

Supplementary Information

Verproside, the most active ingredient in the YPL-001 isolated from *Pseudolysimachion rotundum* var. *subintegrum*, decreases inflammatory response by inhibiting PKC δ activation in human lung epithelial cells

• Supplementary Figures S1 and S2

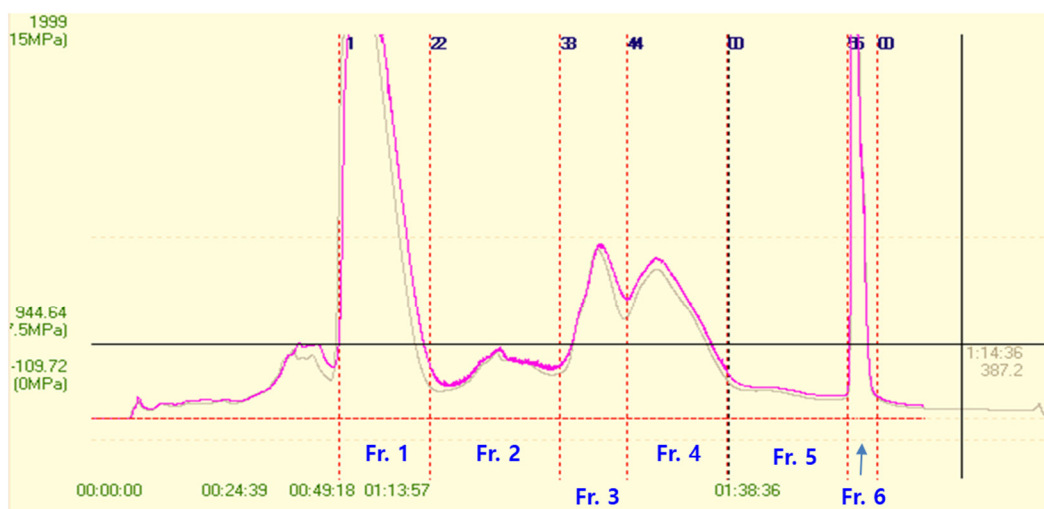
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Experimental Section

¹H, and ¹³C) NMR spectra were obtained on JEOL ECZ500R (¹H NMR at 500 MHz, ¹³C NMR at 125 MHz, Tokyo, JP), Varian UNITY 400 NMR(¹H NMR at 400 MHz, ¹³C NMR at 100 MHz, PaloAlto, USA) and Bruker AVANCE III HD 700 (¹H NMR at 700 MHz, ¹³C NMR at 175 MHz) using acetone-*d*₆ (Cambridge Isotope Laboratories, Andover, MA) as an NMR solvent and tetramethylsilane (TMS) as an internal standard. HRESIMS were measured on an ultraperformance liquid chromatography quadrupole time-of-flight mass spectrometer (UPLC-QToF-MS, Waters, Milford, MA, USA) in the negative-ion mode. Preparative HPLC was performed with a K-Prep LAB-300G instrument (YMC, Kyoto, Japan). Semipreparative HPLC separation (Gilson, Middleton, WI, USA) is comprised of a standard binary pump (321 HPLC pump), UV/Vis detector (172 DAD), evaporative light-scattering detector (ELSD, Varian 380-LC), and injection modules (GX271 liquid handler).



Condition		Method - Gradient		
YPL_001		Time(min)	%A	%B
Instrument	YMC_LAB	(Initial)	73	27
Guard Column	YMC-ODS-AQ-HG-10 um, 298 g YMC-ODS-AQ-HG-10 um, 220 g	10.00	73	27
Solvents	MeOH/D.W	60.00	65	35
Flow Rate	150 ml/min	61.00	0	100
UV	254 nm	76.00	0	100
Loading sample	4 g/9 ml	77.00	73	27
		97.00	73	27

Figure S1-1. Preparative HPLC fractionation of *P. rotundum* var. *subintegrum* extract (YPL-001).

UPLC method

- ① UPLC
Waters Acquity UPLC system
- ② Column
ACQUITY UPLC® BEH C₁₈ 1.7µm 2.1x100mm
- ③ Absorbance
254nm
- ④ Concentration : 1mg/ml
- ⑤ Injection volume : 3 µl
- ⑥ Gradients

Time(min)	Flow(mL/min)	%A(0.1% Formic acid/DW)	%B(0.1% Formic acid/ACN)
0.00	0.4	90	10
1.00	0.4	90	10
10.50	0.4	77	23
12.00	0.4	2	98
13.30	0.4	2	98
13.40	0.4	90	10
15.00	0.4	90	10

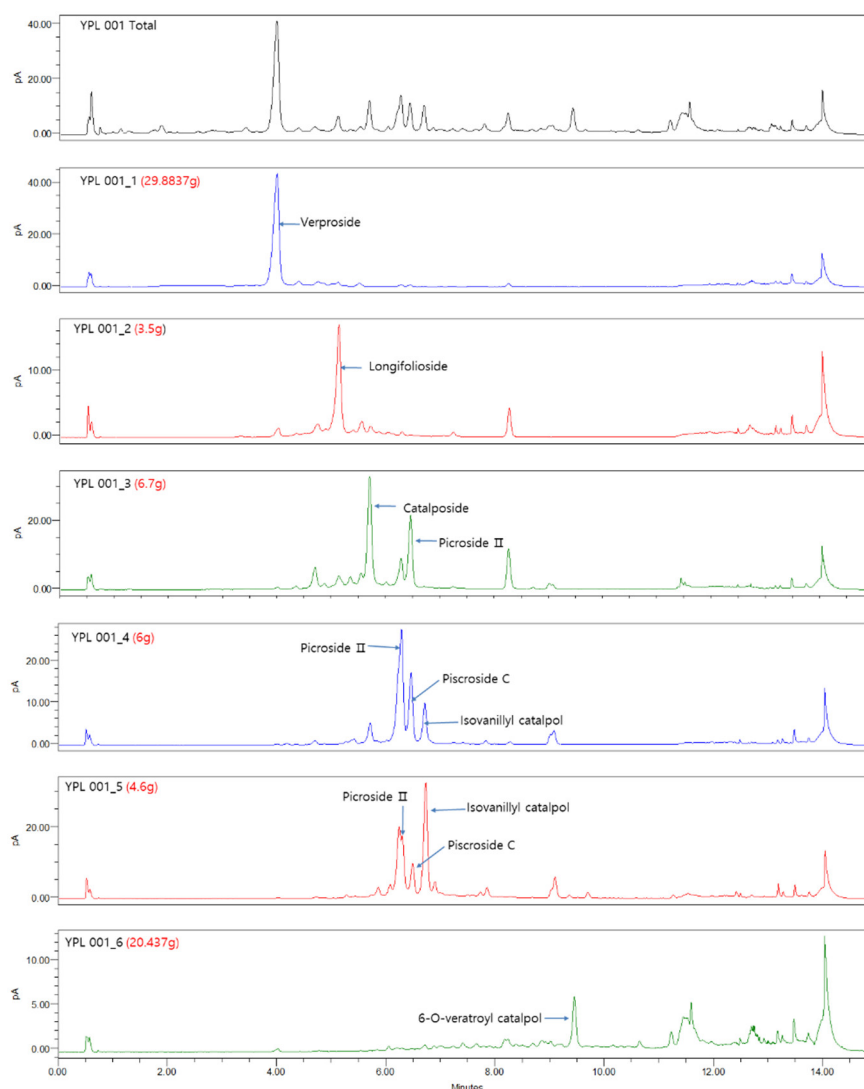


Figure S1-2. UPLC-PDA of fractions of *P. rotundum* var. *subintegrum* extract (YPL-001).

