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SYNTHESIS OF PRODELPHINIDIN TRIMER ISOLATED FROM *CISTUS ALBIDUS* AND ITS GROWTH INHIBITORY ACTIVITY AGAINST HUMAN PROSTATE CANCER CELL LINES

Masataka Mori,[a] Kiriko Matsumoto,[b] Chisato Ishihara,[b] Koichiro Kawaguchi,[c] Sei-ichi Kawahara,[c,d] Yasunao Hattori,[e] Hiroshi Fujii,[b,c,f]* and Hidefumi Makabe[a,c,f]*

[a]Sciences of Functional Foods, Graduate School of Agriculture, Shinshu University, 8304 Minami-minowa, Kami-ina, Nagano 399-4598, Japan
[b]Department of Bioscience and Biotechnology, Faculty of Agriculture, Shinshu University, 8304 Minami-minowa, Kami-ina, Nagano 399-4598, Japan
[c]Interdisciplinary Graduate School of Science and Technology, Shinshu University, 8304 Minami-minowa, Kami-ina, Nagano 399-4598, Japan
[d]St. Cousair Co. Ltd., 1260 Imogawa, Kami-minochi, Iizuna, Nagano 389-1201, Japan
[e]Center for Instrumental Analysis, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8412, Japan
[f]Department of Interdisciplinary Genome Sciences and Cell Metabolism, Institute for Biomedical Sciences, Interdisciplinary Cluster for Cutting Edge Research, Faculty of Agriculture, Shinshu University, 8304 Minami-minowa, Kami-ina, Nagano 399-4598, Japan
*E-mail: hfujii@shinshu-u.ac.jp
*E-mail: makabeh@shinshu-u.ac.jp
M. M. and K. M. equally contributed

Abstract – First synthesis of prodelphinidin trimer (epigallocatechin-gallocatechin-catechin) isolated from *Cistus albidus* was accomplished from monomeric epigallocatechin electrophile and dimeric gallocatechin-catechin nucleophile. The condensation was worked using AgOTf as a Lewis acid and condensed product was successfully converted into titled compound in good yield. Synthesized compound showed significant growth

inhibitory activity against human prostate PC-3 cell lines. Its activity was almost same as prodelphinidin B3.

INTRODUCTION

Cistus albidus (Cistaceae) is a shrub which is widely distributed in south-western Europe and western-north Africa with pink to purple flowers. This plant has been traditionally used as a tanning agent[1] and folk medicine for the treatment of gastrointestinal disorders.[2] In 2003, Qádan and co-workers reported the isolation of prodelphinidin trimers from this plant.[3] Recently we reported that syntheses of prodelphinidin B1 to B4 and C2.[4,5] We also reported that prodelphinidins had significant antitumor activity against human prostate cancer cell lines and found that cytotoxic effects were clearly associated with the presence of the pyrogallol moiety.[4,5] As to the one of the mechanism of action with regards to the anticancer activity, we reported that anticancer activity of prodelphinidins might be due to their blocking cell cycle partly at the G1/G0 phase and activating caspase-3. Thus we concluded that one of the reasons of cell death caused by prodelphinidins was apoptosis.[4] However, synthetic and biological studies on prodelphinidins are still quite limited.[6,7] As to the synthesis of prodelphinidin trimer, only one example was reported by us although synthetic examples of procyanidin trimer have been reported by several groups including us.[8] Thus we have devoted the synthesis and structural activity relationship study of prodelphinidins. Herein we further wish to report the results of our efforts to synthesize prodelphinidin oligomer. The synthetic targeted molecule is prodelphinidin trimer, whose structure is epigallocatechin-gallocatechin-catechin, isolated from *Cistus albidus*.[3] The structural unit of flavan-3-ol framework is different each other, thus it would be challenging to construct this complex molecule (Figure 1).

