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Synthesis of rocaglamide derivatives and evaluation of their Wnt signal inhibitory activities†

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Rocaglamides are bioactive natural compounds which have a cyclopenta[*b*]benzofuran core structure. The total synthesis of a reported natural product, 3'-hydroxymethylrocaglate (5), was achieved using [3 + 2] cycloaddition between 3-hydroxyflavone and methyl cinnamate. We also describe the synthesis of rocaglamide heterocycle derivatives and evaluate their Wnt signal inhibitory activities. Compounds 4, 5, 22a, 22b, 22c and 23c showed potent Wnt signal inhibitory activity.

Introduction

Rocaglamides (= flavaglines, 1–6) are naturally occurring complex molecules with a cyclopenta[*b*]benzofuran core structure that have been isolated from the genus *Aglaiia* and they possess many potent biological activities (Fig. 1),¹ including powerful cytotoxicity against cancer cells,² anti-inflammatory activity³ and neuroprotective activity.⁴ We have recently reported that 1-*O*-formylrocagloic acid (3) and 3'-hydroxy rocagloic acid (4) at low nanomolar concentrations sensitize tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-resistant human gastric adenocarcinoma cells by up-

regulating the expression of death receptors DR4 and DR5 and activating caspase-3/7.⁵ Silvestrol (6)⁶ was reported to exhibit potent cytotoxicity towards human prostate carcinoma LNCaP cells. The rocaglate skeleton comprises five continuous stereocenters and its synthesis has attracted much attention since the first synthesis of rocaglamide (1) by Trost *et al.*⁷ Porco and co-workers reported a oxidopyrylium [3 + 2] cycloaddition approach to the synthesis of rocaglamide derivatives.⁸ Recently, several “remodeling” natural product approaches have been used to synthesize compounds exhibiting new biological activities:⁹ for example, a synthesized rocaglate-derived β-lactone was reported to have serine hydrolase activity.^{8d} The discovery of rocaglamide derivatives exhibiting novel bioactivities would provide new biological tools and drug candidates. We are interested in the role of the heterocyclic structure of rocaglamide derivatives on their bioactivity. Herein, we describe the synthesis of 3'-hydroxymethylrocaglate (5) and rocaglamide heterocycle derivatives and evaluate their Wnt signal inhibitory activity.

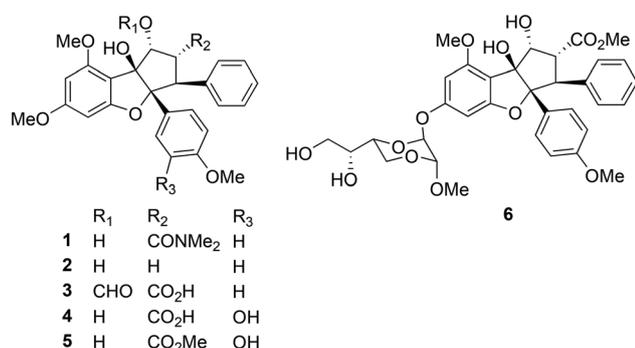


Fig. 1 Representative examples of flavaglines: rocaglamide (1), rocaglaol (2), 1-*O*-formylrocagloic acid (3), 3'-hydroxy rocagloic acid (4), 3'-hydroxymethylrocaglate (5), and silvestrol (6).

