ORGANIC LETTERS

2013
Vol. 15, No. 23
6106–6109

Xylogranin B: A Potent Wnt Signal Inhibitory Limonoid from *Xylocarpus granatum*

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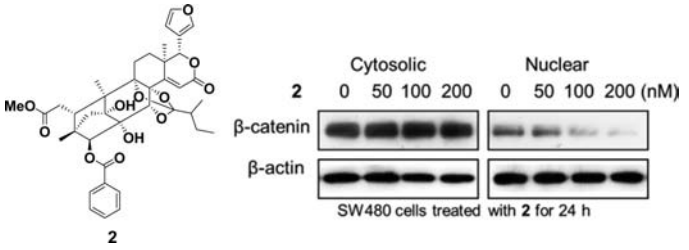
Received October 18, 2013

ABSTRACT



Xylogranin B (2) was isolated from *Xylocarpus granatum* (Meliaceae) leaves, by use of a cell-based luciferase screening system targeting a Wnt signaling pathway. Compound 2 inhibited TCF/β-catenin transcriptional activity (IC_{50} 48.9 nM) and exhibited strong cytotoxicity against colon cancer cell lines. Compound 2 significantly decreased β-catenin protein levels in nuclei but not in the cytosol. These results indicated that a decrease in β-catenin levels in nuclei by 2 resulted in the Wnt signal inhibitory effects of 2.

The Wnt signaling pathway has a crucial role in various cellular processes, including morphology, proliferation, motility, and survival. Aberrant activation of Wnt/β-catenin signaling can lead to tumor formation. It is reported that the high occurrence of Wnt pathway mutations has been found in various type of cancers.[1] The Wnt signal has also been reported to be involved in diabetes and Alzheimer's disease.[2,3] Therefore, small molecules that inhibit Wnt signaling may be useful molecular tools for biological research and lead compounds for therapeutic drugs.

During our studies to search for bioactive natural products targeting Wnt signal inhibitory activity,[4] we examined a number of plant extracts collected in Bangladesh and Thailand. We used a cell-based luciferase assay system to evaluate the inhibition of TCF/β-catenin transcriptional activity (SuperTOP-Flash activity),[5,6] which was measured using the cell line STF/293 (a 293 human embryonic kidney cell line stably transfected with SuperTOP-Flash). With this assay system, we have isolated a series of naphthalene derivatives from an extract of *Eleutherine plamifolia*[7] and *Impatiens balsamina*.[8] We recently identified the strong Wnt inhibitory activity of mangrove *Xylocarpus granatum* and explored its active compounds, leading to the isolation of two new limonoids along with their related compounds. Although a number of limonoids have been isolated from plant resource[9] and several synthetic and natural small molecules were

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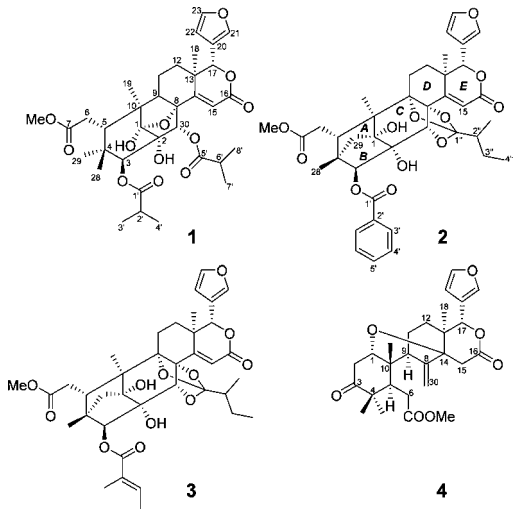
10.1021/ol4029995
Published on Web 11/20/2013

reported to modulate the Wnt signaling pathway,[1] the activity of limonoids on Wnt signaling pathway has not been reported. We herein describe the isolation of the active compounds and their activities. Furthermore, the effects of **2** on the expression of Wnt signaling factors are presented. **2** was shown to be a small molecule natural product that inhibited the accumulation of β-catenin in nuclei.



The MeOH extract of *X. granatum* leaves was partitioned between hexane, EtOAc, *n*-BuOH, and water. Strong activity was found in the hexane-soluble fraction. Activity-guided fractionation of the hexane-soluble fraction by silica gel, ODS, Sephadex column chromatography, and preparative HPLC yielded compounds **1**–**4**. Compounds **3** and **4** were identified as swietephragmin C[10] (**3**) and methyl angolensate[11] (**4**), respectively, by a comparison of their spectroscopic data with values in the literature.