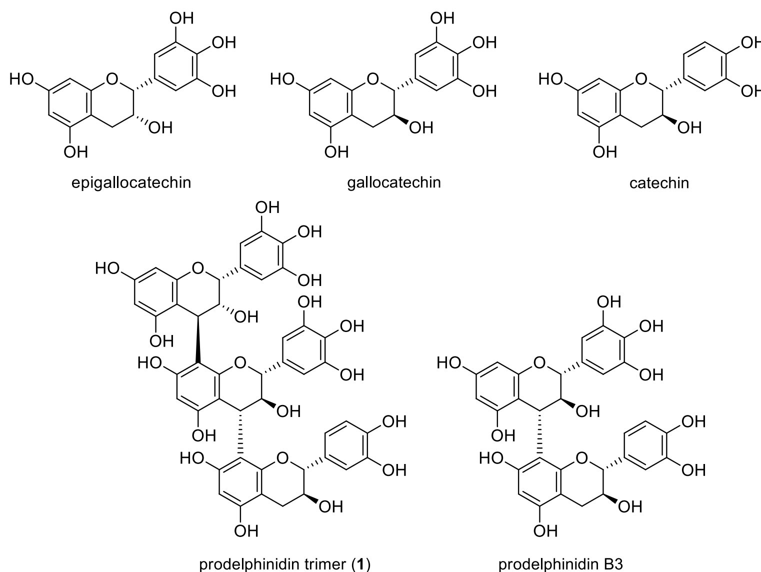
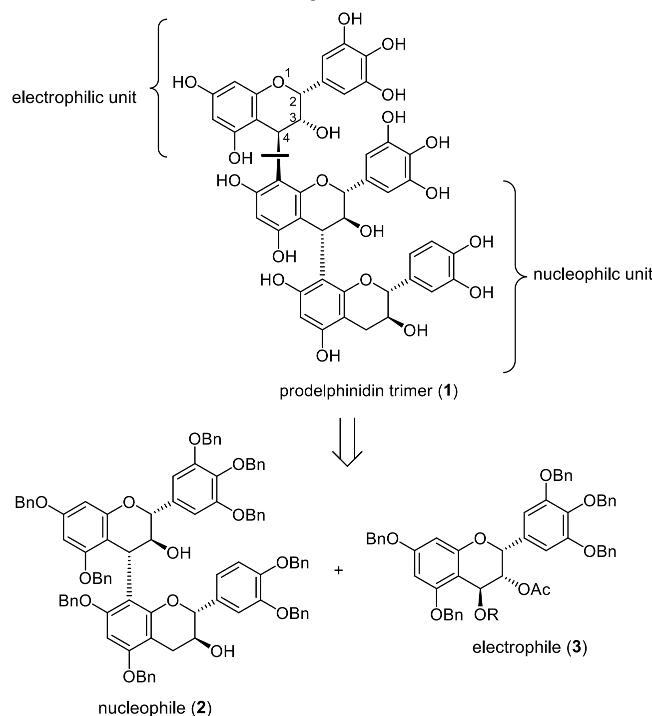


Figure 1. The structures of prodelphinin trimer (**1**) isolated from *Cistus albidus* and related compounds

RESULTS AND DISCUSSION

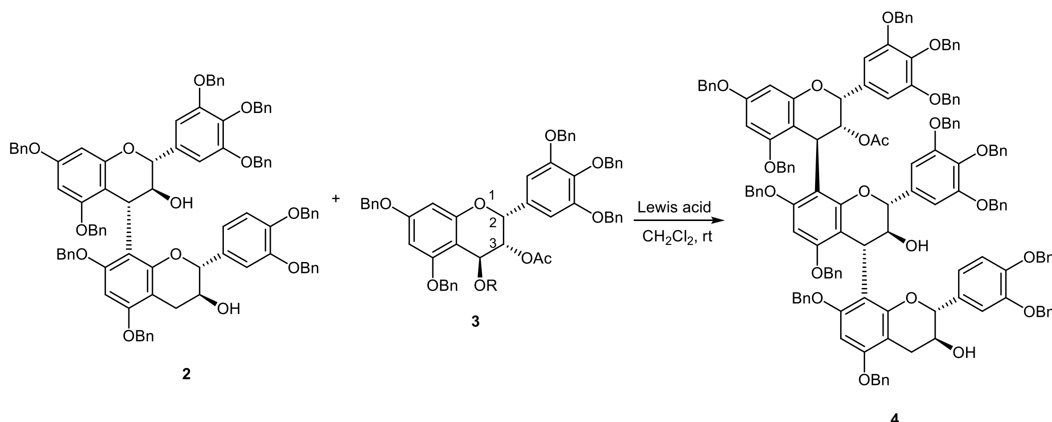
The synthetic strategy is shown in Scheme 1. The targeted molecule, epigallocatechin-gallocatechin-catechin can be divided into two unit. The one is gallocatechin-catechin nucleophile (**2**) which was prepared by us before.[4] The other is epigallocatechin electrophile which was also prepared by us in the synthesis of prodelphinidin B2.[5] Lewis acid mediated condensation between **2** and **3** would give the carbon framework of the targeted molecule (Scheme 1).



