**43**),[19] flavan-3-ol trimers, tetramers, pentamers and a mixture of higher oligomers and polymers. Although flavan-3-ol trimers, tetramers and pentamers assigned by atmospheric pressure ionization mass spectrometry (API-MS) seemed to be mixtures of structural isomers consisting of an epicatechin unit, they were assayed without further purification. The inhibitory activity against HIV-1 protease increased in the order of dimer, trimer, tetramer and pentamer (inhibition at 50 μg/ml: 1—2%, 17%,

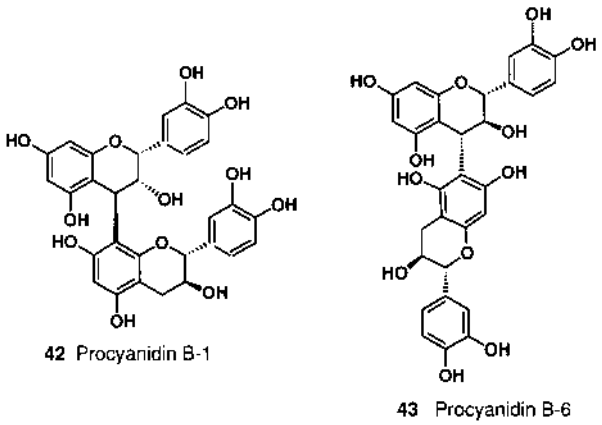

Chart 2

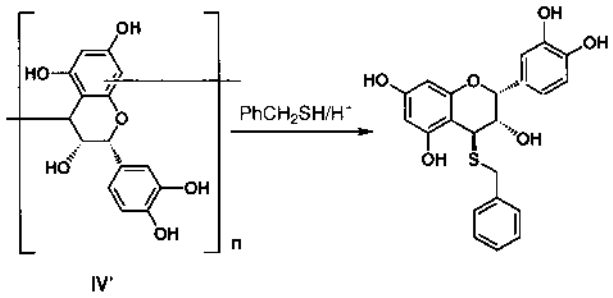

Chart 3

54% and 63%, respectively). Of the higher oligomer and polymer fractions obtained by ultrafiltration with 10000, 30000 and 100000 Dalton filters, the most potent activity was found in fr. IV including the largest molecular weight substances ($IC_{50}=2\,\mu g/ml$).

On the other hand, flavan-3-ol polymers from the EtOH-insoluble part were further fractionated to give frs. I′—IV′ by ultrafiltration. Fraction IV′ was obtained in the largest amount and showed the most potent activity ($IC_{50}=2\,\mu g/ml$). This fraction was subjected to thiolytic degradation to afford benzylthioepicatechin, indicating that the extender flavan unit of the polymers is mainly epicatechin (Chart 3).

From the above results, it can be concluded that the anti-HIV protease principles of the water extract of *C. songaricum* were flavan-3-ol polymers, with epicatechin as the main extender flavan unit.

Experimental

General Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-360 automatic polarimeter. IR spectra were measured with a JASCO FT/IR-230 infrared spectrometer. ^1H- and ^{13}C-NMR spectra were measured with Varian GEMINI 300 (^1H, 300 MHz; ^{13}C, 75 MHz) and Varian UNITY 500 (^1H, 500 MHz; ^{13}C, 125 MHz) spectrometers, the chemical shifts being represented as ppm with TMS as an internal standard. Electron impact (EI) MS were measured with a JEOL JMS-AX 505 HAD mass spectrometer at an ionization voltage of 70 eV. FAB-MS spectra were obtained with a JEOL JMS-DX 300L spectrometer using glycerol as a matrix. API MS was measured with a Perkin-Elmer SCIEX API-III biomolecular mass analyzer. GC-MS was measured on a Shimadzu GC-17A, JEOL automass II spectrometer.

HIV Protease Assay HIV protease assay kits (Bachem Feinchemikalien AG, Bubendorf, Switzerland) were used. Twenty five microliter of HIV-protease assay buffer (50 mM NaOAc, pH 4.9) containing 2.5 μg of the substrate [His–Lys–Ala–Arg–Val–Leu–(pNO_2–Phe)–Glu–Ala–Nle–Ser–NH_2] was mixed with 2.5 μl of a compound solution (using DMSO as a solvent), then 12.5 μl of recHIV-protease (0.02 mg/ml) was added. The reaction mixture was incubated for 15 min at 37 °C and then terminated by addition of 2.5 μl of 10% trifluoroacetic acid (TFA). The hydrolysate (pNO_2–Phe–Glu–Ala–Nle–Ser–NH_2) and the remaining substrate were quantitatively analyzed by reversed-phase HPLC. HPLC conditions: column, YMC-Pack ODS-AP, 4.6×150 mm YMC Co., Kyoto, Japan; solvent, gradient of acetonitrile (20—40%) in 0.1% TFA; flow rate, 1.0 ml/min; detector, UV 280 nm. The substrate and the hydrolysate were eluted at 8.1 and 3.5 min, respectively. The HIV-protease inhibitory activity of a compound was calculated as follows:

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

(where A is relative peak area of the hydrolysate)

HIV protease assay kits (enzyme Lot No. D-00058 and D-00068) were used for the triterpenes and procyanidins, respectively. Acetyl pepstatin was used as a positive control, its IC_{50} being 0.09 μM and 0.3 μM, respectively for the two assay kits.

