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Design, Synthesis, and Structural Optimization of Lycorine-Derived Phenanthridine Derivatives as Wnt/β-Catenin Signaling Pathway Agonists

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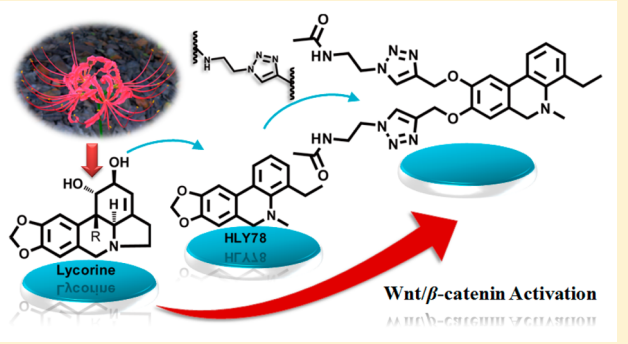
Supporting Information

ABSTRACT: Lycorine is a benzylphenethylamine-type alkaloid member of the Amaryllidaceae family. A lycorine derivative, HLY78, was previously identified as a new Wnt/β-catenin signaling pathway agonist that targets the DAX domain of axin. Herein, the structural optimization of HLY78 and analyses of the structure−activity relationships of lycorine-derived phenanthridine derivatives as agonists of the Wnt/β-catenin signaling pathway are presented. This research suggests that triazole groups are important pharmacophores for Wnt activation; triazole groups at C-8 and C-9 of phenanthridine compounds markedly enhanced Wnt activation. A C-11−C-12 single bond is also important for Wnt activation. On the basis of these findings, two Wnt agonists were designed and synthesized. The results for these agonists indicated that the combination of a 4-ethyldihydrophenanthridine skeleton and a triazole substituent improves Wnt activation. These compounds may be useful in further pharmacological or biological studies.



Wnt/β-catenin is a highly conserved signaling pathway[1,2] that is important for development. Aberrant Wnt signaling is involved in many diseases, such as cancer (in which the pathway is inappropriately activated) and Alzheimer's disease and osteoporosis (in which the pathway is attenuated).[3−5] In recent years, Wnt antagonists have attracted attention due to the potential applications in the clinical treatment of cancer.[6−9] Appropriate activation of Wnt signaling pathways could also be useful in other clinical applications, such as hematopoietic stem cell (HSC) expansion and the treatment of osteoporosis.[10]

Lycorine is a naturally occurring multifunctional benzylphenethylamine alkaloid (Figure 1a) of considerable interest.[11−14] Previous studies have identified that lycorine derivatives are novel HCV (hepatitis C virus) inhibitors that act via novel mechanisms.[15,16] A new small-molecule activator of the Wnt/β-catenin signaling pathway, 4-ethyl-5-methyl-5,6-dihydro[1,3]-dioxolo[4,5-*j*]phenanthridine (HLY78, Figure 1b), which acts in a Wnt ligand-dependent manner, has also been identified. Mechanistic studies have indicated that HLY78 targets the DAX domain of axin and potentiates axin/LRP6 association, which subsequently promotes LRP6 phosphorylation and Wnt signaling transduction.[17] These findings not only provide new insights into the regulation of the Wnt/β-catenin signaling pathway via a Wnt-specific small molecule but also may facilitate therapeutic applications, such as HSC expansion and the treatment of osteoporosis.

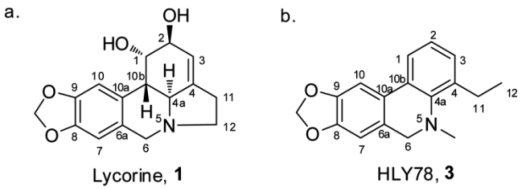


Figure 1. Structures of lycorine and HLY78.

However, the activity of HLY78 is insufficient for further pharmacological study. To identify a Wnt agonist, the structure of HLY78 must be further optimized using rational drug design approaches. Here, the structural optimization of phenanthridine analogues based on X-ray diffraction and molecular docking techniques is described, and an evaluation of derivatives that exhibit Wnt-activating effects in vitro is presented. The structure−activity relationships (SARs) of these derivatives are also analyzed.

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RESULTS AND DISCUSSION

Design and Synthesis of Phenanthridine Derivatives. The well-characterized X-ray crystal structures of axin protein (PDB 1WSP)[18] and HLY78 were examined to predict the binding modes; molecular docking of HLY78 into the axin binding domain, DAX, was analyzed using AutoDock[19] to understand the interaction between HLY78 and axin. A total of 11 conformations of HLY78 docking to the DAX domain were analyzed, and six of the conformations localized to the same cavity formed by the juxtaposition of the residues from two neighboring protomers. The model with the lowest estimated free energy for binding (6.23 kcal/mol) was selected. The modeled complex structure of axin-HLY78 revealed that a group of residues in the cavity contact HLY78 via electrostatic or hydrophobic interactions. The docking results indicated that the binding of the phenanthridine compound to the axin protein likely occurs primarily via two oxygen-bearing substituents at C-8 and C-9. In addition, due to the steric hindrance of the active cavity of axin, substituents at C-11 also affect the binding of the compounds to the protein (Figure 2). Consequently, the design of novel molecules focused on the optimization of the two oxygen-bearing substituents at C-8 and C-9 and the exploration of C-11−C-12 bonds.

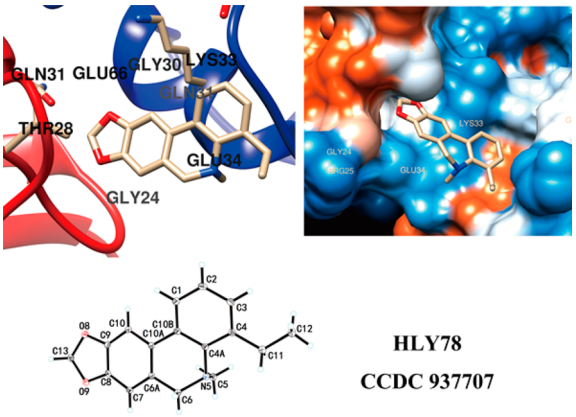

Figure 2. Molecular docking studies of HLY78. The X-ray crystal structure of HLY78 and the schematic representation of HLY78 docked onto the crystal structure of DAX (PDB 1WSP) are shown. The two related protomers of DAX are indicated in red and blue.

Two initial compounds (**4**, **5**) were generated from lycorine using a semisynthetic process; these compounds were further modified to study the effects of structural modifications on Wnt signaling compared to HLY78. Exhaustive methylation of lycorine followed by Hoffman degradation afforded intermediate **2** in 70% yield. Hydrogenation of **2** using 10% Pd/C afforded HLY78 (**3**). Subsequently, compounds **2** and **3** were treated with BBr_3 at −78 °C to obtain compounds **4** and **5**, respectively, which were alkylated using different alkylating agents to afford 14 phenanthridine derivatives (Figure 3).[15]

Activation of the Wnt/β-Catenin Signaling Pathway by Phenanthridine Derivatives. The effects of all derivatives on the reporter gene of the Wnt/β-catenin signaling pathway were evaluated in HEK293T cells. As shown in Table 1, compounds **4**, **5**, **5a**, **5b**, and **5c** activated Wnt signaling, and **5b** was most effective. A concentration of **5b** of 2.5 μM doubled the activity of the Wnt signaling pathway. The remaining phenanthridine derivatives did not activate the Wnt/β-catenin pathway, even at high concentrations. To further confirm the