Scheme 1. Synthetic strategy of epigallocatechin-gallocatechin-catechin (**1**)

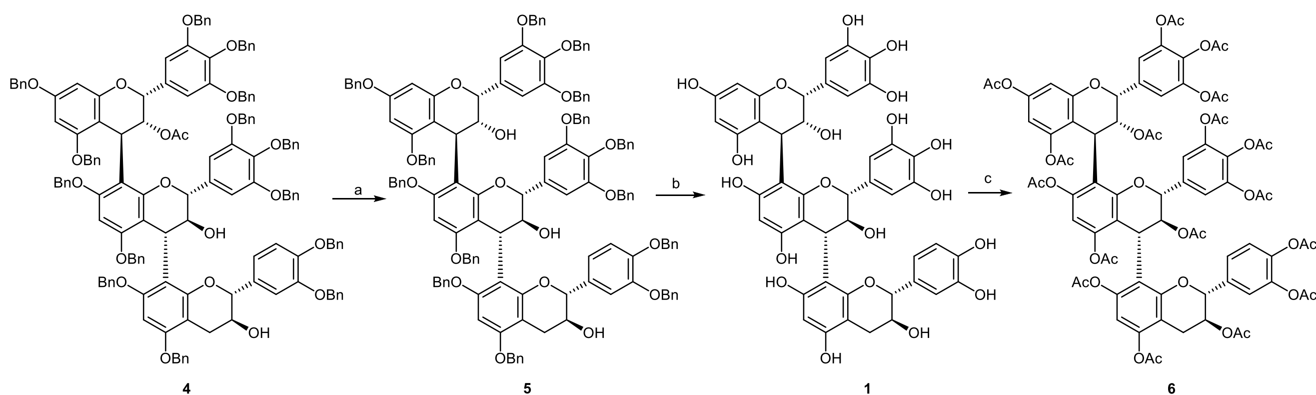
The gallocatechin-catechin nucleophile **2** and epigallocatechin electrophile **3** were prepared as we reported before.[4,5] We examined the condensation between gallocatechin-catechin nucleophile **2** and epigallocatechin electrophile **3**. At first we tried $Yb(OTf)_3$ as a Lewis acid because we have found the condensation between dimeric epicatechin electrophile and monomeric epicatechin nucleophile gave epicatechin trimer derivative in 67% yield as shown in the previous paper.[8c] However, no reaction was observed in this case. $Zn(OTf)_2$ did not furnish condensed product either. Thus we tried to use several Lewis acids and found that silver Lewis acid gave condensed product around 30% yield along with small amount of starting materials and unidentified polymerized products. The reason of the low reactivity of condensation is still unclear. One of the reasons might be a steric factor. The stereochemistry of newly formed interflavan bond was not determined at this stage due to severe overlapping of signals observed in the ^{1}H NMR spectrum. Formation of another diastereomer was not detected (Table 1).