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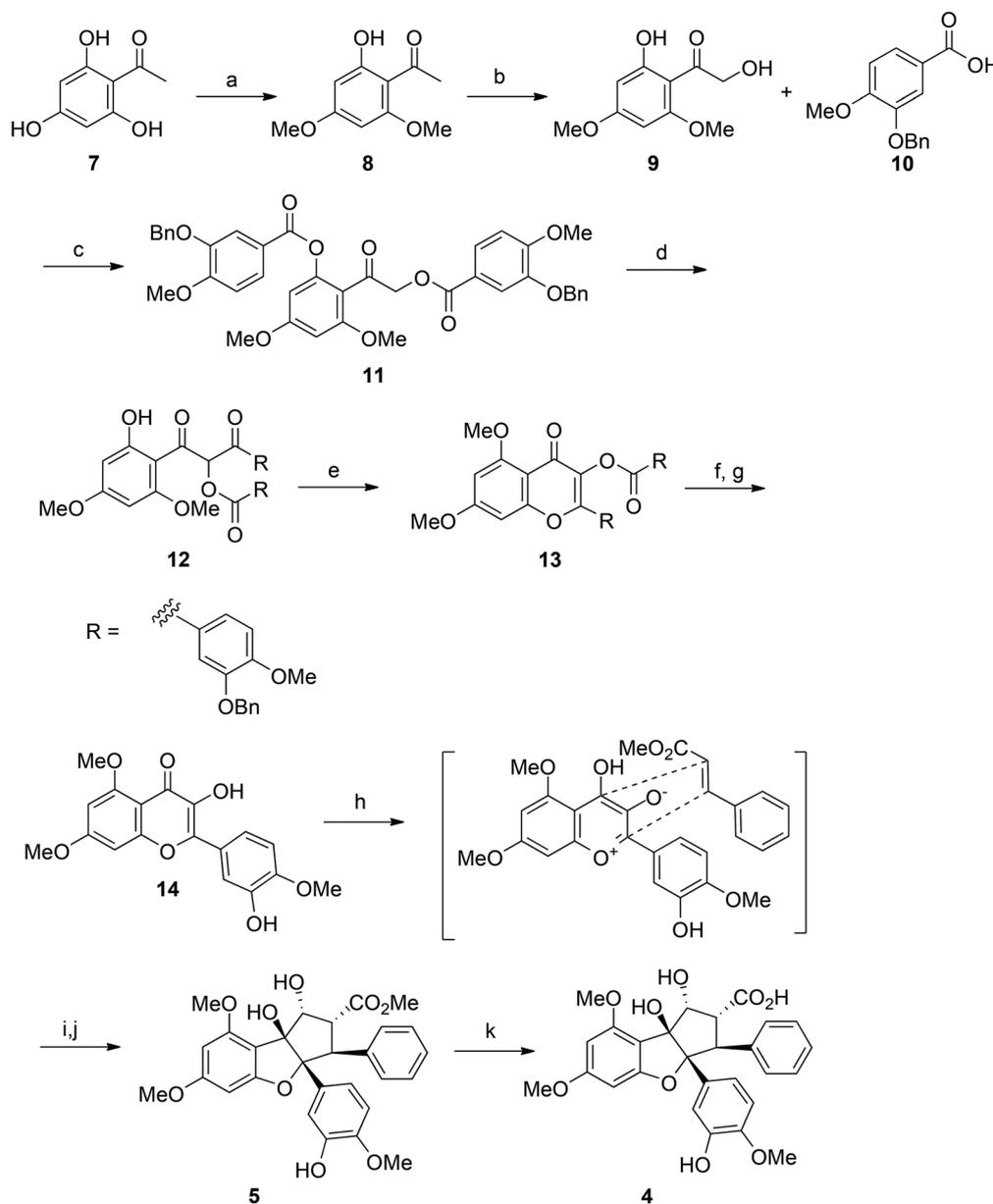
Results and discussion

Synthesis of 3'-hydroxymethylrocaglate (5)

The research was initiated by synthesizing the natural products (4) and (5) previously isolated from *Amoora cucullata*. Starting from trihydroxyacetophenone 7, dimethoxy ether 8 was obtained in 78% yield. Following the preparation of silyl enol ether, the epoxide was generated by a reaction with mCPBA. The reaction of the resulting epoxide under acidic conditions gave alcohol 9 in 69% yield in 3 steps, and coupling between 9 and 10 provided ester 11. The Baker–Venkataraman rearrangement of 11 proceeded to give 12, and under basic conditions a cyclization–dehydration step afforded flavones 13. Hydrolysis of 13 followed by hydrogenolysis gave 14 in good yield. The

irradiation conditions promoted [3 + 2] cycloaddition between methyl cinnamate and the oxidopyrylium from **14** to provide a mixture of the corresponding aglain core products. This mixture was converted to the natural product 3'-hydroxymethylrocaglate (**5**) in two steps: an α -ketol rearrangement and a hydroxyl-directed reduction.^{8b} The ¹H- and ¹³C-NMR spectra of **5** were identical with the reported values.^{10,11} The hydrolysis of **5** gave corresponding acid **4** in good yield. We had previously isolated a natural product exhibiting a strong ability to

suppress the TRAIL resistance⁵ and its structure was proposed to be 3'-hydroxy rocagloic acid (**4**) on the basis of its identical spectroscopic data with the reported values.¹² However, the NMR spectra of the synthesized compound **4** were inconsistent with the literature data for 3'-hydroxy rocagloic acid.¹² Furthermore, the synthesized compound **4** did not suppress the TRAIL resistance, indicating that the reported natural product has a different structure from **4**. Further structure elucidation studies are currently in progress (Scheme 1).