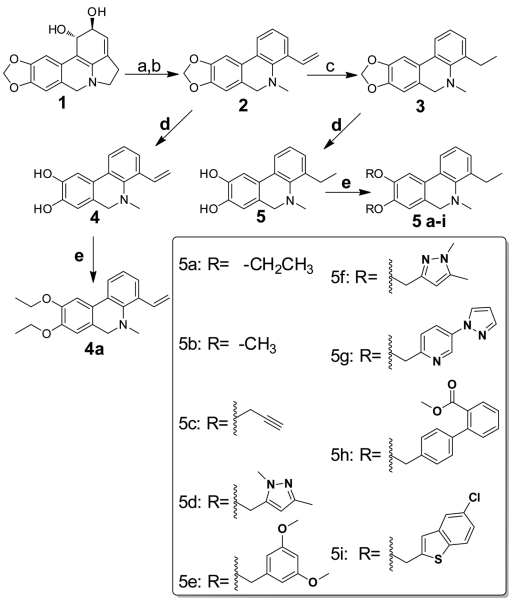

Figure 3. Syntheses of phenanthridine-type derivatives. Reagents and conditions: (a) MeI, DMF, rt, 12 h; (b) *t*-BuOK, *t*-BuOH, N_2, reflux, 4 h, 70%; (c) 10% Pd/C, CH_2Cl_2, rt, 24 h, 90%; (d) BBr_3, CH_2Cl_2, −78 °C, 4 h, 70% (for **4**), 80% (for **5**); (e) RX, NaH, THF, N_2, rt, 24 h, 55−87%.

Table 1. Wnt Activating Efficacy of the Phenanthridine Derivatives

compound	concentration that doubled the activation of Wnt
2	NA
3 (HLY78)	5 μM
4	10 μM
4a	NA
5	5 μM
5a	5 μM
5b	2.5 μM
5c	5 μM
5d	NA
5e	NA
5f	NA
5g	NA
5h	NA
5i	NA

activating function, the effects of compounds **5h** and **5i** on the expression of endogenous Wnt target genes (*Axin2* and *NKD1*) were tested. These compounds did not upregulate the expression of endogenous Wnt target genes (Figure 4).

SAR Analyses of Phenanthridine Derivatives Based on Wnt Activation in Vitro. The bioassays of the phenanthridine derivatives indicated that the C-11−C-12 bond is important for activating the Wnt signaling pathway. A $\Delta^{11,12}$ double bond altered the degree of conjugation within the molecular skeleton and blocked free rotation of the bond, thus increasing steric hindrance and decreasing the activity of the compounds, as shown in Figure 5. Thus, a C-11−C-12 single bond is crucial for the ability of the phenanthridine derivatives to activate Wnt.

Because compound **5** exhibited acceptable Wnt signaling activation, further structural optimization was focused on the C-8 and C-9 hydroxy groups of compound **5**. However, compounds **5d**−**5i** exhibited nearly complete loss of Wnt activation. The above results and docking analysis of HLY78

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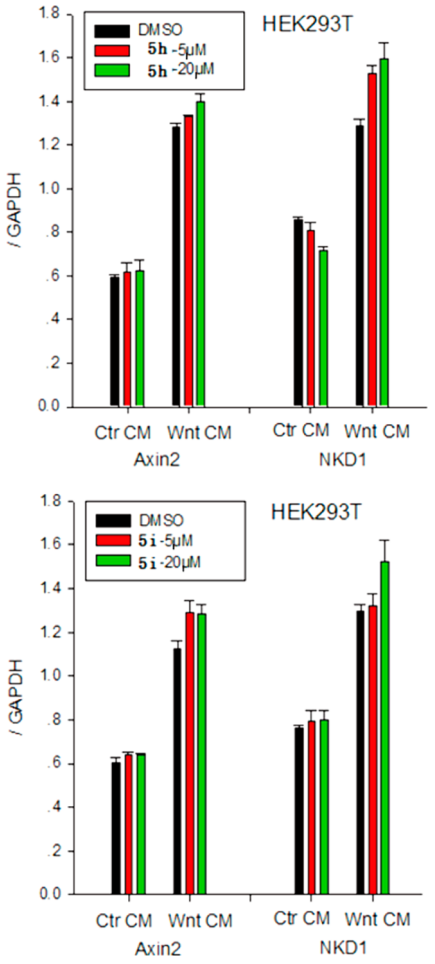

Figure 4. Effects of compounds **5h** and **5i** on the expression of endogenous Wnt target genes (*Axin2* and *NKD1*). Neither **5h** nor **5i** clearly upregulated the expression of endogenous Wnt target genes.

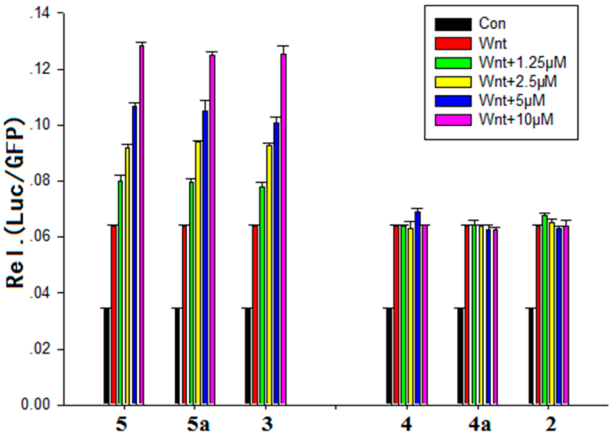

Figure 5. Effects of the C-11−C-12 bond on Wnt activation. A double bond between these two carbons markedly reduced the ability of these phenanthridine derivatives to activate Wnt.

suggested that the cavity or the binding domain of axin may interact with the C-8 and C-9 substituents via electrostatic or hydrophobic interactions to facilitate specific binding and allow only suitable groups to enter this cavity and associate with the protein residues.

Research on "Model Molecules". Interestingly, in a previous study of the activating mechanism of HLY78, a biotinylated derivative (HLY179) that exhibited potent Wnt activation was designed. Specifically, this compound increased the activation of the Wnt signaling pathway by 2-fold at a concentration of 0.325 μM.[17] Inspired by this result, the structural characteristics of HLY179 were analyzed and compared with the precursor, compound **5c**. HLY179 features additional C-8 and C-9 triazole substituents as well as biotin side chains (Figure 6).

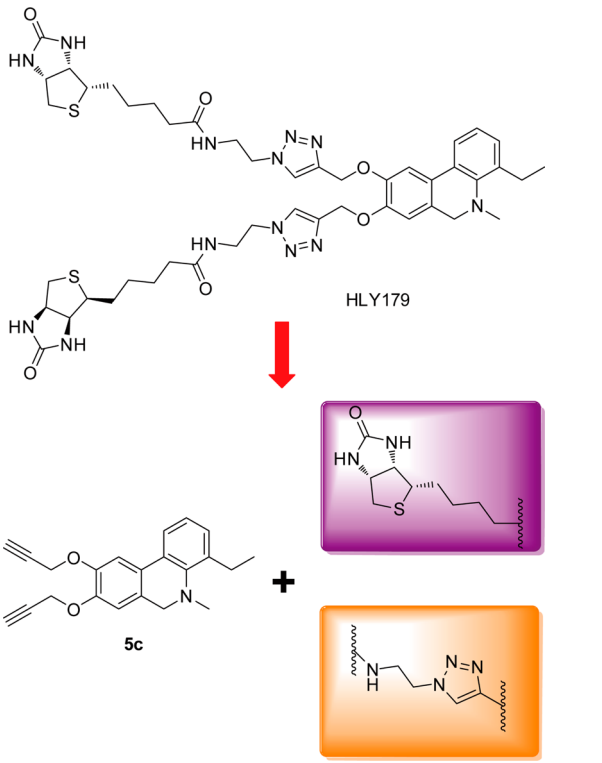

Figure 6. Comparison of HLY179 and the precursor compound **5c**. HLY179 features additional C-8 and C-9 triazole substituents as well as biotin side chains (orange).