YPL001_1: Fr.1 and Fr.2, YPL001_3: Fr.3, YPL001_4: Fr.4, YPL001_5: Fr.5, YPL001_6: Fr.6

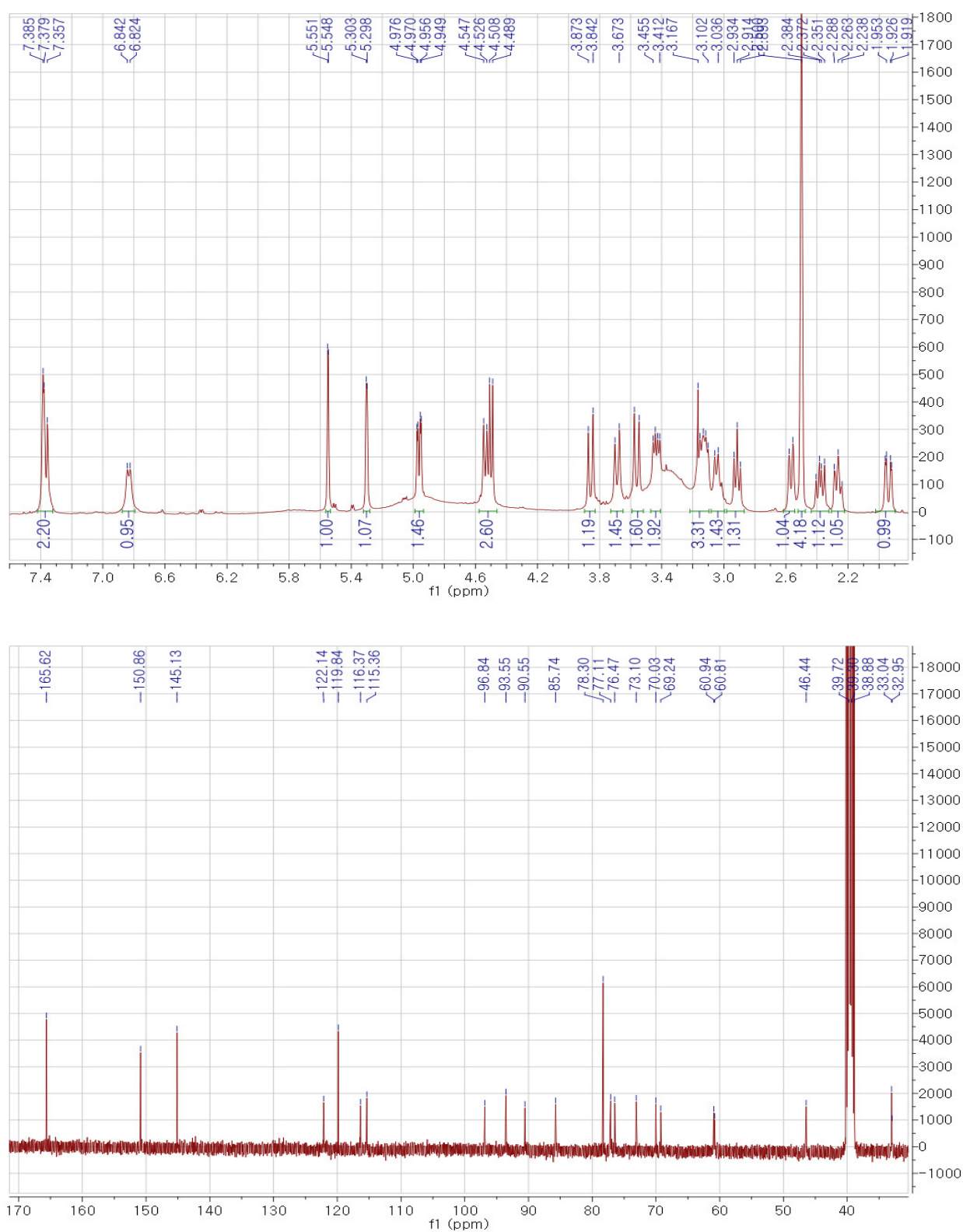


Figure S1-3. ¹H and ¹³C NMR spectrum of Piscroside C (1).