Isolation of Compounds from CH_2Cl_2 and MeOH Extracts of the Stems of *C. songaricum* The stems of *C. songaricum* were collected in May, 1995, in Wulateqianqi, Innermongolia, China. Dried stems (5 kg) were chopped into small pieces and extracted exhaustively with CH_2Cl_2 and then with MeOH at room temperature to obtain the CH_2Cl_2 and MeOH extracts (60 g and 600 g, respectively). The CH_2Cl_2 extract was subjected to silica-gel column chromatography eluting with benzene containing increasing amounts of EtOAc. Fractions eluted from the column were rechromatographed on a silica-gel column using *n*-hexane–benzene or $CHCl_3$–MeOH to obtain **1** (30 mg), **2** (10 mg), **3** (30 mg), **4** (100 mg), **5** (15 mg), **6** (100 mg) and **7** (50 mg). The MeOH extract was suspended in water and successively extracted with EtOAc and BuOH. The EtOAc extract (26 g) was chromatographed on silica gel with a CH_2Cl_2–MeOH–H_2O system. The fractions obtained were further purified by RP-8 medium pressure column chromatography with H_2O–MeOH and MCI gel CH20P with H_2O–MeOH to give **8** (20 mg), **9** (2 g), **10** (1 mg), **11** (3 mg), **12** (12 mg), **13** (6 mg), **14** (2 mg) together with **3** (30 mg) and **6** (100 mg). The BuOH extract (10 g) was subjected to silica gel using EtOAc–EtOH–H_2O as a solvent system. The fractions obtained were further chromatographed on Sephadex LH-20, MCI gel CHP20P and RP-8 to obtain **15** (40 mg), **16** (2 mg), **17** (1 mg) and **18** (9 mg).

***β*-Sitosteryl Oleate (5)** Oil. Compound **5** was treated with 5 M NaOMe in a mixture of benzene–MeOH. The solution was neutralized with 1 N HCl and then partitioned with $CHCl_3$ and water. The $CHCl_3$ layer was applied to GC-MS, which revealed that the fatty acid part of **5** was oleic acid. GC conditions: column, DB-1, J & W Scientific, 0.25 mm×30 m; column temperature, 50—250 °C, 10 °C/min then 250 °C 10 min; carrier gas, He; methyl oleate R_t 19.24 min, MS *m/z*: 296 $[M]^+$, 264, 222, 180, 123, 97, 74, 55 (100).

***β*-Sitosteryl Glucoside 6′-*O*-Aliphatate (7)** Colorless mass. Compound **7** was analyzed with GC-MS in the same manner as described above. The fatty acid part was found to be composed of 44% oleic acid, 37% palmitic acid, 15% linoleic acid and 3% stearic acid. Methyl palmitate: R_t 17.39 min, MS *m/z*: 270 $[M]^+$, 239, 227, 143, 87, 74 (100); methyl linoleate R_t 19.19 min, MS *m/z*: 294 $[M]^+$, 263, 150, 81, 67 (100) and methyl stearate R_t 19.41 min, MS *m/z*: 298 $[M]^+$, 267, 255, 199, 143, 87, 74 (100).

Synthesis of Succinic Acid, Glutaric Acid and 3′,3′-Dimethylglutaric Acid Hemiesters Triterpene (50 mg), dicarboxylic acid anhydride (1 g) and 4-dimethylaminopyridine (0.7 g) in 10 ml of tetrahydrofuran (THF) were stirred for about 4 h at 60 °C. The solution was then poured into ice-water and extracted with $CHCl_3$. The $CHCl_3$ solution was washed with 1 N H_2SO_4 and H_2O, dried over anhydrous Na_2SO_4 and concentrated to dryness under vacuum. The residue was purified by SiO_2-PTLC [developing solvent: benzene–EtOAc (7 : 3)] and RP-2 C.C. eluting the first with MeOH–H_2O (60 : 40) and then with MeOH.