To confirm the effects of the triazole and biotin moieties, a series of "model molecules" were designed and synthesized to simulate different substructures of HLY179 (Figure 7a). Of these molecules, compounds **8** and **9** were used to study the effects of the triazole groups, whereas compounds **10**, **11**, and biotin were used to explore the effects of the biotin chain in HLY179. The abilities of these compounds to activate Wnt signaling were next examined (Figure 7b). Compounds **8** and **9** were clearly Wnt agonists, implying that the triazole moiety markedly enhances the activation of Wnt signaling. In contrast, compounds **10**, **11**, and biotin did not exhibit any activity, indicating that biotin itself does not activate Wnt signaling. However, upon the introduction to the phenanthridine skeleton, biotin improved the activation of Wnt signaling. In addition, a comparison of **5c** with **6** and **7** indicated that the basic phenanthridine framework was crucial for Wnt agonist activity.

Design and Synthesis of Potential Wnt Agonists. The SAR studies prompted us to maintain a C-11−C-12 single bond and to introduce triazole disubstituents at the free C-8 and C-9 hydroxy groups to optimize the activity of these compounds. To further explore compounds with these features, compounds **12** and **14**, which feature a C-11−C-12 single bond and C-8

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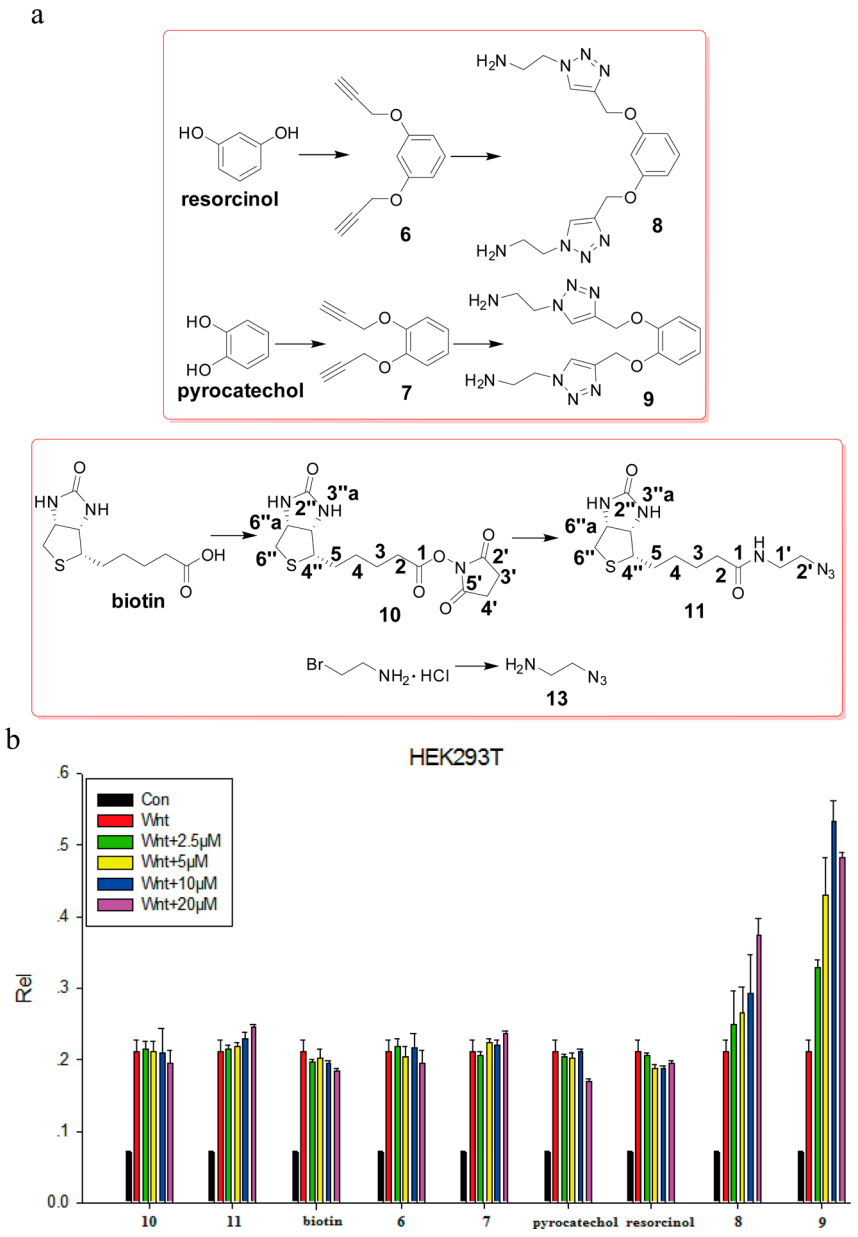

Figure 7. Study of model molecules. (a) Design and syntheses of model molecules. (b) All model molecules except compounds **8** and **9** lacked the ability to activate Wnt signaling.

and C-9 triazole disubstituents (Figure 8a), were designed and synthesized. As expected, compounds **12** and **14** effectively activated Wnt signaling in a reporter gene assay, and **12** exhibited the highest Wnt agonist activity among the synthetic phenanthridine derivatives. Compound **12** doubled the activation of the Wnt signaling pathway at a low concentration of 1.25 μM (Figure 8b). Further evaluation also confirmed the ability of these compounds to activate the expression of endogenous Wnt target genes (Figure 8c). Subsequent docking analysis of **12** indicated that it can associate with the protein residues, in which HLY78 docks, in the axin cavity (Figure 9). This result indicates that the combination of a 4-ethyl-dihydrophenanthridine skeleton and a triazole substituent enhances Wnt activation. Although HLY179 exhibits greater activity than compounds **12** and **14**, the structure makes it more of a molecular probe than a lead candidate, and **12** and **14** are more suitable for further pharmacological or biological studies.

The aforementioned SARs of lycorine-derived HLY78 indicated that the C-11−C-12 single bond is crucial for the activation of Wnt signaling. Moreover, the triazole group appears to serve as a pharmacophore for Wnt activation. Substituting the free C-8 and C-9 hydroxy groups with triazole groups markedly enhanced the activation of Wnt signaling. In addition, biotin itself did not activate Wnt signaling, but when biotin was introduced into the structure of phenanthridine, the abilities of these two derivatives to activate Wnt signaling were improved. Guided by these findings, two good Wnt agonists, **12** and **14**, were designed and synthesized. The activities of these two compounds indicate that the combination of a 4-ethyldihydrophenanthridine skeleton and a triazole substituent optimizes the activation of Wnt. This study not only confirms the predictions obtained from SAR data but also provides suitable lead compounds for use in pharmacological or biological studies.