Scheme 1 Synthesis of 3'-hydroxymethylrocaglate (**5**) and **4**. Reagents and conditions: (a) MeOTf (2.8 equiv.), K₂CO₃ (5.4 equiv.), acetone, reflux, 3 h, 88%; (b) (1) TBSOTf (2.8 equiv.), Et₃N (3.0 equiv.), CH₂Cl₂, 0 °C, 30 min; (2) mCPBA (1.6 equiv.), NaHCO₃ (2.5 equiv.), CH₂Cl₂, rt, 4 h; (3) TsOH·H₂O (0.1 equiv.), THF/H₂O (10/1), reflux, 9 h, 69% in 3 steps; (c) **10** (3.0 equiv.), EDC·HCl (4.5 equiv.), DMAP (0.34 equiv.), CH₂Cl₂, rt, 7 h, 90%; (d) LHMDS (3.0 equiv.), THF, -20 °C, 3 h; (e) H₂SO₄ (5.0 equiv.), AcOH, rt, 28 h; (f) aq. NaOH (2.0 equiv.), EtOH, 80 °C, 4 h, 75% in 3 steps; (g) Pd(OH)₂, H₂, THF, EtOH, rt, 2 h, quant. (h) methyl cinnamate (13 equiv.), MeCN/MeOH, *hν*, 0 °C; (i) NaOMe (2.8 equiv.), MeOH, reflux; (j) NMe₄BH(OAc)₃ (6.0 equiv.), AcOH (10 equiv.), MeCN, rt, 2 h, 35% in 3 steps; (k) LiOH·H₂O (15 equiv.), THF/H₂O (5/1), rt, quant.

Synthesis of heterocyclic rocagloic acid derivatives

We next turned our attention to the synthesis of rocaglamide heterocycle derivatives (Fig. 2). Heterocycles have been incorporated into many biologically active compounds. We envisioned that heterocyclic rocaglamides could provide new biologically active compounds, similar to their natural product parents. As the heterocycles, we chose furan, thiophen units and their Br substituted units, which would be useful for further substitution by a metal catalyzed coupling reaction. The esterification of **9** with the corresponding carboxylic acids **15a–d** provided esters **16a–d** (Scheme 2). The Baker–Venkataraman rearrangement of **17a–d** with sulfuric acid, followed by treatment with a base, afforded the heterocyclic chromones **19a–d**. [3 + 2] cycloaddition between the methyl cinnamate and oxidopyrylium of **19a–d** provided aglain compounds as the major components. The rearrangement and hydroxyl-directed reduction gave the rocaglamide heterocycle derivatives **22a–d** and the rocagloic acid derivatives **23a–d** were obtained by hydrolysis (Scheme 3). The relative stereochemistries of **22a**, **23c** and **23d** were deter-