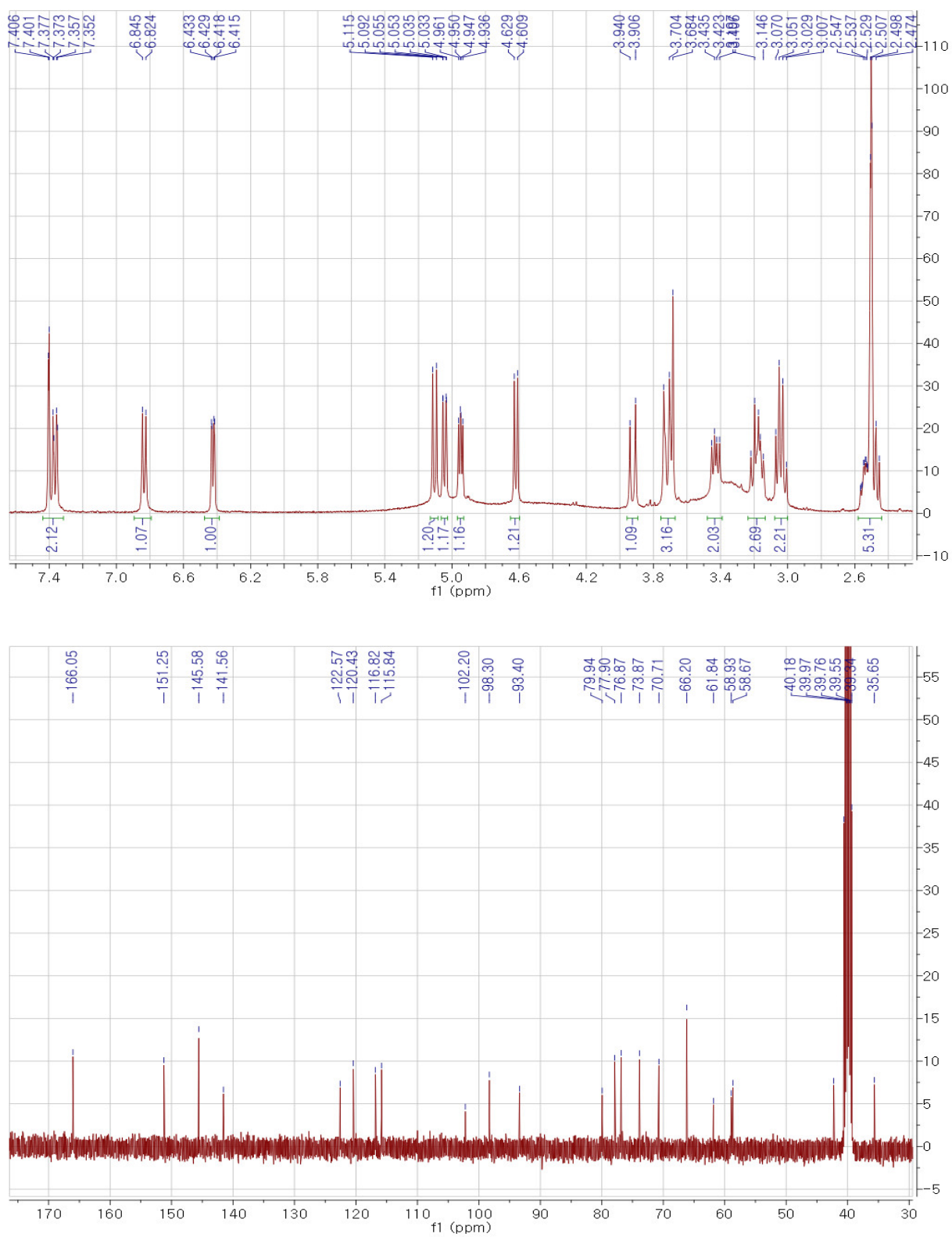


Figure S1-4. ^1H and ^{13}C NMR spectrum of Verproside (2).

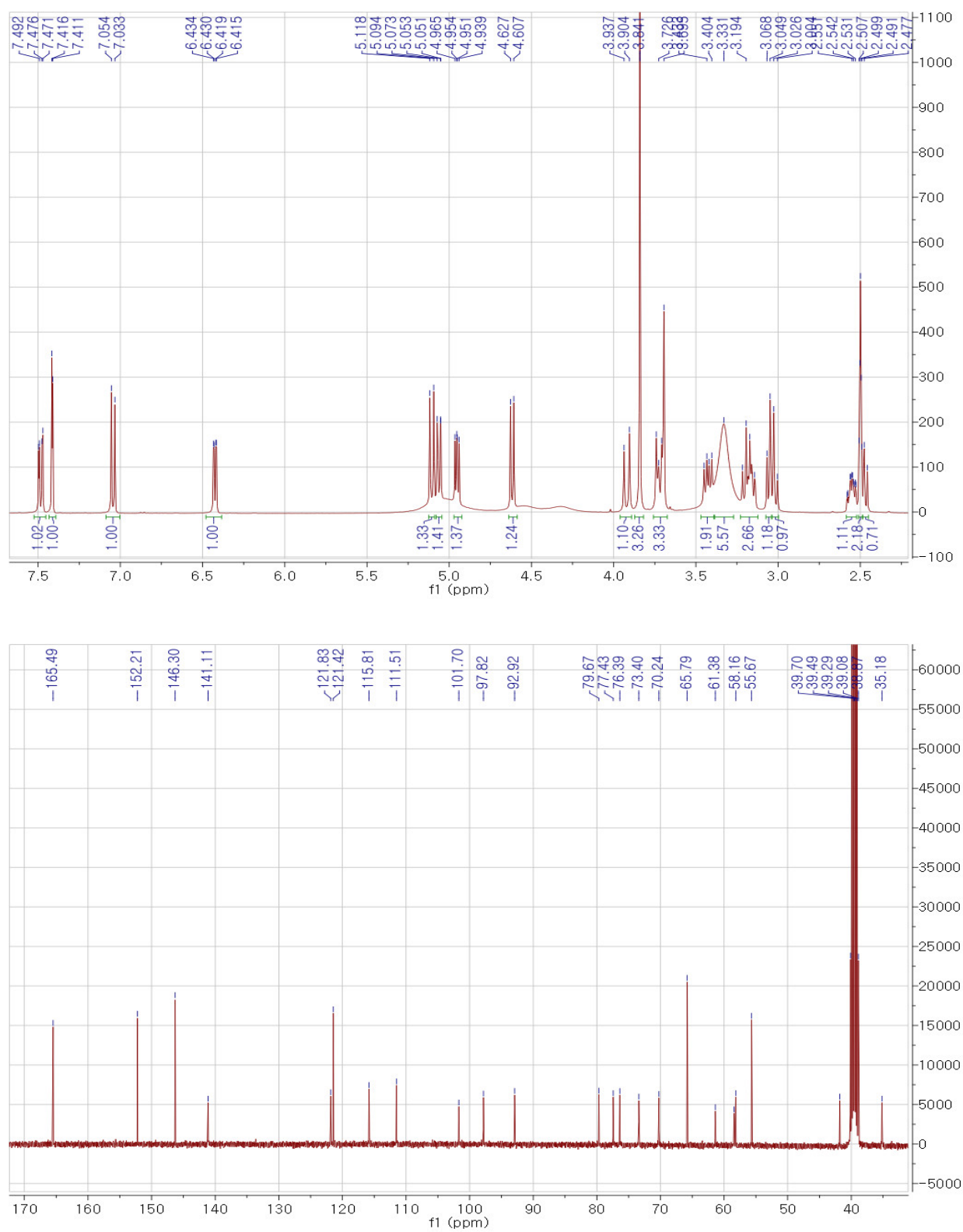


Figure S1-5. ¹H and ¹³C NMR spectrum of Iovanillyl catalpol (3).