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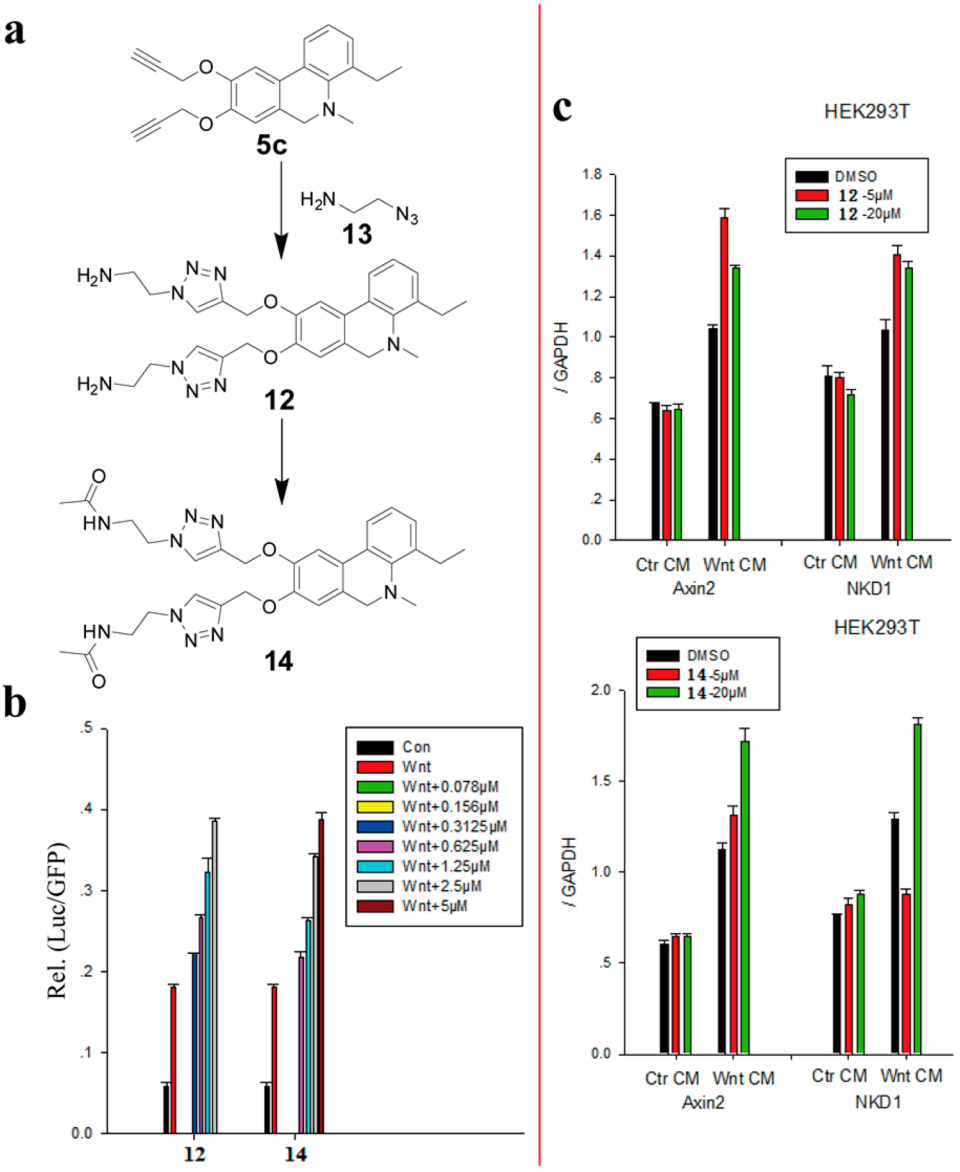

Figure 8. Discovery of the new and effective Wnt agonists compounds **12** and **14**. (a) Syntheses of **12** and **14**. (b) Both **12** and **14** effectively activated Wnt and doubled the activation of the Wnt signaling pathway at concentrations of 1.25 and 2.5 μM, respectively. Compound **12** was the most active Wnt agonist among the examined phenanthridine derivatives. (c) Compounds **12** and **14** clearly activated the expression of endogenous Wnt target genes (*Axin2* and *NKD1*).

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were measured using an X-4 apparatus (Yingyu Yuhua Instrument Factory, Gongyi, Henan Province, P. R. China). ESI and HRMS data were recorded using a Finnigan MAT 90 instrument and a VG Auto Spec-3000 spectrometer, respectively. NMR experiments were conducted on Bruker AM-400, DRX-500, or Avance III 600 spectrometers using residual $CDCl_3$ and DMSO-d_6 or TMS as internal standard. Column chromatography was performed on silica gel (60−80 mesh, 200−300 mesh, 300−400 mesh, Qingdao Haiyang Chemical Co. Ltd., Qingdao, P. R. China). Precoated silica gel 60 GF254 (Merck, Darmstadt, Germany) was used for TLC analyses. Semipreparative HPLC analyses were performed on a Hypersil Gold RP-C_{18} column (i.d. 10 × 250 mm, 5 μm, 5 mL/min) developed with CH_3CN-H_2O at room temperature (rt). All regular solvents and reagents were reagent grade and were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), Acros Organics (Geel, Belgium), and J&K Scientific (Beijing, P. R. China). The purities of all compounds used in biological assays exceeded 95%, as determined by HPLC. HPLC was performed on an X-Bridge RP-C_{18} column (4.6 × 250 mm, 5 μm, 5 mL/min) with CH_3OH-H_2O at rt. All reported yields are for dry compounds that required no further purification for use in other reactions.

Syntheses of Compounds 4 and 5. *5-Methyl-4-vinyl-5,6-dihydro[1,3]dioxolo[4,5-j]phenanthridine* (***2***).[17] A solution of lycorine (300 mg, 1 mmol) in DMF (10 mL) was poured into a round-bottomed flask, followed by the addition of CH_3I (400 μL, 2 mmol). The resulting mixture was stirred at rt for 12 h, after which the mixture was evaporated to remove the DMF. The vessel was charged with *t*-BuOK (1.1 g, 10 mmol) and *t*-BuOH (10 mL), heated to 90 °C, and stirred for 4 h. After cooling the mixture to rt, the reaction was quenched with 50 mL of saturated NH_4Cl and extracted twice with EtO_2 (20 mL). The organic phase was washed with saturated NH_4Cl and brine, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by column chromatography and eluted with petroleum ether−EtOAc (5:1) to yield compound **2** (240 mg, 70% yield). Pale yellow crystals (from $CHCl_3$): mp 168−170 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.58 (1H, d, J = 7.7 Hz), 7.47 (1H, d, J = 7.7 Hz), 7.26 (2H, dt, J = 10.7, 7.1 Hz), 7.16 (1H, d, J = 7.7 Hz, 1), 6.72 (1H, s), 5.99 (2H, s), 5.75 (1H, d, J = 17.8 Hz), 5.32 (1H, d, J = 11.1 Hz), 4.03

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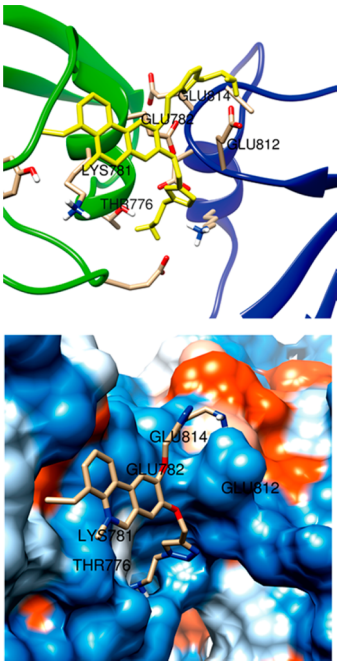

Figure 9. Docking analysis on compound **12**. The results indicated that **12** effectively associates with the axin cavity in which HLY78 docks.