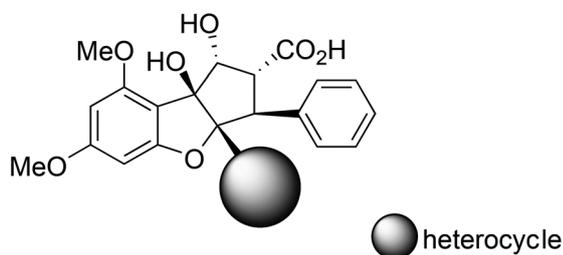


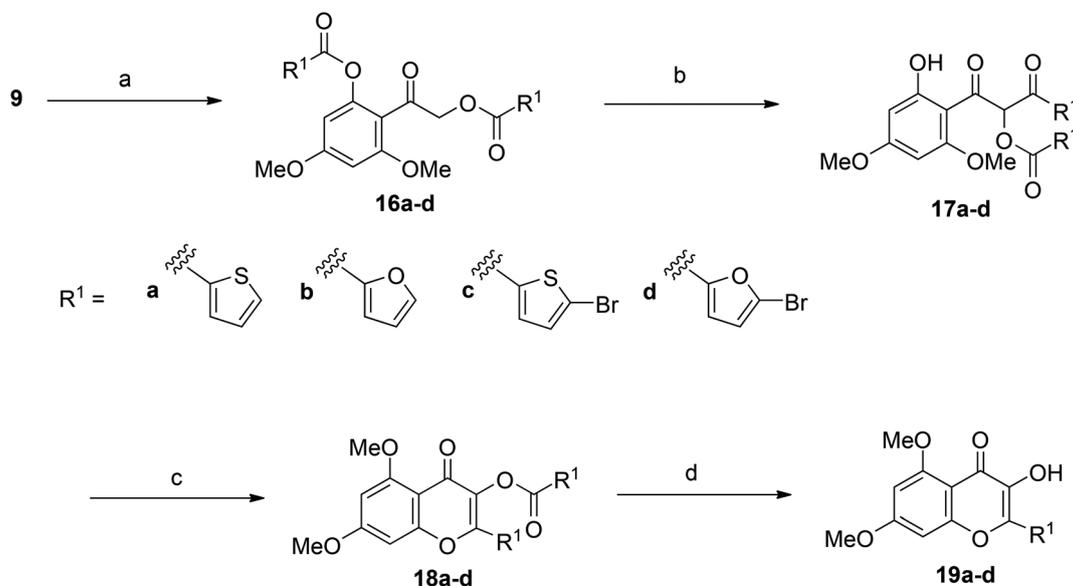
Fig. 2 Design of the rocaglate with heterocycles.

mined by X-ray crystallographic analysis¹³ and revealed that the *endo* cycloadducts were the main products¹⁴ (Fig. 3).

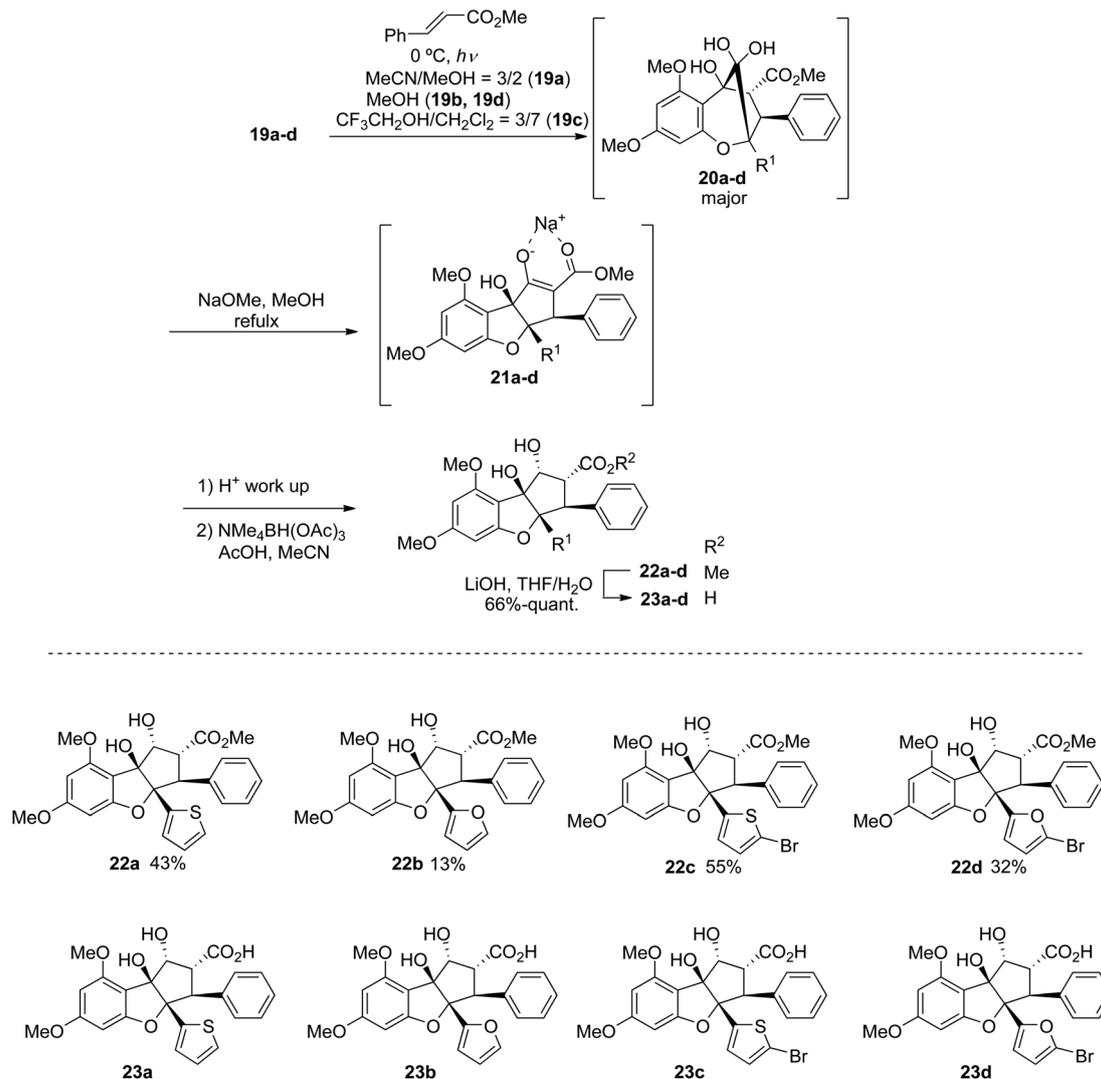
Evaluation of the Wnt signal inhibitory activity of synthetic rocaglamide derivatives