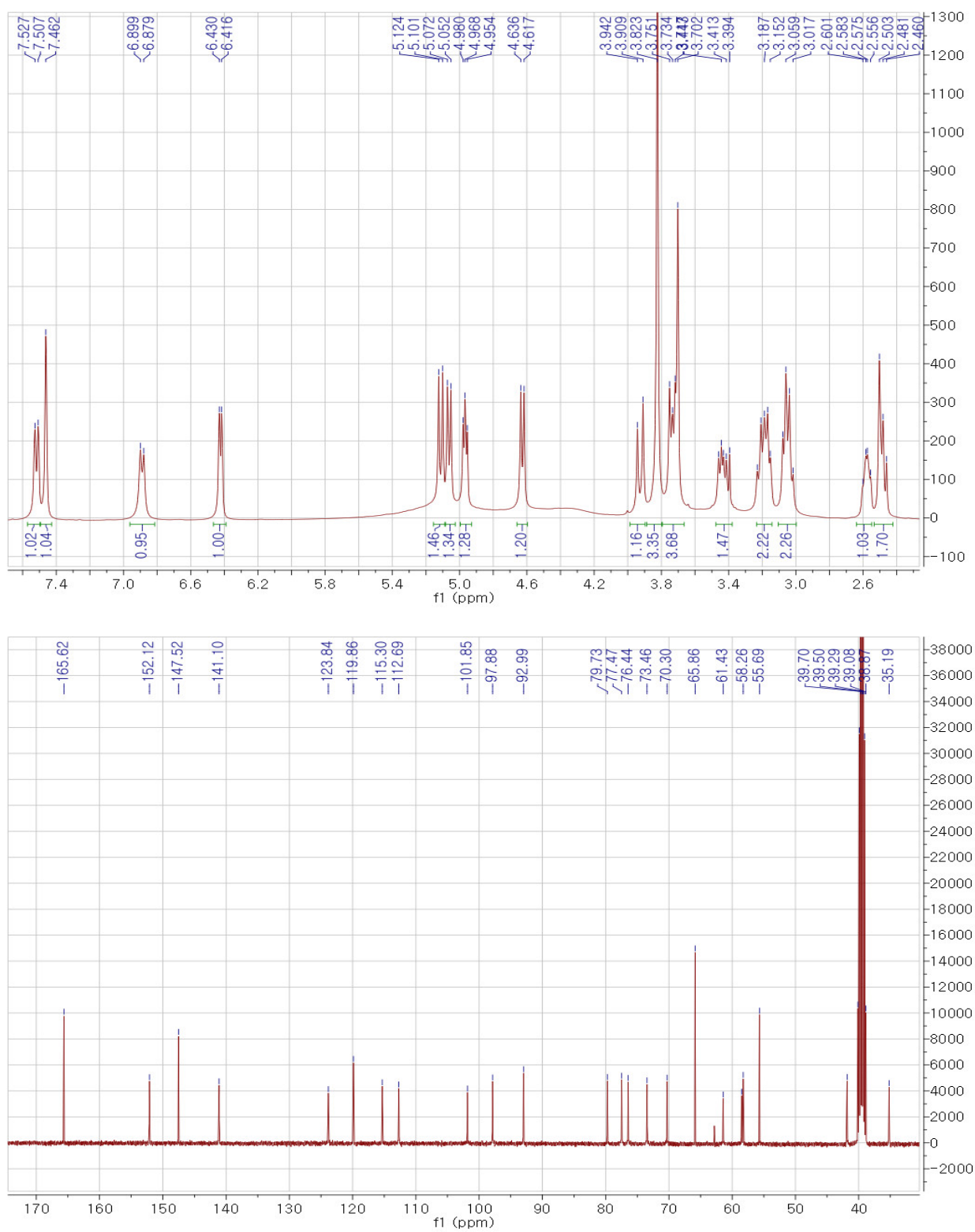


Figure S1-6. ¹H and ¹³C NMR spectrum of Picroside II (4).

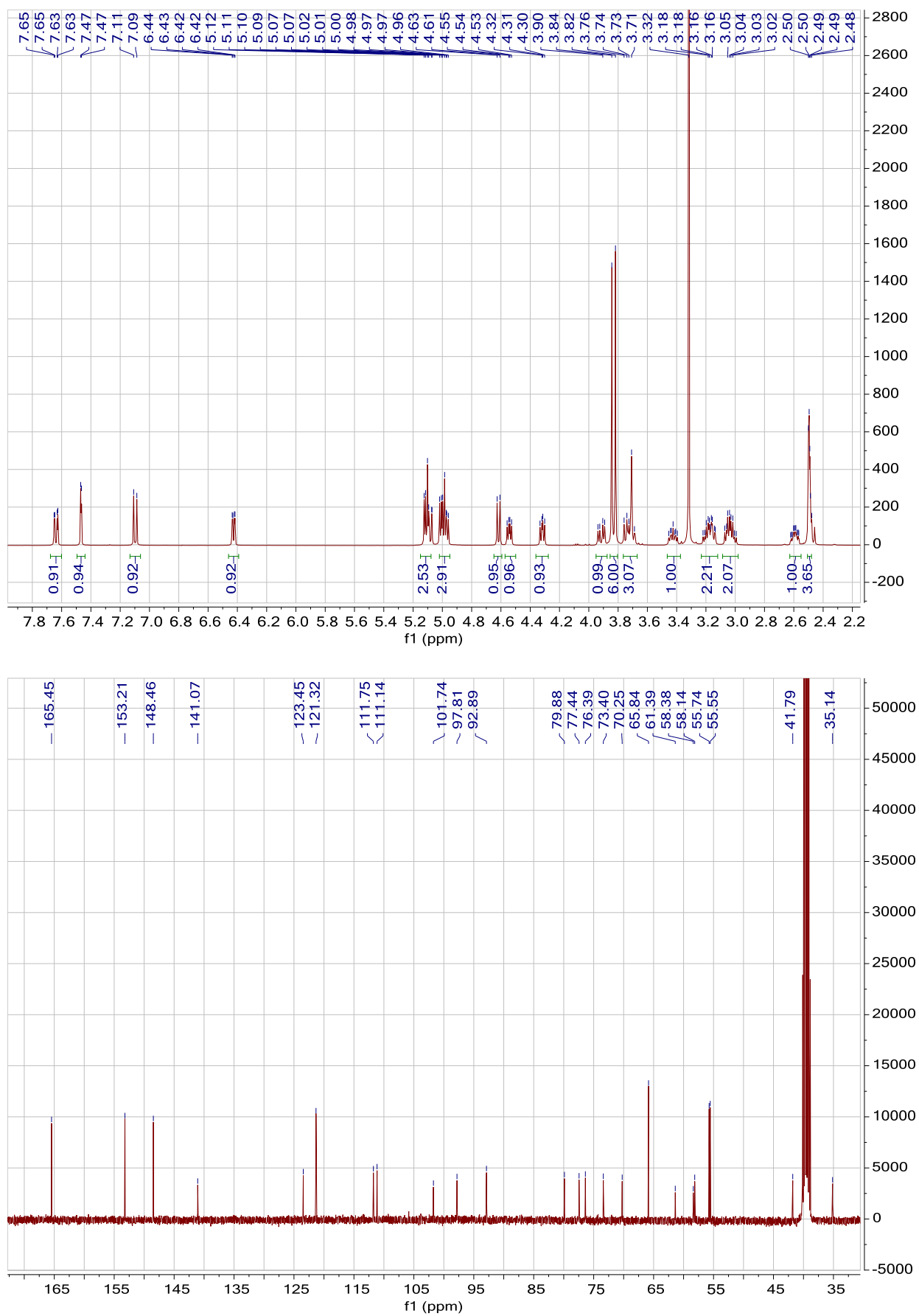


Figure S1-7. ¹H and ¹³C NMR spectrum of 6-O-Veratroyl catalpol (5).

UPLC-QToF-MS analysis

P. rotundum var. *subintegrum* extract (YPL-001) profiling was performed using an ACQUITY UPLCTM system (Waters Corporation, Milford, MA, USA) equipped with a binary solvent delivery manager and a sample manager coupled to a Micromass Q-TOF Premier™ mass spectrometer (Waters Corporation) with electrospray ionization (ESI) interface with MassLynex V4.1 software. Chromatographic separation was performed using an ACQUITY BEH C18 chromatography column (2.1 × 100 mm, 1.7 μm). The column temperature was maintained at 35°C, and the mobile phases A and B were water with 0.1% formic acid and acetonitrile with 0.1% formic acid, respectively. The gradient elution program was as follows: 0.0-1.0 min, 10% B; 1.0-10.5 min, 10-23% B; 10.5-12.0 min, 23-98% B; wash for 1.4 min with 100% B; and a 1.6 min recycle time. The injection volume was 2.0 μL, and the flow rate was 0.4 mL/min. The mass spectrometer was operated in positive ion mode. N₂ was used as the desolvation gas. The desolvation temperature was 350°C, the flow rate was 500 L/h, and the source temperature was 110°C. The capillary and cone voltages were 2300 V and 50 V, respectively. The Q-TOF Premier™ was operated in v mode with a 9000 mass resolving power. The data were collected for each test sample from 100 to 1500 Da with 0.25-s scan time and 0.01-s interscan delay over the 15 min analysis time. Leucine-enkephalin was used as the reference compound (*m/z* 554.2661 in the negative mode).