(2H, s), 2.51 (3H, s); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 147.43 (C), 145.16 (C), 133.43 (CH), 133.28 (C), 129.21 (C), 126.43 (C), 125.82 (C), 124.96 (CH), 124.35 (CH), 122.74 (CH), 114.32 (CH_2), 107.17 (CH), 103.61 (CH), 100.93 (CH_2), 54.86 (CH_2), 41.55 (CH); HREIMS m/z 265.1107 (calcd for $C_{17}H_{15}NO_2$, 265.1103).

*4-Ethyl-5-methyl-5,6-dihydro-[1,3]dioxolo[4,5-j]phenanthridine (**3**).*[17] A solution of **2** (27 mg, 0.1 mmol) and 10% Pd/C (30 mg) in CH_2Cl_2 (5 mL) was stirred under an atmosphere of H_2 for 24 h. The organic layer was filtered and concentrated. The residue was purified by column chromatography and was eluted with petroleum ether−EtOAc (15:1) to afford compound **3** (22 mg, 90% yield). Pale yellow crystals (from $CHCl_3$): mp 159−160 °C; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.51 (1H, t, J = 8.4 Hz), 7.28 (2H, d, J = 6.4 Hz), 7.20 (2H, t, J = 7.9 Hz), 6.75 (1H, s), 6.01 (1H, s), 4.01 (2H, s, 2.83 (2H, q, J = 7.5 Hz), 2.50 (3H, s), 1.32 (3H, dd, J = 15.7, 8.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 147.3 (C), 147.12 (C), 145.43 (C), 139.45 (C), 129.33 (C), 127.71 (CH), 126.65 (C), 126.32 (C), 124.64 (CH), 121.02 (CH), 107.26 (CH), 103.76 (CH), 100.95 (CH_2), 55.22 (CH_2), 41.02 (CH), 23.13 (CH_2), 14.85 (CH_3); HREIMS m/z 267.1261 (calcd for $C_{17}H_{17}NO_2$, 267.1259).

Syntheses of Compounds 4 and 5.[17] Compound **2** or **3** (0.2 mmol) was dissolved in 10 mL of CH_2Cl_2. The solution was cooled to −78 °C, and BBr_3 (200 μL, 0.4 mmol) was added. The mixture was stirred for 10 h and diluted in 50 mL of saturated $NaHCO_3$, followed by extraction with CH_2Cl_2 (2 × 20 mL). The organic layer was washed with brine and concentrated. The residue was purified by column chromatography using $CHCl_3$−MeOH (**4**, 25:1; **5**, 20:1) as the eluent to afford **4** (35.7 mg, 70% yield) and **5** (40.8 mg, 80% yield), respectively.

*5-Methyl-4-vinyl-5,6-dihydrophenanthridine-8,9-diol (**4**).*[17] Pale yellow, amorphous powder (from $CHCl_3$): ^{1}H NMR ($CDCl_3$, 800 MHz) δ 7.55 (1H, d, J = 7.7 Hz, H-1), 7.44 (1H, d, J = 7.7 Hz, H-3), 7.21 (2H, m, H-10, H-11), 7.13 (1H, t, J = 7.7 Hz, H-2), 6.73 (1H, s, H-7), 5.72 (1H, d, J = 17.7 Hz, H-12), 5.30 (1H, d, J = 10.9 Hz, H-12), 3.99 (2H, s, H-6), 2.49 (3H, s, NMe); ^{13}C NMR ($CDCl_3$, 200 MHz) δ 145.0 (C, C-9), 143.4 (C, C-8),142.7 (C, C-4a), 133.2 (CH, C-11), 133.0 (C, C-6a), 128.7 (C, C-10a),125.5 (C, C-4), 124.8 (C, C-10b), 124.6 (CH, C-1), 124.1 (CH, C-3), 122.4 (CH, C-2), 114.2 (CH_2, C-12), 113.4 (CH, C-7), 110.1 (CH, C-10), 54.0 (CH_2, C-6),41.5 (CH_3, NMe); HREIMS m/z 253.1109 (calcd for $C_{16}H_{17}NO_2$, 253.1103).

*4-Ethyl-5-methyl-5,6-dihydrophenanthridine-8,9-diphenol (**5**).*[17] Pale yellow, amorphous powder (from $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.46 (1H, d, J = 7.1 Hz), 7.27 (1H, d, J = 2.7 Hz), 7.17 (1H, dt, J = 15.0, 7.5 Hz), 7.00 (1H, s), 6.75 (1H, s), 3.96 (2H, s), 2.82−2.76 (2H, m), 2.31 (3H, s), 1.28 (3H, dd, J = 14.7, 7.1 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 143.83 (C), 143.05 (C), 141.22 (C), 139.43 (C), 128.85 (C), 127.66 (CH), 126.32 (C), 125.18 (C), 124.67 (CH), 120.74 (CH), 113.82 (CH), 110.44 (CH), 53.67 (CH_2), 40.21 (CH_3), 23.26 (CH_2), 14.85 (CH_3); HREIMS m/z 255.1250 (calcd for $C_{16}H_{17}NO_2$, 255.1259).

Alkylation of Compounds 4 and 5. *Syntheses of Compounds **4a** and **5a**−**5i**.* The lycorine derivatives (0.1 mmol) were dissolved in dry THF (10 mL), and NaH (50 mg, 2 mmol) and the appropriate haloalkane (1 mmol) were added. The mixture was stirred at rt for 24 h and quenched with H_2O (50 mL) in an ice bath. The solution was evaporated to remove the THF and extracted with CH_2Cl_2 (2 × 30 mL). The organic layer was washed with saturated $NaHCO_3$ and brine, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by column chromatography using petroleum ether−EtOAc as the eluent to afford compounds **4a** and **5a**−**5i**.

*8,9-Diethoxy-5-methyl-4-vinyl-5,6-dihydrophenanthridine (**4a**).* Yield: 20.1 mg, 65%. Colorless, amorphous powder (from $CHCl_3$): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.96 (d, J = 7.8 Hz, 1H), 7.84 (s, 1H), 7.51 (s, 1H), 7.49 (d, J = 7.5 Hz, 1H), 7.18 (m, 1H), 7.13 (dd, J = 17.3, 10.9 Hz, 1H), 5.71 (d, J = 17.3 Hz, 1H), 5.58 (d, J = 17.9 Hz, 1H), 4.28 (s, 2H), 3.81 (q, J = 7.4 Hz, 2H), 3.76 (q, J = 7.1 Hz, 2H), 2.85 (s, 3H), 1.49−1.43 (m, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 162.4 (C), 151.4 (C), 150.3 (C), 137.6 (CH), 130.9 (CH), 127.3 (C), 126.1 (C), 121.9 (CH), 121.3 (CH), 120.5 (C), 118.9 (C), 117.2, (CH), 114.8 (CH_2), 103.4 (CH), 59.4 (CH_2), 55.3 (CH_2), 54.6 (CH_2), 38.9 (CH_3), 16.5 (CH_3), 16.4 (CH_3); HREIMS m/z 309.1735 $[M]^+$ (calcd for $C_{20}H_{23}NO_2$, 309.1729).