Table 1. Condensation between dimeric epigallocatechin-gallocatechin nucleophile **2** with catechin electrophile **3**



Entry	R	Lewis acid (eq.)	Time (h)	Yield of **4** (%)
1	EE	$Yb(OTf)_3$ (2.0)	5.0	0
2	EE	$Zn(OTf)_2$ (1.0)	6.5	0
3	EE	AgOTf (1.0)	6.5	34
4	Me	AgOTf (1.0)	6.5	33
5	Me	$AgBF_4$ (1.0)	6.5	27

Because the condensed product **4** was obtained, the next attention was turned towards the synthesis of prodelphinidin trimer (**1**). Removal of the acetyl groups of **4** was achieved by treatment with n-Bu_4NOH to afford triol **5**. Finally, deprotection of all benzyl groups using $Pd(OH)_2$/C in the presence of hydrogen atmosphere in THF-MeOH-H_2O followed by lyophilization afforded prodelphinidin trimer (**1**) in good yield. The specific rotation value of synthetic **1** was close to that of the reported one.[3] We confirmed synthetic **1** was pure by HPLC analysis.[9] We also prepared peracetate of **1** (**6**) using Ac_2O and DMAP in



Scheme 2. Synthesis of epigallocatechin-gallocatechin-catechin (**1**). Reagents and conditions: (a) n-Bu_4NOH, THF, 47 h, rt, 78%; (b) H_2, $Pd(OH)_2$-C, THF/MeOH/H_2O (20:20:1), 5 h, rt, quant; (c) Ac_2O, pyridine, DMAP, 1 h, rt, 24%.

pyridine. The ^{1}H and ^{13}C NMR data of synthetic **6** were in good accordance to those of the reported values.[3] Thus newly formed chiral center in the condensation could be confirmed to be 3,4-*trans*. The reason for forming 3,4-*trans* stereochemistry can be explained by the neighboring group participation of 3-position of the acetyl group of **3** (Scheme 2).

Our interest was focused on examining the antitumor activities of the synthesized prodelphinidin trimer **1**. We investigated the antitumor activity of compound **1** against PC-3 prostate cancer cell lines together with prodelphinidin B3 (PDB3) which was previously prepared by us.[4] Results were obtained by cell count measurement and MTT assay. PDB3 was used as a positive control. As shown in Figure 3, prodelphinidin trimer **1** showed significant cytotoxic effects and its activity was almost same as prodelphinidin B3 (Figures 2 and 3).

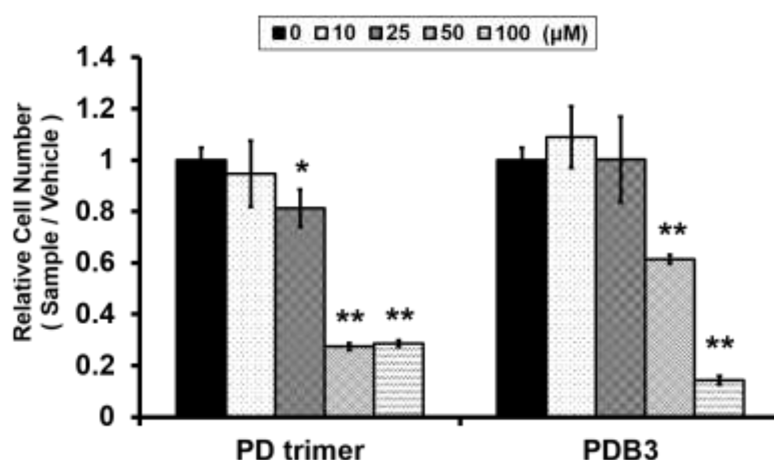


Figure 2. Effects of various concentrations of test compounds on cell proliferation using cell count method. After treatment of cells with prodelphinidin trimer (PD trimer, **1**) or PDB3 for 48 h, the cell proliferation was determined by cell count as shown in supplementary data. The values were represented as the rate of inhibition of cell proliferation by the treated sample compared to the untreated control (vehicle). Values are means ± S.Ds. for three independent experiments. Asterisks indicated a significant difference between the control- and test-compound-treated cells, as analyzed by Student's test (* $P<0.05$, ** $P<0.01$).

The MTT assay of compound **1** showed almost same tendency (Figure 3).