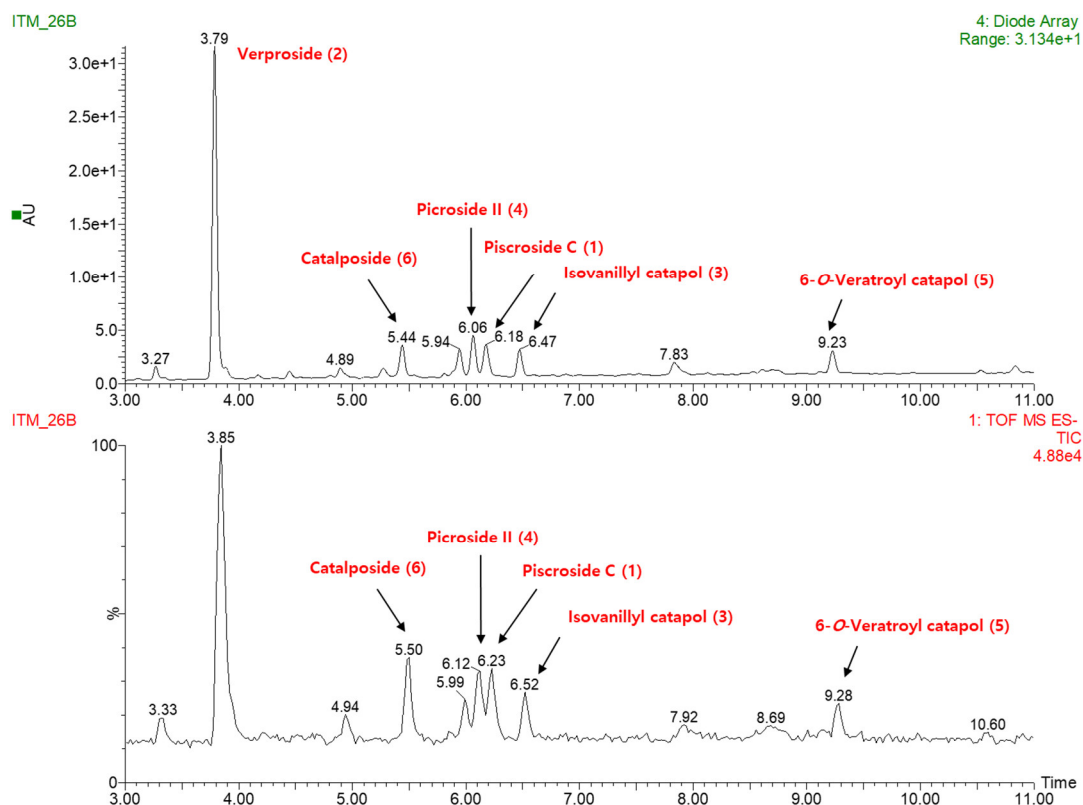


Figure S2-1. UPLC-QToF-MS chromatogram of *P. rotundum* var. *subintegrum* extract (YPL-001).

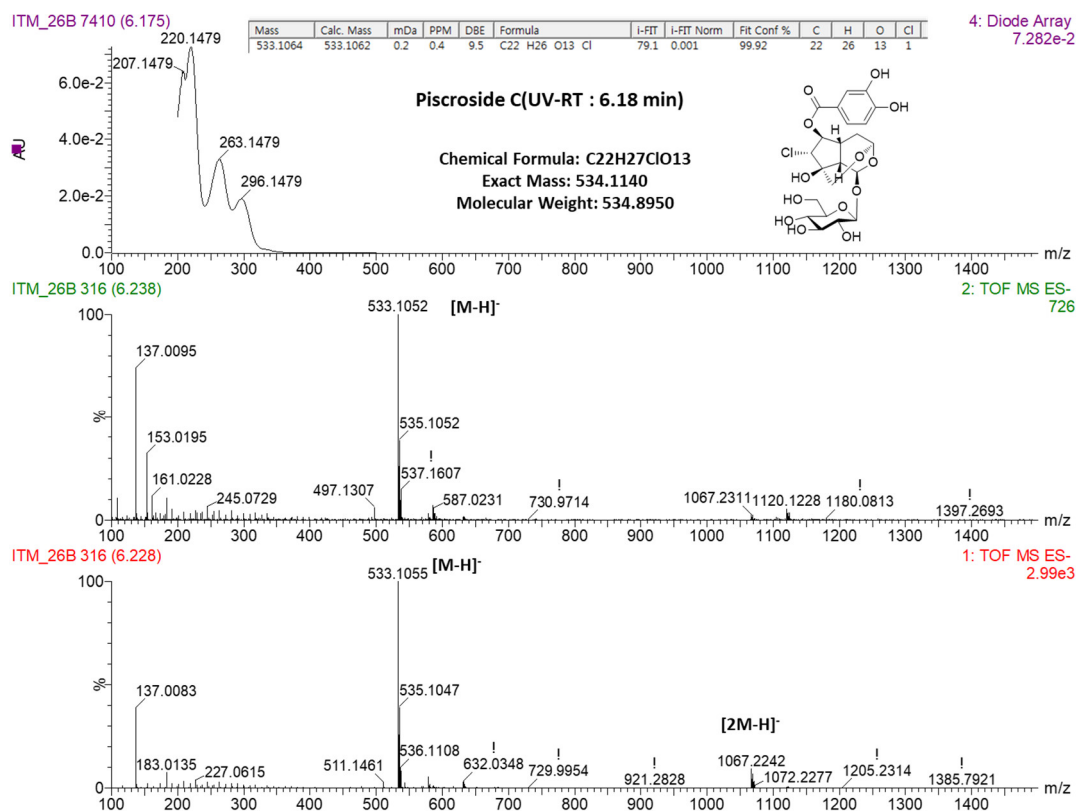


Figure S2-2. UV, MS/MS, MS and HREIMS data of Piscroside C (1).