*8,9-Diethoxy-4-ethyl-5-methyl-5,6-dihydrophenanthridine (**5a**).* Yield: 24.9 mg, 80%. Colorless, amorphous powder (from $CHCl_3$): ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.57 (1H, d, J = 7.8 Hz), 7.47 (1H, t, J = 7.8 Hz), 7.41 (1H, s), 7.33 (1H, d, J = 7.9 Hz), 6.83 (1H, s), 4.13 (2H, s), 3.46 (2H, q, J = 7.1 Hz), 3.39 (2H, q, J = 7.1 Hz), 2.58 (3H, s), 2.35−2.31 (2H, m), 1.53−1.43 (6H, m), 1.29 (3H, t, J = 7.3 Hz); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 157.32 (C), 148.43 (C), 141.41 (C), 139.75 (C), 133.36 (C), 132.11 (CH), 130.83 (C), 129.14 (C), 124.10 (CH), 121.12 (CH), 107.66 (CH), 106.27 (CH), 58.39 (CH_2), 58.26 (CH_2), 54.03 (CH_2), 40.31 (CH_3), 28.55 (CH_2), 16.84 (CH_3), 16.62 (CH_3), 15.81 (CH_3); HREIMS m/z 311.1874 (calcd for $C_{20}H_{25}NO_2$, 311.1885).

*4-Ethyl-8,9-dimethoxy-5-methyl-5,6-dihydrophenanthridine (**5b**).* Yield: 23.9 mg, 87%. Colorless, amorphous powder (from $CHCl_3$): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.57 (1H, d, J = 7.1 Hz), 7.53 (1H, s), 7.49 (1H, s), 7.18 (1H, d, J = 6.9 Hz), 6.75 (1H, m), 4.06 (2H, s), 4.02 (3H, s), 3.98 (3H, s), 2.96 (2H, q, J = 7.4 Hz), 2.41 (3H, s), 1.23 (3H, t, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 153.44 (C), 146.52 (C), 140.53 (C), 138.81 (C), 132.96 (C), 131.73 (CH), 130.04 (C), 129.01 (C), 123.23 (CH), 120.56 (CH), 108.92 (CH), 103.18 (CH), 52.32 (CH_2), 56.83 (CH_3), 56.41 (CH_3), 39.95 (CH_3), 28.58 (CH_2), 15.80 (CH_3); HREIMS m/z 283.1567 (calcd for $C_{18}H_{21}NO_2$, 283.1572).

*4-Ethyl-5-methyl-8,9-bis(prop-2-yn-1-yloxy)-5,6-dihydrophenanthridine (**5c**).* Yield: 22.3 mg, 70%. Pale yellow, amorphous powder (from $CHCl_3$): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.55 (1H, dd, J = 7.0, 2.1 Hz), 7.47 (1H, s), 7.19−7.14 (2H, m), 6.92 (1H, s), 4.85−4.82 (4H, m), 4.03 (2H, s), 2.85−2.77 (2H, m), 2.59−2.54 (2H, m), 2.51 (3H, s), 1.30 (3H, q, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 147.43 (C), 146.82 (C), 145.61 (C), 139.58 (C), 128.97 (C), 127.94 (CH), 126.82 (C), 126.34 (C), 124.61 (CH), 121.03 (CH), 113.15 (CH), 110.51 (CH), 78.68 (CH), 78.44 (CH), 75.91 (C), 75.89 (C), 55.22 (CH_2), 56.9 3 (CH_2), 54.81 (CH_2), 41.36 (CH_3), 23.13 (CH_2), 14.87 (CH_3); HREIMS m/z 331.1579 (calcd for $C_{22}H_{21}NO_2$, 331.1572).

*8,9-Bis[(1,3-dimethyl-1H-pyrazol-5-yl)methoxy]-4-ethyl-5-methyl-5,6- dihydrophenanthridine (**5d**).* Yield: 26.8 mg, 57%. Pale yellow powder (from $CHCl_3$): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.50 (1H, dd,

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J. Nat. Prod. XXXX, XXX, XXX−XXX

J = 7.3, 1.6 Hz), 7.36 (1H, s), 7.23−7.12 (2H, m), 6.85 (1H, s), 6.05 (2H, s), 5.04 (2H, s), 5.02 (2H, s), 4.00 (2H, s), 3.82 (3H, s), 3.81 (3H, s), 2.80 (2H, q, J = 7.5 Hz), 2.47 (3H, s), 2.24 (3H, s), 2.24 (3H, s), 1.30 (3H, t, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 148.40 (C), 147.79 (C), 147.21 (C), 147.18 (C), 145.64 (C), 139.67 (C), 137.99 (C), 137.80 (C), 128.70 (C), 128.08 (CH), 127.26 (C), 126.79 (C), 124.67 (CH), 120.97 (CH), 114.06 (CH), 111.55 (CH), 106.82 (CH), 106.81 (CH), 62.51 (CH_2), 62.07 (CH_2), 54.76 (CH_2), 41.31 ($2CH_3$), 36.32 (CH_3), 23.15 (CH_2), 14.83 (CH_3), 13.37 ($2CH_3$); HREIMS m/z 417.2635 (calcd for $C_{28}H_{33}N_5O_2$, 471.2634).

*8,9-Bis[(3,5-dimethoxybenzyl)oxy]-4-ethyl-5-methyl-5,6-dihydrophenanthridine (**5e**).* Yield: 33.3 mg, 60%. Colorless, amorphous powder (from $CHCl_3$): ^{1}H NMR ($CDCl_3$, 400 MHz,) δ 7.49 (1H, dd, J = 7.1, 1.9 Hz), 7.37 (1H, s), 7.22−7.10 (2H, m), 6.83 (1H, s), 6.68 (2H, d, J = 2.2 Hz), 6.66 (2H, d, J = 2.2 Hz), 6.41 (2H, d, J = 1.9 Hz), 5.17 (2H, s), 5.14 (2H, s), 3.99 (2H, s), 3.77 (12H, overlap), 2.80 (2H, q, J = 7.5 Hz), 2.47 (3H, s), 1.31 (1H, t, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$, 101 MHz,) δ 160.91 (4C), 148.88 (C), 148.25 (C), 145.61 (C), 139.80 (C), 139.61 (C), 139.54 (C), 129.09 (C), 127.70 (CH), 126.31 (C), 125.82 (C), 124.57 (CH), 120.93 (CH), 113.07 (CH), 110.41 (CH),104.99 (2CH), 104.92 (2CH), 99.87 (CH), 99.79 (CH), 71.62 (CH_2), 71.13 (CH_2), 55.27 ($4CH_3$), 54.85 (CH_2), 41.30 (CH_3), 23.16 (CH_2), 14.85 (CH_3); HRESIMS m/z 556.2696 (calcd for $C_{34}H_{38}NO_6$, 556.2699).

*8,9-Bis[(1,5-dimethyl-1H-pyrazol-3-yl)methoxy]-4-ethyl-5-methyl-5,6- dihydrophenanthridine (**5f**).* Yield: 28.3 mg, 60%. Pale yellow, amorphous powder (from $CHCl_3$): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.51 (1H, dd, J = 6.5, 2.7 Hz), 7.47 (1H, s), 7.18−7.11 (2H, m), 6.89 (1H, s), 6.16 (1H, s), 6.15 (1H, s), 5.18 (2H, s), 5.14 (2H, s), 3.96 (2H, s), 3.77 (6H, s), 2.79 (2H, q, J = 7.5 Hz), 2.45 (3H, s), 2.26 (3H, s), 2.24 (3H, s), 1.29 (3H, t, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$, 101 MHz) δ 148.91 (C), 148.14 (C), 147.57 (C), 147.40 (C), 145.54 (C), 139.47 (C), 139.44 (C), 139.38 (C), 129.33 (C), 127.41 (CH), 125.92 (C), 125.39 (C), 124.52 (CH), 121.09 (CH), 113.09 (CH), 110.67 (CH), 104.97 (CH), 104.95 (CH), 65.87 (CH_2), 65.58 (CH_2), 54.82 (CH_2), 41.32 (CH_3), 35.96 ($2CH_3$), 23.11 (CH_2), 14.86 (CH_3), 11.26 ($2CH_3$); HREIMS m/z 471.2621 $[M]^+$ (calcd for $C_{28}H_{33}N_5O_2$, 471.2634).