Synthesis of Oxalic Acid and Malonic Acid Hemiesters Triterpene (0.1 g) dissolved in 20 ml of THF was slowly added into oxalyl chloride (0.2 g) or malonyl chloride (0.3 g) [the molar ratio of triterpene to acid chloride was 1 : 7 to 1 : 10] with stirring at room temperature. Five minutes later, the reaction mixture was poured into ice water and extracted with $CHCl_3$. The $CHCl_3$ solution was washed with water, dried over anhydrous Na_2SO_4 and then concentrated to dryness. Compounds **30** and **37** were further purified by RP-2 C.C. with 40% MeOH–H_2O and then with MeOH. The MeOH eluate was subjected to silica gel C. C. with $CHCl_3$–MeOH (93 : 7) for further purification.

Acetylation and Methylation Acetylation was carried out in the usual manner. Methylation was carried out by treatment of triterpenes with trimethylsilyldiazomethane in a mixed solvent of MeOH and benzene.

Isolation and Fractionation of Procyanidins from a Water Extract of the Stems of *C. songaricum* The stems of *C. songaricum* (5 kg, purchased from a drug store in Huhhot, Innermongolia of China in Oct., 1997) were extracted three times (1.5 l each) with H_2O at room temperature. The extract was then passed through a Diaion HP-20 column (9×55 cm) eluted with H_2O and then with MeOH. The MeOH eluate (100 g) was put into 600 ml of EtOH and filtered. The EtOH soluble part (40 g) was chromatographed on Sephadex LH-20 with EtOH (fr. 1, 11.0 g) and then with MeOH (frs. 2—4, 8.8 g, 5.7 g, 5.1 g respectively). Fractions 2 and 3 were rechromatographed on Sephadex LH-20 with H_2O–MeOH and MCI gel CHP20P with H_2O–MeOH to give **42** (30 mg), **43** (17 mg), flavan-3-ol trimers (100 mg), tetramers (62 mg) and pentamers (60 mg). A portion of fr. 4 (150 mg) was fractionated by ultrafiltration to give four fractions whose molecular sizes increased in the order of I—IV (37 mg, 9 mg, 65 mg and 25 mg, respectively) [devices used: 1. Centriprep-10, 2. Centriprep-30 (Amicon, U.S.A.), 3. Cintricon Plus-20 Biomax-100 (Millipore, U.S.A.), corresponding to 10000, 30000 and 100000 Daltons of molecular weight, respectively]. A portion of the EtOH insoluble part (1.2 g from 60 g) was separated to frs. I′—IV′ (170 mg, 176 mg, 360 mg, 450 mg, respectively) by using the same ultrafiltration method.

Procyanidin B1 (42) Amorphous powder; $[\alpha]_D^{21}$ +35.7° (c=0.55, acetone); API-MS (positive) *m/z*: 601 $[M+Na]^+$; (negative) *m/z*: 577 $[M-H]^-$.

Procyanidin B6 (43) Amorphous powder; $[\alpha]_D^{21}$ −153.0° (c=0.79, EtOH); API-MS (positive) *m/z*: 601 $[M+Na]^+$; (negative) *m/z*: 577 $[M-H]^-$.

Flavan-3-ol Trimers Amorphous powder; API-MS (positive) *m/z*: 889 $[M+Na]^+$; (negative) *m/z*: 865 $[M-H]^-$.

Flavan-3-ol Tetramers Amorphous powder; API-MS (positive) *m/z*: 1177 $[M+Na]^+$; (negative) *m/z*: 1153 $[M-H]^-$.

Flavan-3-ol Pentamers Amorphous powder; API-MS (positive) *m/z*: 1465 $[M+Na]^+$; (negative) *m/z*: 1441 $[M-H]^-$.

Thiolytic Degradation of IV′ Fraction IV′ (52 mg) was added to a mix-

ture of EtOH (10 ml), AcOH (2 ml) and benzylthiol (1 ml). The mixture was stirred at 70 °C for 48 h then concentrated *in vacuo*. The residue was applied to a Sephadex LH-20 column with benzene and then eluted with MeOH. The MeOH eluate was further purified by RP-2 C.C. with MeOH–H_2O (1 : 1) to obtain benzylthioepicatechin (2 mg). ^{1}H-NMR (MeOH-d_4) δ: 7.41 (2H, dd, J=7.0, 2.0 Hz, H-2″, 6″), 7.29 (2H, t, J=7.0 Hz, H-3″, 5″), 7.21 (1H, tt, J=7.0, 2.0 Hz, H-4″), 6.93 (1H, d, J=1.8 Hz, H-2′), 6.75 (1H, d, J=8.2 Hz, H-5′), 6.67 (1H, dd, J=8.2, 1.8 Hz, H-6′), 5.96 (1H, d, J=2.1 Hz, H-6), 5.90 (1H, d, J=2.1 Hz, H-8), 5.23 (1H, br s, H-2), 4.05 (1H, d, J=2.3 Hz, H-4), 3.95 (2H, s, –SCH_2–), 3.85 (1H, dd, J=2.3, 1.2 Hz, H-3).

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