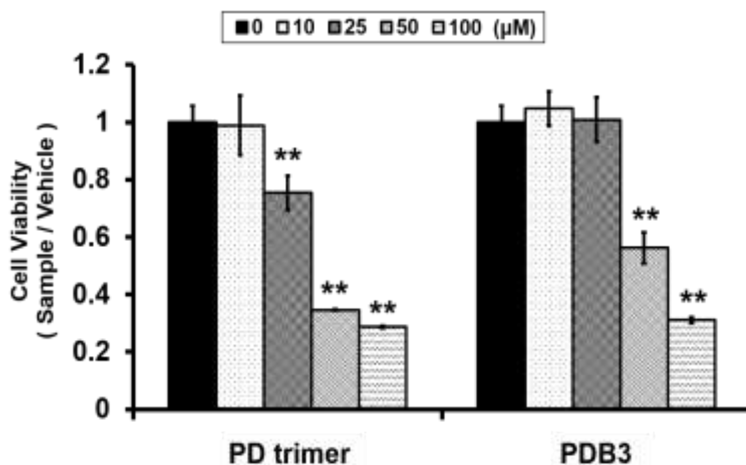
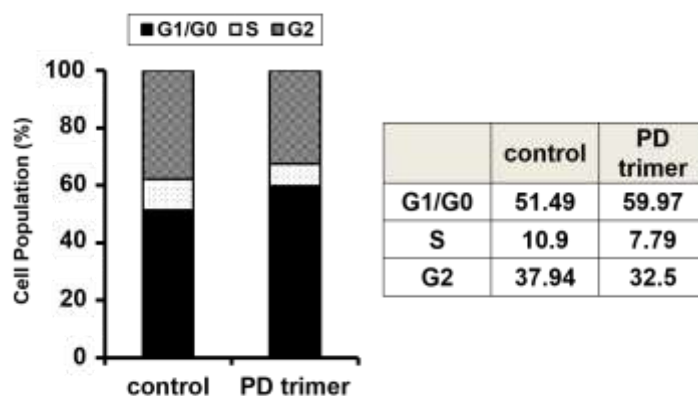


Figure 3. Effects of various concentrations of test compounds on cell proliferation using MTT assay. After treatment of cells with PD trimer (**1**) or PDB3 for 48 h, the cell proliferation was determined by MTT assay as shown in supplementary data. The values were represented as the rate of inhibition of cell proliferation by the treated sample compared to the untreated control (vehicle). Values are means ± S.Ds. for three independent experiments. Asterisks indicated a significant difference between the control- and test-compound-treated cells, as analyzed by Student's test (** $P<0.01$).

In the previous study,[4] we reported that PDB3 and prodelphinidin C2 caused G1/G0 arrest in PC-3 prostate cancer cell cycle. In this study, treatment of PC-3 prostate cancer cells with 50 μM of PD trimer (**1**) for 48 h induced a G1/G0 phase population increase from 51.49% to 59.97% and an S phase fraction decreased from 10.90% to 7.79%. Obviously, PD trimer (**1**) blocked the PC-3 prostate cancer cell cycle partly at the G1/G0 phase within 48 h. We confirmed that proanthocyanidins which have pyrogallol moiety cause G1/G0 arrest in the cell cycle (Figure 4).



	control	PD trimer
G1/G0	51.49	59.97
S	10.9	7.79
G2	37.94	32.5

Figure 4. Effects of test compounds on cell cycle distribution. The cells treated with PD trimer for 48 h were collected and stained with propidium iodide using a BD Cycletest^{TM} Plus DNA Reagent Kit (Becton Dickinson and Company BD Biosciences) obtained from Phoenix Flow Systems. Following FACS analysis, cell cycle distributions were further analyzed by Cell Quest software. The phase fraction (%) is shown in the graph. The experimental data are shown in supplementary data.

CONCLUSION

In conclusion, prodelphinidin trimer, epigallocatechin-gallocatechin-catechin (**1**), was synthesized from gallocatechin-catechin nucleophile **2** and epigallocatechin electrophile **3**. The condensation between **2** and **3** was succeeded to afford **4** using AgOTf as a Lewis acid. Compound **4** was successfully converted to targeted molecule **1** in good yield. The antitumor activities of **1** suggested that this compound showed significant cytotoxic activity. The activity was almost same as PDB3. This activity of prodelphinidin trimer might be due to its blocking cell cycle at the G1/G0 phase.