*8,9-Bis{[5-(1H-pyrazol-1-yl)pyridin-2-yl]methoxy}-4-ethyl-5-methyl-5,6- dihydrophenanthridine (**5g**).* Yield: 37.0 mg, 65%. Pale yellow powder (from $CHCl_3$): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.54 (2H, s), 8.47 (2H, s, 2H), 8.02−7.96 (2H, m), 7.92 (2H, s), 7.72 (2H, s), 7.48 (1H, d, J = 6.7 Hz), 7.38 (1H, s), 7.15 (2H, m), 6.85 (1H, s), 6.45 (2H, s), 5.20 (2H, s), 5.18 (2H, s), 3.98 (2H, s), 2.78 (2H, d, J = 7.6 Hz), 2.45 (3H, s), 1.28 (3H, t, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 148.96 (C), 148.38 (C), 147.76 (CH), 147.68 (CH), 146.08 (C), 142.59 (CH), 142.55 (CH), 140.09 (C), 138.82 (CH), 138.76 (CH), 130.78 (C), 130.59 (C), 129.61 (C), 129.20 (C), 128.46 (CH), 127.52 (2CH), 127.43 (C), 127.00 (C), 125.10 (CH), 121.38 (CH), 114.10 (CH), 112.70 (CH), 112.68 (CH), 111.46 (CH), 108.33 (CH), 108.30 (CH), 69.68 (CH_2), 69.20 (CH_2), 55.24 (CH_2), 41.76 (CH_3), 23.59 (CH_2), 15.28 (CH_3); HREIMS m/z 568.2510 $[M]^+$ (calcd for $C_{34}H_{31}N_7O_2$, 568.2503).

*8,9-Bis{[methyl-(1,1′-biphenyl)-2-carboxylate]methoxy}-4-ethyl-5-methyl-5,6-dihydrophenanthridine (**5h**).* Yield: 49.2 mg, 70%. Colorless, amorphous powder (from $CHCl_3$): ^{1}H NMR ($CDCl_3$/methanol-d_4, 400 MHz) δ 7.85−7.78 (m, 2H), 7.58−7.29 (m, 16H), 7.21−7.10 (m, 2H), 6.87 (s, 1H), 5.28 (s, 2H), 5.26 (s, 2H), 3.99 (s, 2H), 3.61 (s, 3H), 3.55 (s, 3H), 2.79 (q, J = 7.5 Hz, 2H), 2.47 (s, 3H), 1.30 (t, J = 7.5 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 169.38 (C), 169.30 (C), 149.04 (C), 148.32 (C), 145.48 (C), 142.09 (C), 142.06 (C), 140.91 (C), 140.88 (C), 139.51 (C), 136.35 (C), 136.12 (C),131.37 (2CH), 131.34 (2CH), 130.74 (CH), 130.71 (CH), 129.79 (CH), 129.75 (CH), 129.04 (2C), 128.47 (2CH), 127.79 (2CH), 127.25 (2CH), 127.22 (2CH), 127.15 (CH), 126.33 (C), 125.89 (C), 124.69 (CH), 120.95 (CH), 113.44 (CH), 110.93 (CH), 71.71 (CH_2), 71.22 (CH_2), 54.82 (CH_2), 51.96 (CH_3), 51.91 (CH_3), 41.26 (CH_3), 23.10 (CH_2), 14.82 (CH_3); HRESIMS m/z 704.3021 $[M + H]^+$ (calcd for $C_{46}H_{42}NO_6$, 704.3012).

*8,9-Bis[(5-chlorobenzothiophen-2-yl)methoxy]-4-ethyl-5-methyl-5,6- dihydrophenanthridine (**5i**).* Yield: 36.9 mg, 60%. Colorless, amorphous powder (from $CHCl_3$): ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.94 (1H, d, J = 1.9 Hz), 7.90 (1H, d, J = 3.1 Hz), 7.75 (2H, dd, J = 8.6, 4.6 Hz), 7.52−7.46 (3H, m), 7.43 (1H, s), 7.33−7.29 (2H, m), 7.24−7.13 (2H, m), 6.91 (1H, s), 5.36 (1H, s), 5.34 (2H, s), 4.01 (2H, s), 2.80 (2H, q, J = 7.5 Hz), 2.48 (2H, s), 1.30 (3H, t, J = 7.5 Hz); ^{13}C NMR ($CDCl_3$, 126 MHz) δ 148.89 (C), 148.27 (C), 145.70 (C), 139.66 (C), 139.12 (C), 139.02 (C), 138.76 (C), 138.74 (C), 131.75 (C), 131.47 (C), 130.69 (C), 128.91 (C), 127.99 (CH), 127.12 (CH), 127.08 (C), 127.02 (CH), 126.58 (C), 125.06 (CH), 125.04 (CH), 124.70 (CH), 123.82 (CH), 123.79 (CH), 121.97 (CH), 121.85 (CH), 121.05 (CH), 113.92 (CH), 111.38 (CH), 66.74 (CH_2), 66.31 (CH_2), 54.87 (CH_2), 41.39 (CH_3), 23.21 (CH_2), 14.89 (CH_3); HREIMS m/z 615.0863 $[M]^+$ (calcd for $C_{34}H_{27}Cl_2NO_2S_2$, 615.0860).

Syntheses of "Model Molecules". *Syntheses of Compounds **6** and **7**.*[14] Pyrocatechol or resorcinol (0.1 mmol) was dissolved in dry acetone (3 mL), and K_2CO_3 (0.4 mmol) and propargyl bromide (20 μL, 0.25 mmol) were added. The mixture was stirred at 60 °C for 24 h and quenched with H_2O (50 mL) in an ice bath. The solution was evaporated to remove acetone and extracted with CH_2Cl_2 (2 × 30 mL). The organic layer was washed with saturated $NaHCO_3$ and brine, dried over $MgSO_4$, filtered, and concentrated. The residue was purified by column chromatography with $CHCl_3$−MeOH (100:1) to afford compounds **6** and **7**, respectively.

*1,3-Bis(prop-2-yn-1-yloxy)benzene (**6**).* Yield: 16.7 mg, 90%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.24−7.20 (1H, m), 6.64−6.62 (3H, m), 6.39 (1H, t, J = 2.9 Hz), 4.67 (4H, s), 2.56−2.55 (2H, m); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 158.69 (2C), 129.96 (CH), 107.84 (2CH), 102.39 (CH), 78.49 (2C),75.73 (2CH), 55.82 ($2CH_2$); ESIMS m/z 187 $[M + H]^+$.

*1,2-Bis(prop-2-yn-1-yloxy)benzene (**7**).* Yield: 17.7 mg, 95%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.06−6.95 (4H, overlap), 4.72 (4H, s), 2.52−2.51 (2H, m); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 159.21 (2C), 126.38 (2CH), 111.26 (2CH), 79.04 (2C), 76.21 (2CH), 56.13 ($2CH_2$); ESIMS m/z 187 $[M + H]^+$.