Xylogranin A (**1**) was isolated as a white amorphous solid, the molecular formula of which was $C_{35}H_{46}O_{12}$ on the basis of HRESIMS data (*m*/*z* 681.2866, calcd for $C_{35}H_{46}O_{12}Na$, $[M + Na]^+$, Δ −2.1 mmu), requiring 13 degrees of unsaturation. Analysis of its ^1H and ^{13}C 1D and 2D NMR spectra revealed the presence of a β-furan moiety, four carbonyl carbons, an sp^2 methine, an sp^2 quaternary carbon, three oxymethines, two hydroxyl groups, a methoxy, and eight methyls. Since 8 out of 13 unsaturation degrees were thus accounted for, the remaining unsaturation indicated the presence of five other rings in **1**. A detailed analysis of the $^1H-^1H$ COSY and HMBC results indicated **1** to be a limonoid (Table S1 and Figure S1, Supporting Information).[9] The ^1H and ^{13}C NMR spectroscopic data of **1** (Table S1, Supporting Information) were analogous to that of xyloccensin X[12] ($C_{36}H_{48}O_{12}$), which was obtained from the same genus *Xylocarpus molluccensis*, with the only difference being the presence of an isobutyryl group in **1** instead of a 2-methylbutanoyl group at O-3 for xyloscensin X. The ^1H NMR spectroscopic data of **1** also closely resembled that of xylorumphiin A[13] ($C_{35}H_{48}O_{12}$), having a single bond between C-14 and C-15. The detailed comparison of the ^1H NMR spectra as well as the observation of the same NOE correlations (e.g., from H-5 to H_β-11, H_3-28, and H-30) as observed in xylorumphiin A indicated these two compounds had the same relative configurations. Thus, the structure of xylogranin A was revealed as **1**.

Xylogranin B (**2**) was shown to have the molecular formula $C_{39}H_{44}O_{12}$ by HRESIMS (*m*/*z* 727.2751, calcd for $C_{39}H_{44}O_{12}Na$, $[M + Na]^+$, Δ +2.0 mmu). ^1H and ^{13}C NMR and HMQC indicated the presence of three deshielded signals attributable to three carbonyl carbons, a monosubstituted benzene ring, a typical β-substituted furan ring, a trisubstituted olefin, three oxymethine signals, a methoxy, two hydroxyl groups, and five methyls. Since 11 out of 18 unsaturation degrees were accounted for, the remaining unsaturation indicated the presence of seven other rings in **2**. By means of 2D NMR analysis (Figure S1, Supporting Information), the planar structure of **2** was elucidated as shown, and the structural assignment of **2** was also supported by a comparison of the ^1H and ^{13}C NMR spectrum of **2** with that of known phragmalin, 3β-*O*-detigloyl-3β-*O*-benzoyl-12α-acetoxyswietephragmin C.[14] (Table S2, Supporting Information)

The relative and absolute configuration of **2** were elucidated by considering NOESY and CD spectra. NOESY correlations H_2-29/H-3, H_2-29/H_3-19, and H_2-6/H_3-19 indicated that these protons were located on the α-side and the *O*-benzoyl group at C-3 was β-oriented. In contrast NOESY cross peaks H_β-12/H-5, H_β-12/H-17, and H-5/H-17 indicated a β-orientation for these three protons. NOESY correlation H-15/H-30 and H-11/H_3-18 and model consideration showed that the formation of a less strained 8,9,30-*ortho*-2-methylbutanoate moiety was allowed only on the α-side, which suggested that **2** was present in a folded conformation containing a quasichair C ring, as shown in Figure S2 (Supporting Information). Thus, the structure of **2** was elucidated as 3β-*O*-detigloyl-3β-*O*-benzoyl-swietephragmin C. Similar cotton effects on the CD spectrum (Figure S10, Supporting Information) for **2** ($\Delta\varepsilon_{238}$ +13.7) and **3** ($\Delta\varepsilon_{231}$ +22.0) indicated that these compounds shared the same absolute configuration. On the basis of the comparison of the CD spectra and the biogenetic considerations of the limonoids ever isolated,[9] the absolute configuration of xylogranin B was deduced as **2**.