EXPERIMENTAL

General. ^{1}H and ^{13}C NMR spectra were measured with a Bruker DRX 500 FT-NMR spectrometer in $CDCl_3$ at 500 and 125 MHz, respectively. Chemical shifts were relative to tetramethylsilane as an internal standard. The coupling constants were given in Hz. Mass spectra were obtained on Shimadzu LCMS-IT-TOF mass spectrometer. IR spectra were recorded with JASCO FT-IR 480 Plus infrared spectrometer. Optical rotations were determined with a JASCO DIP-1000 polarimeter.

[4,8:4",8"]-2,3-*cis*-3,4-*trans*: 2",3"-*trans*-3",4"-*trans*: 2''',3'''-*trans*-3-Acetoxy-tetradeca-*O*-benzyl-(−)-epigallocatechin-(+)-gallocatechin-(+)-catechin (4): To a solution of nucleophile **2** (31 mg, 0.022 mmol) and electrophile **3** (20 mg, 0.022 mmol) in CH_2Cl_2 (5.0 mL) was added AgOTf (5.8 mg, 0.022 mmol) at room temperature. After being stirred for 6 h, the reaction was quenched with water. The mixture was extracted with EtOAc (10 mL x 2) and the organic layer was washed with water, brine, dried over Na_2SO_4, and concentrated. The residue was purified with preparative TLC (toluene : EtOAc = 8 : 1) to afforded **4** (17 mg, 34%) as a pale yellow oil. $[\alpha]^{20}_{D}$ −24.3 (*c* 1.03, $CHCl_3$), IR (film): 3568, 3062, 3011, 2925, 1741, 1597, 1497, 1454, 1428, 1373, 1227, 1118, 1028, 736, 696 cm^{-1}, ^{1}H NMR (500 MHz, $CDCl_3$, 0.90:0.10 mixture of rotational isomer, major isomer) δ: 7.45-7.10 (63H, m), 6.75 (2H, d, J = 7.5 Hz), 6.68 (2H, d, J = 7.5 Hz), 6.62 (2H, s), 6.35 (2H, s), 6.25 (2H, s), 6.09 (1H, s), 5.97 (1H, s), 5.75 (1H, s), 5.62 (1H, s), 5.59 (1H, s), 5.16-4.53 (30H, m), 4.52 (2H, d, J = 11.0 Hz), 4.35 (1H, d, J = 11.5 Hz), 4.10 (1H, m), 3.43 (1H, d, J = 9.5 Hz), 2.98-2.94 (1H, m), 2.35-2.25 (3H, m), 2.07-1.96 (1H, m), 1.64 (3H, s), 1.60 (1H, brs), 1.45 (2H, brs), ^{13}C NMR (125 MHz, $CDCl_3$, 0.90:0.10 mixture of rotational isomer, major isomer) δ: 169.0, 158.3, 156.3, 156.0, 155.9, 155.5, 155.3, 155.2, 154.1, 153.6, 152.7, 152.4, 149.0, 148.8, 137.9-136.7, 134.5, 134.1, 131.6, 128.8-126.9, 120.0, 114.7, 113.8, 111.8, 111.0, 105.4, 104.2, 101.8, 93.0, 92.9, 92.3, 91.3, 82.6, 80.6, 75.2, 75.0, 74.3, 74.1, 72.1, 71.2, 71.0, 70.9, 70.5, 70.4, 70.1, 69.8, 69.1, 68.3, 37.5, 33.2, 29.3, 27.9, HRMS-ESI: *m/z* $[M+Na]^{+}$: calcd for $C_{145}H_{124}O_{21}Na$; 2223.8527, found: 2223.8537.