Novel bioactive rocaglamide derivatives were identified using a previously described cell-based assay for Wnt signal inhibitors.¹⁵ The Wnt signal plays crucial roles in the differentiation and proliferation of many types of cells.¹⁶ In normal cells, the β -catenin levels are controlled by a degradation system that involves the action of a destruction complex consisting of glycogen synthase kinase 3 β (GSK-3 β), adenomatous polyposis coli (APC), and casein kinase 1 α . Inactivation of this system due to mutation of the components causes aberrant activation of the Wnt signal associated with many cancers and diseases.¹⁷ Therefore, inhibitors of this signal could be drug candidates or useful biological tools. Several Wnt signal inhibitors have been reported,^{18a,b} including windorphen,^{18c} XAV939,^{18d} IWP-2,^{18e} and ICG-001,^{18f} but more potent inhibitors with novel structures are required. We have recently reported several Wnt signal inhibitors.^{15,18b,19} Even though the synthesized rocaglamide derivatives are racemates, it would be useful to evaluate the Wnt signal inhibitory activities of these unique complex structures.

We have investigated Wnt signal inhibition using the TOP-Flash assay, a cell-based reporter luciferase assay for TCF/ β -catenin transcription in the STF/293 cell line,¹⁵ and previously constructed a cell assay using LiCl as a GSK-3 β inhibitor for inducing β -catenin accumulation. Interestingly, as shown in Fig. 4, several synthetic rocaglamide derivatives showed potent inhibition of TCF/ β -catenin transcription activity and low toxicity. To the best of our knowledge, this is



Scheme 2 Reagents and conditions: (a) carboxylic acids R¹COOH (**15a–d**) (3.0 equiv.), EDC-HCl (4.5 equiv.), DMAP (0.34 equiv.), CH₂Cl₂, reflux, 91%-quant.; (b) LHMDS (3.0 equiv.), THF, –20 °C, 65–85% in 2 steps; (c) H₂SO₄ (5.0 equiv.), AcOH, 55–85%; (d) 1 N NaOH (2.0 equiv.), EtOH, 80 °C, 55–92%.



Scheme 3 Synthesis of rocgamide derivatives.

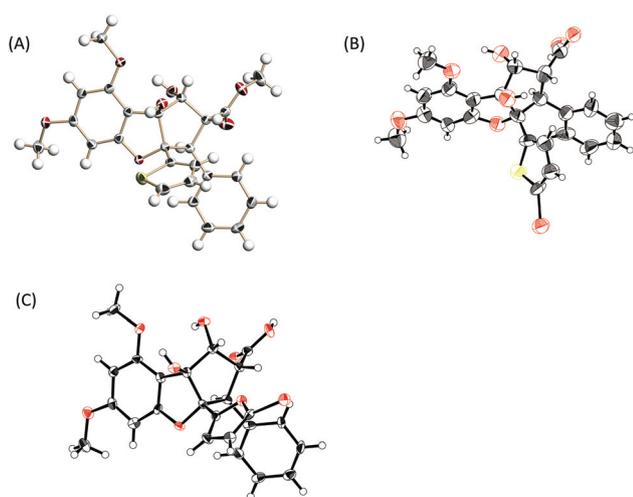


Fig. 3 X-ray structure of (A) 22a, (B) 23c and (C) 23d. Solvent molecules are omitted for clarity.