The isolated compounds were tested for TCF/β-catenin transcriptional activity using a cell-based luciferase reporter gene assay system with STF/293 cells. Wnt signaling activates gene transcription by forming a complex between the DNA-binding protein of the TCF/LEF family and β-catenin, and SuperTOP-Flash, a β-catenin-responsive

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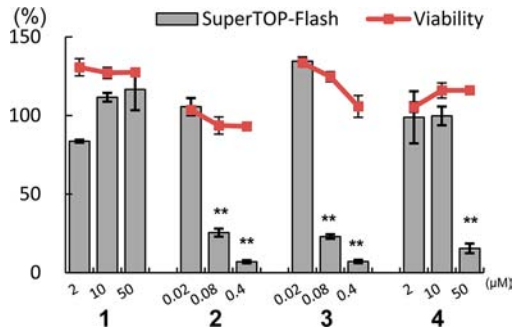

Figure 1. Effects of **1**–**4** on the SuperTOP-Flash activity and viability of STF/293 cells.

reporter plasmid with multiple (seven) TCF-binding sites (CCTTTGATC), is activated under this condition. The viability of STF/293 cells was also examined, because a decrease in cell number may contribute to a decrease in TCF/β-catenin transcriptional activity. **2** and **3** with a phragmalin *ortho*-ester structure potently inhibited TOP activity with IC_{50} values of 48.9 and 54.2 nM, while **4** exhibited weak inhibition (IC_{50} 25.9 μM). **1** did not decrease TOP activity at 2–50 μM. Assays were conducted at concentrations that did not show any cytotoxicity (>80% viability) against STF/293 cells (Figure 1). A DFT calculation was conducted to compare structural differences between active compound **2** and an inactive compound **1** in TOP activity. A comparison of the lowest energy structure of **2** with that of **1** indicated that the conformation with the phragmalin *ortho*-ester of the molecules may be important in inhibiting TOP activity. As shown in Figure S3 (Supporting Information), the active structure of **2** had a folded shape, while **1** was less folded. Furthermore, the distance between H-5 and H-17 of **2** was 2.8 Å, in contrast to that of **1**, which was 6.9 Å. The direction of a methoxy group connecting at C-7 of **2** was at the same side of H-17 (in the concave side of the molecule), while that of **1** was at the opposite side of H-17 (in the convex side). These structural differences may contribute to the difference in activities.

To investigate the effects of **2** and **3** on human colorectal cancer cells, cytotoxicity against DLD1, HCT116, and SW480 cells, in which the Wnt signal pathway is known to be activated,[15,16] were evaluated together with human embryonic kidney 293 cells as a noncancer cell line. Compounds **2** and **3** showed potent cytotoxicities against HCT116 and SW480; however, they exhibited weak toxicities against 293 and DLD1 cells (Table 1).

To exclude the probability of the false positive TCF/β-catenin transcriptional inhibitory activities of **2** and **3**, FOP activity was also evaluated. SuperFOP-Flash, a reporter plasmid with eight mutated TCF-binding sites (CCTTTGGCC), was used in this assay system, which has been widely applied to determine false positive activity.[17] C3H10T1/2 cells transiently transfected with SuperTOP-Flash or SuperFOP-Flash plasmid gene were employed. As shown in Figure S4 (Supporting Information), **2** and **3** inhibited TOP activity with IC_{50} values of 270 and 330 nM, respectively, without significant FOP inhibition (>73%) or a decrease in cell viability (>89%). Thus, **2** and **3** were revealed to be potent TCF/β-catenin transcriptional inhibitors.

Table 1. IC_{50} Values for the Cytotoxicities of **2** and **3**

	IC_{50} (μM)			
compound	SW480	HCT116	DLD1	293
2	0.26	0.05	3.75	5.58
3	0.32	0.06	9.19	4.00

To investigate the effect of **2** on Wnt/β-catenin signaling-related factors, β-catenin protein levels were examined with Western blot analysis using SW480 cells. The translocation of β-catenin into the nucleus was required for its transcriptional activity as a coactivator. Because β-catenin exists in both the cytosol and nucleus, cytosolic and nuclear lysates from **2**-treated SW480 cells were prepared, and β-catenin protein levels in both lysates were analyzed. Dose-dependent decreases in β-catenin were observed in the nuclear lysate when treated with **2** (50–200 nM), whereas β-catenin protein levels remained unchanged in the cytosol (Figure 2A). As shown in Figure 2B, treating SW480 cells with **2** also decreased β-catenin levels in nuclei under immunofluorescence microscopy. From these results, **2** was suggested to inhibit the accumulation of β-catenin in nuclei. Treatment of SW480 cells with **2** at 200 nM decreased protein and mRNA levels of c-myc and PPARδ, target of the Wnt signaling pathway (Figure 2C and Figure S5, Supporting Information). Taken together, these results indicated that inhibiting the accumulation of β-catenin in nuclei was one of the responsible factors for the inhibitory effects of **2** on the transcription of TCF/β-catenin.