[4,8:4",8"]-2,3-*cis*-3,4-*trans*: 2",3"-*trans*-3",4"-*trans*: 2''',3'''-*trans*-Tetradeca-*O*-benzyl-(–)-epigallocatechin-(+)-gallocatechin-(+)-catechin (5): To a solution of **4** (22 mg, 9.8 μmol) in THF (5.0 mL) was added *n*-Bu_4NOH (40% in water, 0.32 mL, 0.49 mmol). After being stirred for 47 h, the reaction mixture was diluted with water. The mixture was extracted with EtOAc (10 mL x 2) and the organic layer was washed with water, brine, dried over $MgSO_4$, and concentrated. The residue was purified with preparative TLC (toluene : EtOAc = 12 : 1) to afforded **5** (16.5 mg, 78%) as a pale yellow oil. $[\alpha]^{19}_D$ –35.7 (*c* 0.825, $CHCl_3$), IR (film): 3567, 3062, 3031, 2925, 1597, 1497, 1454, 1428, 1374, 1338, 1217, 1117, 735, 696 cm^{-1}, ^1H NMR (500 MHz, $CDCl_3$, 0.90:0.10 mixture of rotational isomer, major isomer) δ: 7.45-6.60 (70H, m), 6.32 (2H, brs), 6.28 (1H, s), 6.62 (2H, s), 5.95 (1H, s), 5.65 (1H, s), 5.59 (1H, s), 5.15-4.50 (30H, m), 4.35 (1H, d, *J* = 11.5 Hz), 4.15 (1H, brs), 4.08 (1H, m), 3.69 (2H, s), 3.43 (1H, d, *J* = 9.5 Hz), 3.00-2.93 (1H, m), 2.38-2.25 (2H, m), 2.07-1.94 (1H, m), 1.71 (1H, brs), 1.68 (1H, brs), 1.46 (1H, brs), 1.36 (1H, brs), ^{13}C NMR (125 MHz, $CDCl_3$, 0.90:0.10 mixture of rotational isomer, major isomer) δ: 158.4, 156.5, 156.1, 155.5, 155.2, 154.9, 153.6, 153.1, 152.4, 148.9, 148.8, 138.0-136.7, 134.1, 131.7, 128.7-126.8, 120.0, 114.7, 114.7, 113.7, 111.8, 111.6, 111.3, 105.09, 104.0, 101.8, 93.3, 92.9, 92.6, 91.1, 82.5, 80.6, 75.2, 75.1, 74.2, 72.7, 71.2, 71.0, 70.8-69.8, 69.0, 68.8, 68.3, 37.4, 35.3, 29.7, HRMS-ESI: *m/z* $[M+Na]^+$: calcd for $C_{143}H_{122}O_{20}Na$; 2181.8422, found: 2181.8413.

(–)-Epigallocatechin-(+)-gallocatechin-(+)-catechin (1): A solution of **5** (15.6 mg, 7.2 μmol) in THF/MeOH/H_2O (20/20/1) (4.1 mL) was hydrogenated over 20% $Pd(OH)_2/C$ (13 mg) for 5 h at room temperature. The mixture was filtered and the filtration residue was washed with MeOH (10 mL). The combined filtrates were evaporated, and the residue was taken up in distilled water (5.0 mL). The solution was filtered and lyophilized to give **1** (7.4 mg, quant.) as a fluffy amorphous solid, $[\alpha]^{19}_D$ –139 (*c* 0.57, MeOH), {lit. $[\alpha]^{20}_D$ –176 (*c* 0.10, MeOH)},[3] IR (KBr): 3365, 1610, 1520, 1451, 1211, 1141, 1041, 981cm^{-1}, ^1H NMR (500 MHz, CD_3OD) δ: 6.86-5.50 (11H, m), 4.80-3.30 (6H, m), 2.75-2.35 (4H, m), ^{13}C NMR (125 MHz, CD_3OD) δ: 158.6, 157.9, 157.2, 156.9, 155.9, 155.8, 146.7, 146.6, 146.3, 146.2, 145.3, 132.2, 131.8, 120.2, 120.1, 119.5, 116.3, 116.2, 116.1, 115.3, 108.5, 108.3, 107.9, 107.2, 101.7, 100.8, 97.4, 96.9, 96.3, 95.5, 84.3, 83.0, 82.9, 82.0, 73.5, 68.8, 68.6, 38.7, 30.8, 28.6; HRMS-ESI: *m/z* $[M+H]^+$: calcd for $C_{45}H_{39}O_{20}$; 899.2035, found: 899.2004.