the first report of the Wnt signal inhibitory activity of rocgamide derivatives. For example, 4, 5, 22a, 22b, 22c and 23c exhibited inhibition activity with IC₅₀ values of 9.3 μM, 32 nM, 9.4 μM, 9.4 μM, 0.19 μM and 1.0 μM, respectively. To determine if these inhibitions are selective for the Wnt signal pathway, we next examined their potency for β-catenin/TCF transcriptional inhibition using the FOP-Flash assay. The FOP-Flash plasmid has mutated TCF binding sites. The compounds that are Wnt signal inhibitors affect only the TOP-Flash activity, which has the native TCF binding sites. As shown in Fig. 5, 4, 5, 22a, 22b, 22c and 23c showed potent inhibition of TOP-Flash activity whereas FOP-Flash activities were unaffected, indicating that these inhibitors are potent Wnt signal inhibitors.

Because there is a report showing that Wnt expressing cells suppress TRAIL-induced apoptosis,²⁰ Wnt signal inhibitors might show TRAIL resistance overcoming activity. As we expected, compounds 5, 22c and 23c showed good TRAIL resistance overcoming activity (see the ESI†).

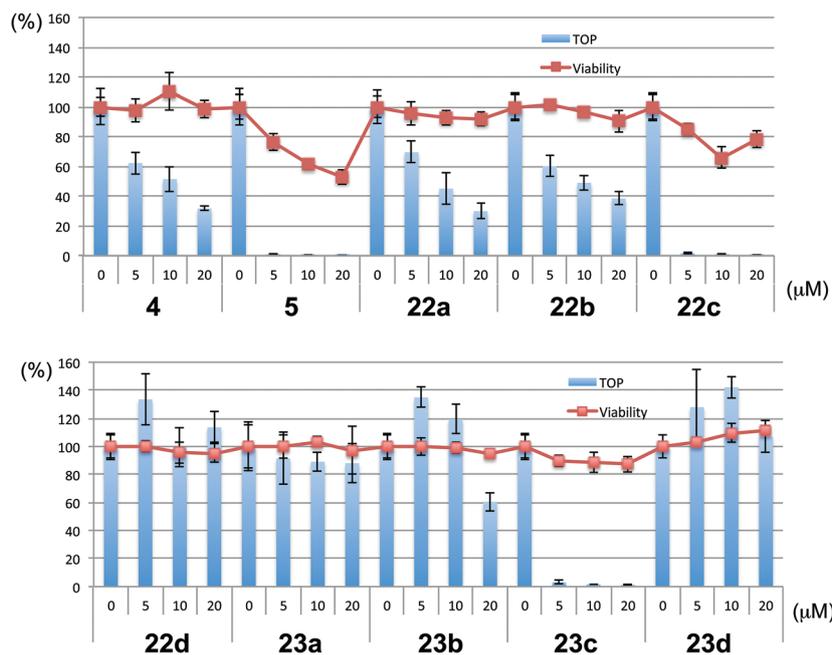


Fig. 4 Inhibition of TCF/β-catenin transcriptional activity by the synthesized compounds. The results of the Super TOP-Flash (blue bars) assay and cell viability (red) are shown.