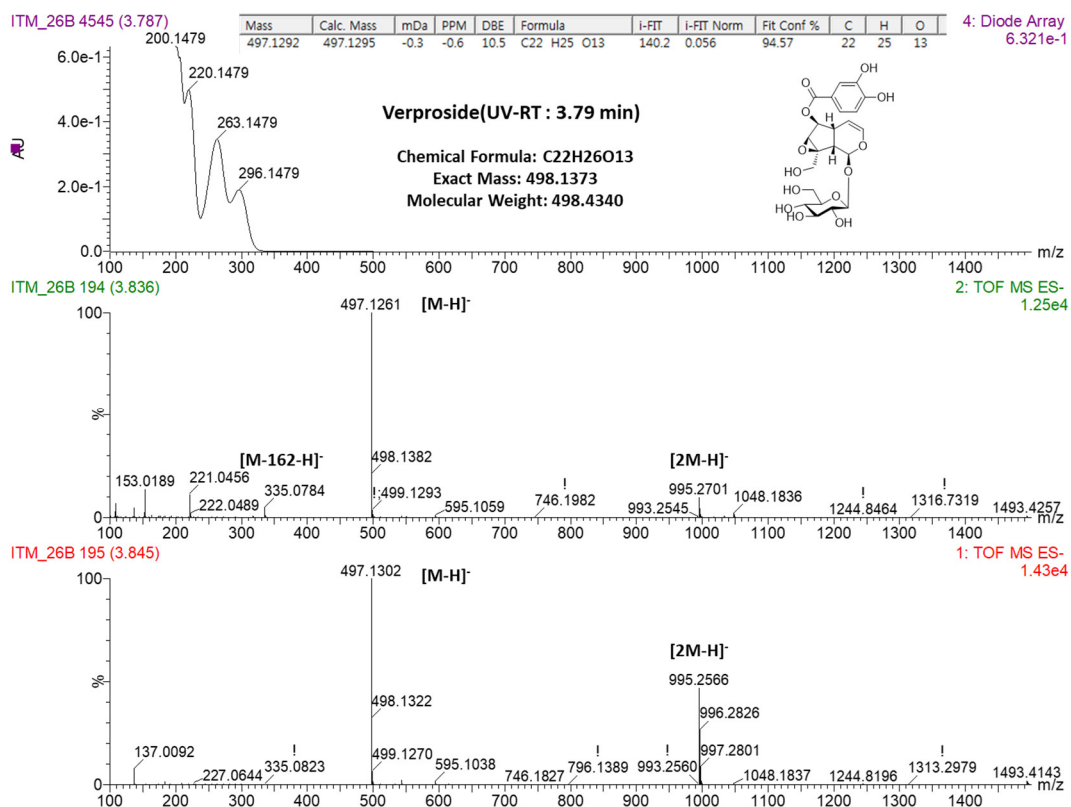


Figure S2-3. UV, MS/MS, MS and HREIMS data of Verproside (2).

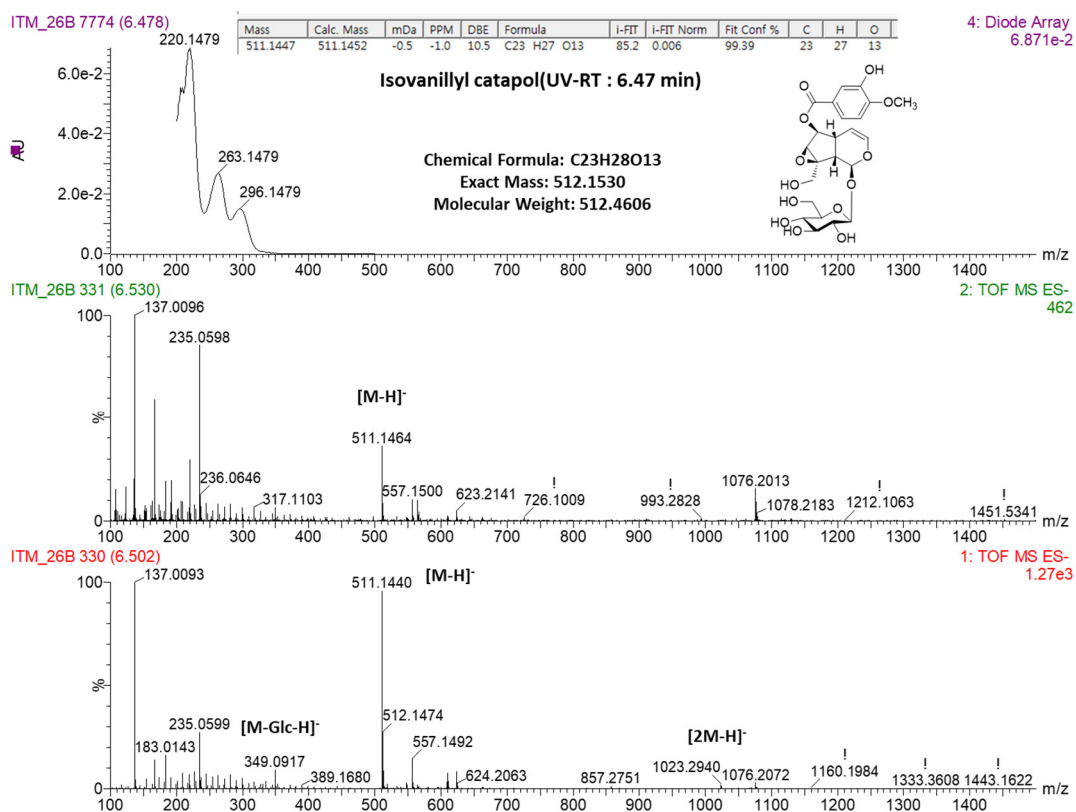


Figure S2-4. UV, MS/MS, MS and HREIMS data of Isovanillyl catapol (3).

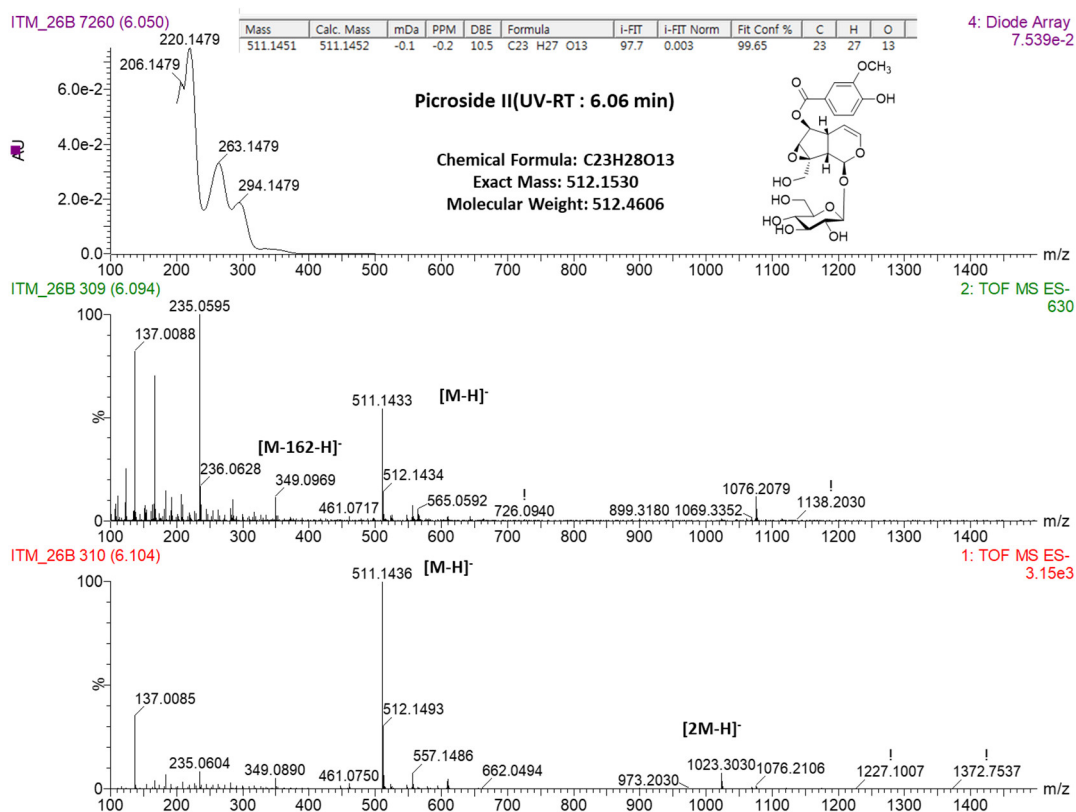


Figure S2-5. UV, MS/MS, MS and HREIMS data of Picroside II (4).