Peracetate of 1 (6). To a solution of **1** (18 mg, 20 μmol) in pyridine (0.25 mL) was added Ac_2O (0.25 mL, 3.1 mmol) and DMAP (0.2 mg). After being stirred for 1 h, the reaction was quenched with saturated aqueous $NaHCO_3$ solution (2 mL). The mixture was extracted with EtOAc (10 mL x 2) and the organic layer was washed with water, 0.5 M phosphate buffer, saturated aqueous $NaHCO_3$, dried over $MgSO_4$,

and concentrated. The residue was purified with preparative TLC (hexane : EtOAc = 1 : 5) to afforded **6** (7.8 mg, 24%) as a pale yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.03 (1H, d, *J* = 8.5 Hz), 6.89 (2H, s), 6.83 (2H, brs), 6.69 (1H, s), 6.64 (1H, s), 6.46 (1H, d, *J* = 2.0 Hz), 6.30 (1H, d, *J* = 2.5 Hz), 6.22 (1H, dd, *J* = 8.5, 2.0 Hz), 6.04 (1H, d, *J* = 2.0 Hz), 5.59 (1H, dd, *J* = 10.5, 10.5 Hz), 5.41 (1H, brs), 5.20 (1H, m), 5.14 (1H, brs), 4.94 (1H, brs), 4.39 (1H, brs), 4.38 (1H, d, *J* = 11.0 Hz), 4.29 (1H, d, *J* = 10.5 Hz), 2.63 (1H, m), 2.28 (1H, m), 2.40-1.65 (51H, m), ^{13}C NMR (125 MHz, $CDCl_3$) δ: 171.28-166.24, 155.48, 155.10, 150.68, 149.19, 148.38, 148.06, 147.79, 147.49, 147.05, 143.14, 142.90, 141.93, 141.61, 135.19, 135.03, 134.59, 134.40, 134.28, 124.28, 122.56, 119.38, 119.23, 117.99, 117.03, 116.96, 114.47, 109.97, 108.58, 108.28, 107.00, 78.98, 76.42, 72.65, 71.21, 68.68, 67.03, 36.33, 33.78, 29.69, 21.12-20.18.

Biochemical methods

Cell lines, cell culture and reagents. Human prostate cancer cell, PC-3, was purchased from the Health Science Research Resources Bank. The cells were maintained in monolayer culture at 37 °C and 5% CO_2 in RPMI-1640 (SIGMA, R8755) supplemented with 10% charcoal-stripped fetal bovine serum (Biological Industries, No. 04-201-1), 1% antibiotic-antimycotic mixed stock solution (Nacalai Tesque, No. 09366-44). The cells were treated with various concentrations of prodelphinidin B3 (PDB3) or prodelphinidin trimer (PD trimer, **1**).

Cell count. Cells were plated in 12-well plates (1×10^4 cells/well) and grew to reach 50% confluent. The cells were treated with the indicated concentrations of EGCG, PDB3 or PD trimer (**1**) for 48 h. The cells treated with the above test compounds were trypsinized. After adding the culture medium to each well, they were agitated by pipetting. The number of cells was measured with the hemocytometer.

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. The degree of cell proliferation was evaluated by Cell Count Reagent SF (Nacalai Tesque, No. 07553-15), according to the manufacturer's protocol. The cells were plated in 96-well plates and treated with the indicated concentrations of PDB3 or PD trimer (**1**) for 48 h. Absorbance at 450 nm was measured using the microplate reader after the addition of the Cell Count Reagent.

Statistical analysis. Each experiment was performed at least three times. Data were expressed as the means ± standard deviation (S. D.). Statistical analysis was performed using Student's t-test. $P < 0.05$ was considered to be significant.

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9. HPLC measurement condition: column; InertSustain C18, 250×4.6 mm GL-Sciences, eluent MeCN-0.2%AcOH (5:95~25:75), flow rate: 0.8 mL/min, detection: UV 280 nm, retention time: 5.85 min.