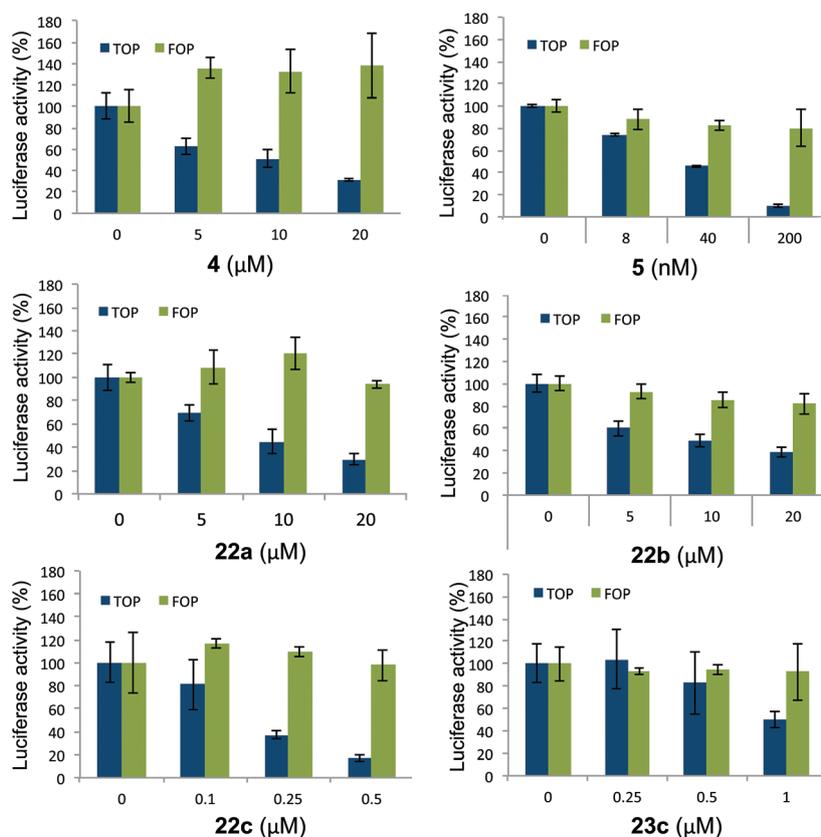


Fig. 5 Inhibition of TCF/β-catenin transcriptional activity by 4, 22a, 22c and 23c. The results of the TOP-Flash (blue bars) and FOP-Flash (green bars) assays are shown. The FOP-Flash assay was carried out as a transient luciferase reporter assay by using Super FOP-Flash reporter and control pRL-CMV vectors, and HEK293 cells. The control (DMSO) luciferase activity was normalized to 100%. $N = 3$. Error bars represent SD.

Conclusions

In conclusion, we synthesized a natural product, 3'-hydroxy-methylrocaglate (**5**), and eight rocaglamide heterocycle derivatives and investigated their Wnt signal inhibitory activities. We found that **4**, **5**, **22a**, **22b**, **22c** and **23c** are potent Wnt signal inhibitors. To the best of our knowledge, this is the first report of the Wnt signal inhibitory activity of rocaglamide derivatives. The synthesis of more complex derivatives exhibiting more effective Wnt signal inhibition is in progress.

Experimental section

General experimental procedure

NMR spectra were recorded on JEOL ECP400 and ECP600 spectrometers in a deuterated solvent whose chemical shift was used as an internal standard. Mass spectra were obtained on an AccuTOF LC-plus JMS-T100LP (JEOL). ATR-IR spectra were measured on a JASCO FT-IR 230 spectrophotometer. Column chromatography was performed using silica gel PSQ100B (Fuji Silysia Chemical Ltd, Kasugai, Japan) and silica gel 60N (Kanto Chemical Co., Inc., Tokyo, Japan). Photochemical reactions were carried out using HL-400B-8 (400 W, 33 Å; mercury lamp) and HB400P-1 (400 W) (SEN Light Co., Osaka, Japan) lamps and the cooling system consisted of TRL-117ST and TC-107E (THOMAS, KAGAKU Co., Ltd, Tokyo, Japan) units. The mercury lamp was cooled using a glass container (Pyrex) (USHIO Inc., Tokyo, Japan) filled with water.

General procedure for compounds **11**, **16a–d**

To a solution of **9** (300 mg, 1.4 mmol) in CH_2Cl_2 (14 mL) was added **15a** (538 mg, 4.2 mmol), DMAP (59 mg, 0.48 mmol) and EDC-HCl (1.2 g, 6.3 mmol). The reaction mixture was stirred at rt for 8 h and then diluted with water. The mixture was extracted with CH_2Cl_2 and the organic layer was dried over Na_2SO_4 . After filtration and concentration, the resulting residue was purified by silica gel chromatography (hexane : AcOEt = 3 : 1) to afford **16a** (605 mg, 1.4 mmol, quant.).

General procedure for compounds **12**, **17a–d**

To a solution of **16a** (578 mg, 1.3 mmol) in THF (38 mL), LHMDs (1.3 M, 3.1 mL, 4.0 mmol) was added dropwise at $-20\text{ }^\circ\text{C}$. The reaction mixture was stirred for 2 h and the reaction was quenched using sat. aq. NaHCO_3 . The resulting mixture was extracted using EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4 and filtered. The solvent was evaporated *in vacuo* and the resulting residue was purified by silica gel chromatography (hexane : AcOEt = 3 : 1) to afford **17a** (432 mg, 1.0 mmol, 75%).