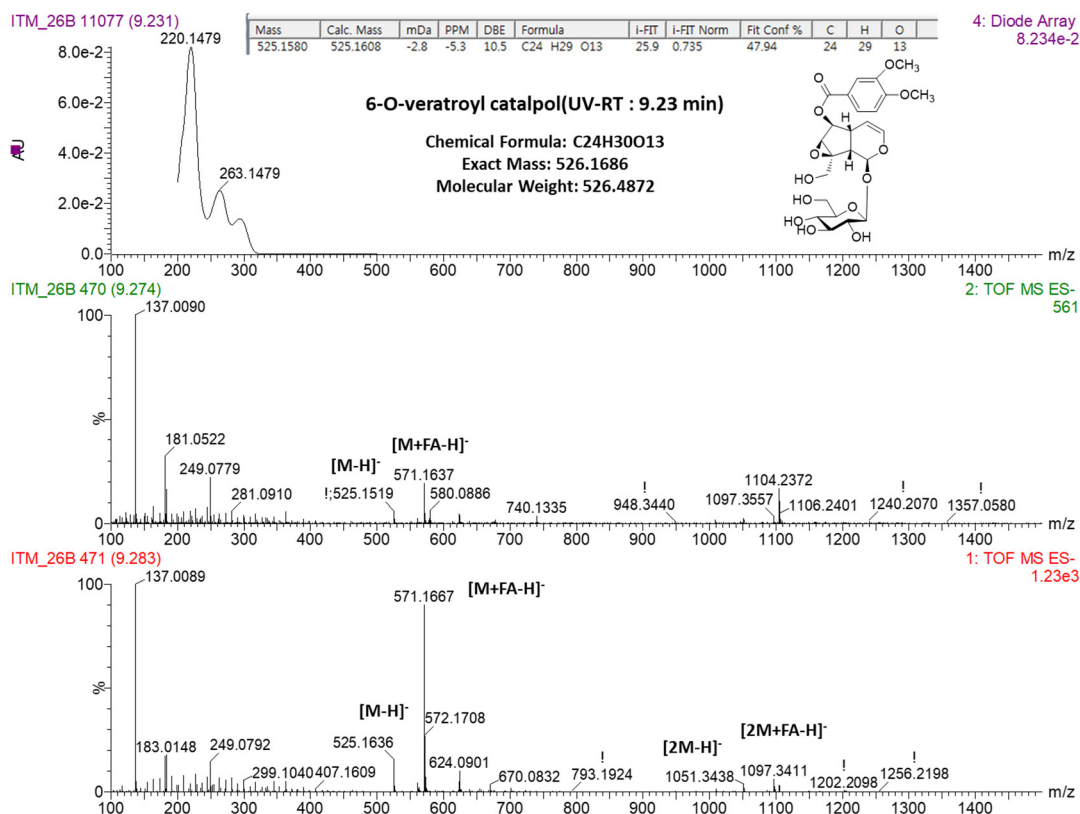


Figure S2-6. UV, MS/MS, MS and HREIMS data of 6-O-Veratroyl catalpol (5).

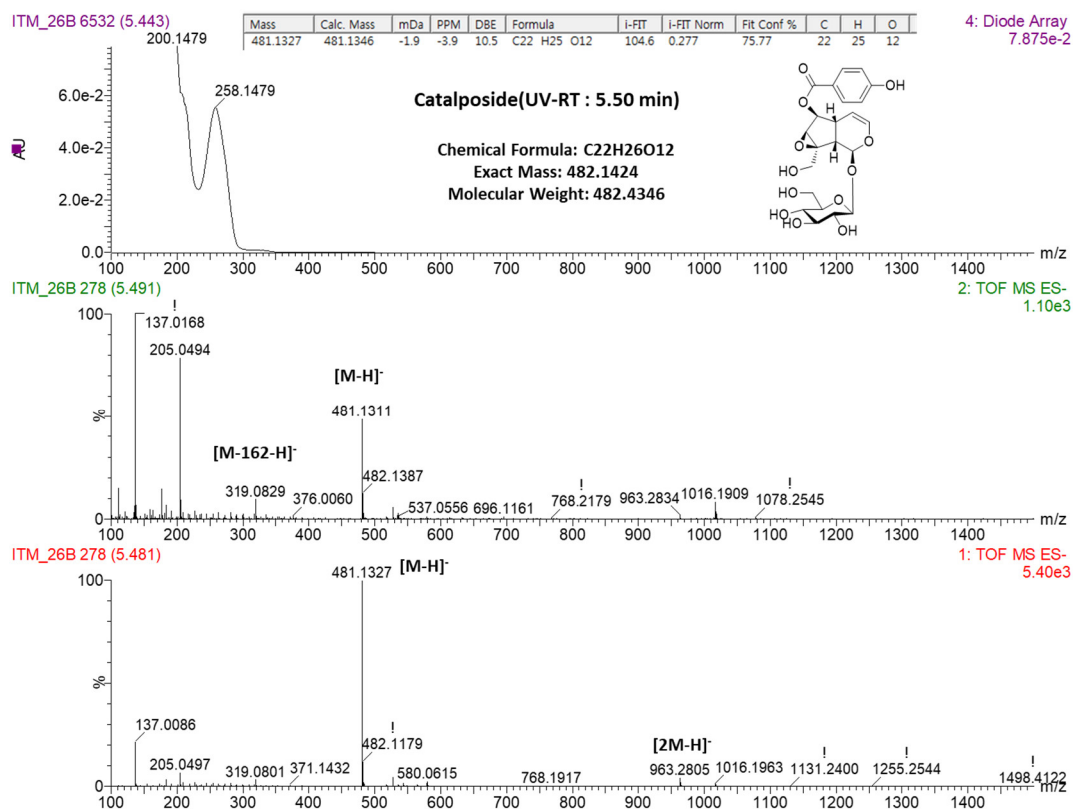


Figure S2-7. UV, MS/MS, MS and HREIMS data of Catalposide (6).

Western blot analysis

Western blots were performed as described in the "Materials and Methods" section. Here, we show the uncropped and unprocessed original image of the western blot image shown in our data figures.