In conclusion, the activity-guided separation of *Xylocarpus granatum* leaves led to the isolation of two new limonoids (**1** and **2**), together with two related limonoids (**3** and **4**). Of these, **2** and **3** showed potent TCF/β-catenin transcriptional activity and potent cytotoxicity against SW480 and HCT116 colon cancer cells, while **2** and **3** exhibited moderate cytotoxicity against DLD1 colon cancer cells and 293 human embryonic kidney cells. Although it is known that SW480 and DLD1 cells contain APC mutation[15] and HCT116 cells have β-catenin mutation,[16] these three cells are known to be Wnt-signaling-dependent cells. While the reason for moderate cytotoxicity of **2** and **3** against DLD1 cells comparing with SW480 and HCT116 cells is still unclear, these differences may indicate the

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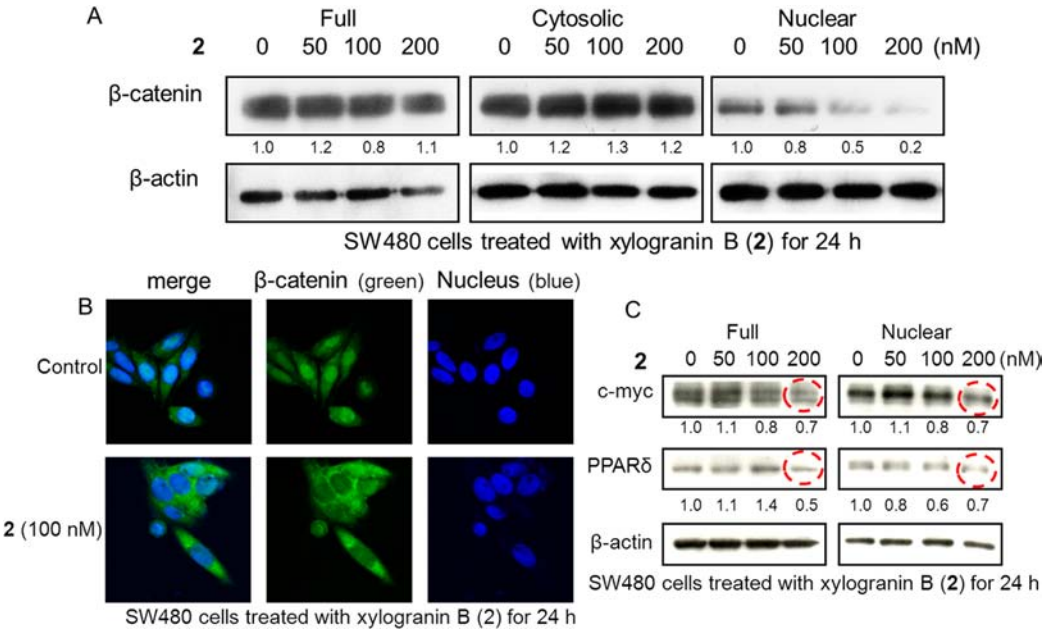

Figure 2. Effects of **2** on SW480 cell. (A) Western blot analysis of **2** on β-catenin levels in SW480 cells. (B) Immunofluorescence analysis of **2** on β-catenin levels in SW480 cells. (C) Western blot analysis of **2** on the expression of target gene products in SW480 cells. The quantity of each protein was expressed as a ratio of control (0 nM), normalized with respect to β-actin.

involvement of other signal pathways on cell survival in DLD1. These results indicate that the cytotoxicities of **2** and **3** may be mediated by the down-regulation of Wnt/β-catenin signaling, at least in SW480 and HCT116 cells. A significant decrease in β-catenin protein levels in nuclei and the reduced expression of c-myc and PPARδ by **2** in SW480 cells confirmed the inhibitory effects of **2** on TCF/β-catenin transcription. To the best of our knowledge, this is the first report of limonoids that can strongly inhibit the Wnt signaling pathway.

Acknowledgment. We thank Prof. J. Nathans (John Hopkins University School of Medicine) for the STF/293 cells and Prof. R. T. Moon (University of Washington) for the SuperFOP-Flash plasmid. This study was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS), a Grant-in-Aid for Scientific Research on Innovative Areas "Chemical Biology of Natural Products" from The Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, the Cosmetology Research Foundation, AstraZeneca R&D Grant, Japan Antibiotics Research Association, and Sekisui Chemical Innovations Inspired by Nature Research Support Program.

Supporting Information Available. Supporting figures, experimental methods, spectroscopic data, and NMR data of new compounds. These materials are available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.