General procedure for compounds **13**, **19a–d**

To a solution of **17a** (404 mg, 0.93 mmol) in 12 mL of glacial acetic acid was added 246 μL of sulfuric acid. The resulting mixture was stirred at rt for 22 h. The reaction was quenched with cool water and filtered, then EtOH was added to the

resulting residue and the mixture was stirred for several hours under reflux. The reaction mixture was concentrated and the resulting crude product **18a** (307 mg) was used for the next reaction without further purification. To a solution of crude product **18a** (307 mg) in EtOH (3.7 mL) was added 1 N NaOH (890 μL). The reaction mixture was heated at $80\text{ }^\circ\text{C}$ and stirred for 5 h. The reaction was quenched with 1 N HCl and filtered through a Kiriya funnel. The residue was washed with cool EtOH and the solvent was evaporated *in vacuo* to afford **19a** (149 mg, 0.5 mmol, 66% from **17a**).

General procedure for compounds **5**, **22a–d**

To a solution of 3-hydroxychromone (**19a**; 91 mg, 0.30 mmol) in dry acetonitrile (4.7 mL) and dry MeOH (3.2 mL) was added methyl cinnamate (610 μL , 3.9 mmol). The reaction mixture was irradiated (400 W mercury lamp) at $0\text{ }^\circ\text{C}$ for 2 h. The solvent was removed *in vacuo* and the resulting residue was purified by silica gel column chromatography (hexane : AcOEt = 10 : 1 \rightarrow 2 : 1 \rightarrow 1 : 1) to afford a mixture containing **20a** (95.7 mg). To a mixture containing **20a** (95.7 mg) in dry MeOH (7 mL) was added NaOMe (31 mg, 0.57 mmol). The reaction mixture was stirred for 2 h under reflux, quenched with sat. aq. NH_4Cl and then extracted with EtOAc. The combined organic layer was washed with brine and dried over Na_2SO_4 . After filtration and concentration, the resulting residue was purified by silica gel column chromatography (hexane : AcOEt = 2 : 1) to afford the inseparable keto–enol isomers of **21a** (55.9 mg, 0.12 mmol, 53% in 2 steps from **19a**). A mixture of tetramethylammonium triacetoxymethylborohydride (189 mg, 0.72 mmol) and acetic acid (70 μL , 1.2 mmol) in dry acetonitrile (3.1 mL) was stirred at rt for 5 min, added to a solution of keto–enol tautomers **21a** (55.9 mg, 0.12 mmol) in dry acetonitrile (2.1 mL) and stirred at rt for 2 h. The reaction was quenched with sat. aq. NH_4Cl and the mixture was extracted with CH_2Cl_2 . The combined organic layer was washed with brine and dried over Na_2SO_4 . After filtration and concentration, the resulting residue was purified by silica gel column chromatography (hexane : AcOEt = 3 : 2) to afford **22a** (45.9 mg, 0.098 mmol, 82%).

General procedure for compounds **4**, **23a–d**

Rocaglamide derivative **22a** (10.3 mg, 0.022 mmol) was dissolved in 4.7 μL of a 5 : 1 mixture of dry THF and distilled water. Lithium hydroxide monohydrate (13.8 mg, 0.33 mmol) was added and the reaction mixture was stirred at rt for 23 h. The mixture was diluted with CH_2Cl_2 and washed with 1 N HCl and the organic layer was extracted with CH_2Cl_2 . The combined organic layer was dried over Na_2SO_4 and filtered. Concentration *in vacuo* gave rocagloic acid **23a** (9.7 mg, 0.021 mmol, 97%).

Reporter gene assay and transfection for the Wnt signal inhibitory activity

A previously described cell-based assay method¹⁵ was used to evaluate the TCF/ β -catenin transcriptional activity. Assay cells (STF/293 cells) were seeded into 96-well plates (3×10^4 cells per

well). After 24 h, the cells were treated for 24 h with the synthesized compounds mixed with 15 mM LiCl. The cells were then lysed, and the luciferase activity was measured using the Luciferase Assay System (Promega) on a Luminoskan Ascent Microplate Luminometer (Thermo). The FOP activity was also evaluated to eliminate nonspecific inhibition of TOP activity. HEK293 cells were plated on 24-well plates (1×10^5 cells per well) and incubated for 24 h. Using Lipofectamine 2000, the cells were transiently transfected with 500 ng per well of a luciferase reporter construct (SuperFOPflash), and 25 ng per well of pRL-CMV (Promega) for normalization. The compounds mixed with 15 mM LiCl were added to the cells 12 h post-transfection. After incubation for 24 h with the compounds, the cells were lysed and the luciferase activity was measured using the PICAGENE Dual Sea Pansy assay system (Toyo Ink) and a Luminoskan Ascent Microplate Luminometer.

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