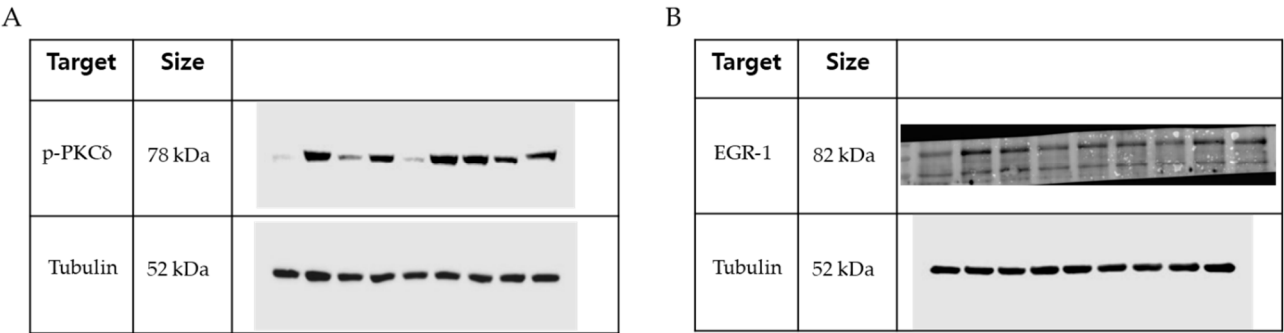
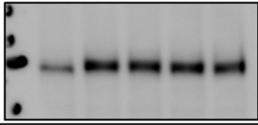
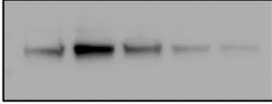
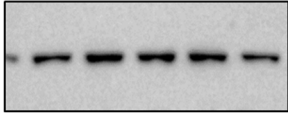
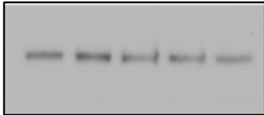
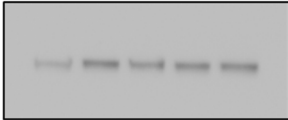
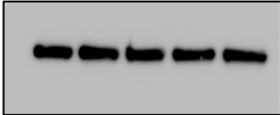


Figure S3. Original western blots in Figure 5. (A) Uncropped and unprocessed images of Figure 5E. (B) Original images of Figure 5F.

A

Target	Size	
p-PKC (pan)	78 kDa	
p-PKC δ (Thr505)	78 kDa	
p-PKC θ (Thr538)	78 kDa	
p-PKC α/β II (Thr638/641)	78 kDa	
p-PKC μ (Ser744/748)	129 kDa	
Tubulin	52 kDa	

B

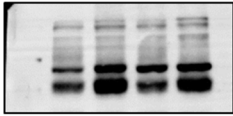
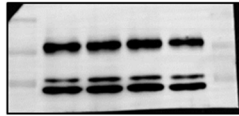
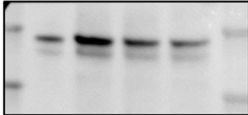
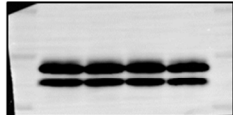
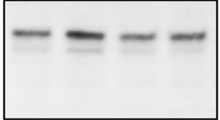
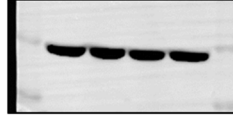
Target	Size	
p-PKC δ	78 kDa	
PKC δ	78 kDa	
p-ERK	40/42 kDa	
ERK	40/42 kDa	
EGR-1	82 kDa	
Actin	52 kDa	

Figure S4. Original western blots in Figure 6. (A) Uncropped and unprocessed images of Figure 6A. (B) Original images of Figure 6B.

Methods for Supplementary Table S1

Protein structure and compound preparation for computational calculations

We prepared protein kinase of PKC δ for molecular docking simulation. Since the PKC δ structure is unavailable in Protein Data Bank (PDB), we performed a homology modeling simulation based on the given PKC δ sequence via PQR-SA (Pseudo Quadratic Restraint with Simulated Annealing)[PMID: 22648914]. The best template structure of 3PFQ was used with a total of 10 template structures to generate homology restraint potentials (distances and dihedral angles), and the potent maximum sequence identity from template structures is 0.84. We performed a simulated annealing method as a global optimizer with the generated restraint potentials to get the energy optimal structure. We picked the best energy structure from the generated structures. The CHARMM program (Chemistry at HARvard Macromolecular Mechanics: <http://www.charmm.org>) was used for these processes. For docking simulations using the structure of the six compounds, the 2D structure of each compound was downloaded from the PubChem site (<https://pubchem.ncbi.nlm.nih.gov>), and the 3D structure for each compound was formed using the MarvinSketch program (ChemAxon; <http://www.chemaxon.com>; 5.11.4, 2012).

Protein-compound docking simulations

Protein-compound docking simulations were conducted using the AutoDock Vina program (<http://vina.scripps.edu>). In order to select the docking position of the compounds, the C α coordinate of the protein backbone was used as the docking center. The box size of the docking grid was 20Å×20Å×20Å. Flexible ligand docking simulations were performed 20 times with different random seeds in each box. After the compounds were docked at each docking center, ligand clustering was performed again to classify the docked ligand structures by orientations and positions. The ligand clustering calculated the ligand's center of geometry (COG) and classified the ligand where the COG is located within 1Å. In order to select the ligand structure with the lowest binding energy in each cluster, the free energy ($\Delta G = \text{lowest energy} + (-kT \ln N)$, where k is Boltzmann constant, T is room temperature

(300K), and N is the number of cluster structures) reflecting the population size was calculated. The lowest energy means the lowest docking energy in each cluster. After that, we sorted them in the order of the docking energy, and the results, including the best two structure energies, are tabulated in **Table S1**.

Table S1. Molecular docking energy of PKC δ with six components (1-6)

	Compounds	Docking energy in kcal/mol between PKC δ and compounds	
		The lowest energy	The second-lowest energy
1	Picroside C	-7.2 ^a (ATP) ^b	-7.1 (ATP)
2	Verproside	<i>-7.5 (Surface)</i>	-7.4 (ATP)
3	Isovanillyl catalpol	-7.3 (Near)	-7.2 (ATP)
4	Picroside II	-7.3 (ATP)	-7.2 (ATP)
5	6-O-Veratroyl catalpol	-7.3 (ATP)	-7.0 (ATP)
6	Catalposide	-7.4 (ATP)	-7.0 (ATP)

^a two energies are tabulated (the lowest and the second-lowest energy). ^b the locations of the compound on the kinases. ATP: a chemical on the ATP binding site. Near: a chemical near the ATP binding site (it could be inhibitory on the entrance). Surface: a chemical on a surface far from the ATP binding site (it could be allosteric inhibition). A molecular docking simulation shows that verproside has the lowest docking energy (italics), suggesting that verproside directly binds to PKC δ .

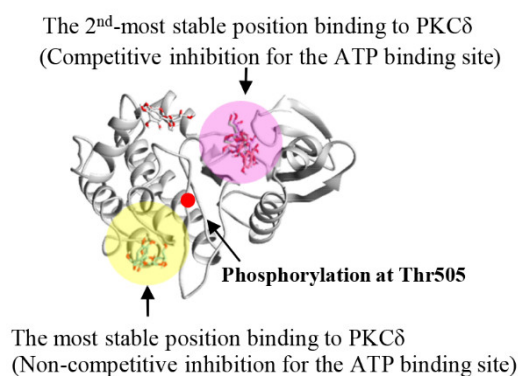


Figure S5. Molecular docking simulation of PKC δ and verproside. (A) The PKC δ homology model structure is depicted as a gray cartoon image. The adenosine triphosphate (ATP)-binding site is shaded pink. Verproside is drawn as a stick model colored in red or green. Phosphorylation of PKC δ at Thr505 is marked with a red spot. Two positions in PKC δ were predicted as direct binding sites for verproside: 1) the ATP-binding site (shaded in pink) and 2) the surface area far from the ATP-binding site (shaded in yellow). The most stable binding between PKC δ and verproside was observed on the yellow surface of PKC δ (Table S1, red

letters), suggesting its effect on PKC δ activity could involve allosteric inhibition at the ATP-binding site (pink area) or at phosphorylation sites such as Thr505.