*Syntheses of Compounds **8** and **9**.* Compound **6** or **7** (66.0 mg, 0.2 mmol) was added to compound **13** (22 μL, 0.25 mmol) in H_2O/*t*-BuOH (2 mL, 1:1), followed by the addition of $CuSO_4$ (3.0 mg) and a sodium ascorbate solution (50 μL, 1 M solution). The mixture was stirred for 15 h at rt and concentrated in vacuo; the resultant residue was purified by column chromatography with $CHCl_3$−MeOH (50:1) to yield compounds **8** and **9**, respectively.

*2,2′-{[(1,3-Phenylenebis(oxy))bis(methylene)]bis(1H-1,2,3-triazole-4,1-diyl)}bis(ethan-1-amine) (**8**).* Yield: 17.9 mg, 50%. ^{1}H NMR (methanol-d_4, 400 MHz) δ 7.41 (2H, s), 7.21−7.17 (1H, m), 6.46−6.40 (3H, m), 5.07 (4H, s), 4.29−4.21 (4H, m), 3.21−3.14 (4H, m); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 157.13 (2C), 143.68 (2C), 128.67 (CH), 126.64 (2CH),109.34 (2CH), 103.45 (CH), 62.23 ($2CH_2$), 57.44 ($2CH_2$), 54.39 ($2CH_2$); ESIMS m/z 359 $[M + H]^+$.

*2,2′-{[(1,2-Phenylenebis(oxy))bis(methylene)]bis(1H-1,2,3-triazole-4,1-diyl)}bis(ethan-1-amine) (**9**).*[17] Yield: 16.1 mg, 45%. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.41 (2H, s), 7.13−6.99 (4H, overlap), 5.11 (4H, s), 4.29−4.22 (4H, m), 3.21−3.14 (4H, m); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 159.21 (2C), 144.38 (2C), 127.71 (2CH), 126.92 (2CH), 112.06 (2CH), 61.18 ($2CH_2$), 58.68 ($2CH_2$), 55.19 ($2CH_2$); ESIMS, m/z 359 $[M + H]^+$.

*2-Azidoethylamine (**13**).*[17] 2-Bromoethylamine hydrobromide (500 mg, 2.44 mmol) and sodium azide (475.9 mg, 7.32 mmol) were dissolved in H_2O (2 mL); the solution was heated to 75 °C, stirred for 21 h, and cooled to 0 °C. To this mixture were added KOH (800 mg) and Et_2O (2 mL), and the solution was extracted with Et_2O (2 × 10 mL) and concentrated in vacuo. The resultant residue was purified by column chromatography with $CHCl_3$−MeOH (20:1) to afford compound **13** (171 mg, 82% yield). Colorless liquid (from ethyl ether): ^{1}H NMR ($CDCl_3$, 400 MHz) δ 3.30 (2H, t, J = 5.7 Hz, CH_2NH_2), 2.80−2.84 (2H, m, CH_2N_3), 1.27 (2H, s, NH_2); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 54.6 (CH_2N_3), 41.2 (CH_2NH_2); ESIMS m/z 87 $[M + H]^+$.

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*2′,5′-Dioxopyrrolidin-1-yl-5-[(3″aS,4″S,6″aR)-2″-oxohexahydro-1H- thieno(3″,4″-d)imidazol-4″-yl]pentanoate (**10**).*[17] A solution of D-biotin (24 mg, 0.1 mmol) in DMF (10 mL) was added to pyridine (2 mL) and dicyclohexylcarbodiimide (DCC) (41 mg, 0.2 m). The mixture was stirred for 0.5 h and charged with *N*-hydroxysuccinimide (13.8 mg, 0.12 mmol). The resultant solution was stirred for 24 h at rt and concentrated in vacuo. The residue was recrystallized from propanol to give **10** (22 mg, 65% yield). Colorless solid (from $CHCl_3$): mp 204−206 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 6.42 (1H, s, NH), 6.36 (1H, s, NH), 4.27−4.32 (1H, m, H-6″a), 4.11−4.16 (1H, m, H-3″a), 3.06−3.12 (1H, m, SCH), 2.78−2.85 (5H, m, CH_2CH_2 (succinyl), SCH_2), 2.66 (2H, t, J = 7.3 Hz, H-2), 2.57 (1H, d, J = 11.4 Hz, SCH_2), 1.58−1.68 (3H, m, H-3, H-5), 1.35−1.54 (3H, m, H-4, H-5); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 170.34 ($N(CO)_2$), 168.91 (CO_2), 162.73 ($(HN)_2CO$), 61.05 (C-3″a), 59.23 (C-6″a), 55.27 (SCH), 39.94 (SCH_2), 30.08 (C-2), 27.85 (C-4), 27.65 (C-5), 25.44 (CH_2CH_2 (succinyl)), 24.37 (C-3); ESIMS (m/z) 342[M + H]$^+$.

*N-(2′-Azidoethyl)-5-[(3″aS,4″S,6″aR)-2″-oxohexahydro-1H-thieno(3″,4″-d)imidazol-4″-yl]pentanamide (**11**).*[17] Compounds **10** (34 mg, 0.1 mmol) and **13** (17 mg 0.2 mmol) were dissolved in DMF (2 mL), and Et_3N (12 mg, 1.2 mmol) was added. The solution was stirred for 24 h at rt and concentrated in vacuo. The resultant residue was purified by column chromatography using $CHCl_3$−MeOH (20:1) to yield **11** (23 mg, 70% yield). Colorless waxy substance (from $CHCl_3$): ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.03 (1H, t, J = 5.3 Hz, CONH), 6.42 (1H, s, NH), 6.35 (1H, s, NH), 4.26−4.32 (1H, m, H-6″a), 4.08−4.14 (1H, m, H-3″a), 3.31 (2H, d, J = 7.6 Hz, CH_2N_3), 3.19−3.24 (2H, m, $CH_2CH_2N_3$), 3.05−3.11 (1H, m, SCH), 2.80 (1H, dd, J = 12.4, 5.1 Hz, SCH_2), 2.56 (1H, d, J = 12.9 Hz, SCH_2), 2.06 (2H, t, J = 7.3 Hz, H-2), 1.55−1.65 (1H, m, H-5), 1.39−1.55 (3H, m, H-3, H-5), 1.20−1.38 (2H, m, H-4); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 172.43 (CONH), 162.74 ($(HN)_2CO$), 61.06 (C-3″a), 59.23 (C-6″a), 55.45 (SCH), 50.01 (CH_2N_3), 39.97 (SCH_2), 38.10 ($CH_2CH_2N_3$), 35.13 (C-2), 28.22 (C-4), 28.07 (C-5), 25.26 (C-3); HRESIMS m/z 313.1442 [M + H]$^+$ (calcd for $C_{12}H_{21}N_6O_2S$, 313.1446).

*2,2′-{[((4-Ethyl-5-methyl-5,6-dihydrophenanthridine-8,9-diyl)bis-(oxy))bis(methylene)]bis(1H-1,2,3-triazole-4,1-diyl)}bis(ethan-1-amine) (**12**).*[17] Compound **5c** (66.0 mg, 0.2 mmol) was added to compound **13** (22 μL, 0.25 mmol) in H_2O/*t*-BuOH (2 mL, 1:1), followed by the addition of $CuSO_4$ (3.0 mg) and a sodium ascorbate solution (50 μL, 1 M solution). The resultant solution was stirred for 20 h at 60 °C and concentrated in vacuo; the resultant residue was purified by column chromatography with $CHCl_3$−MeOH (9:1) to afford **12** (60 mg, 55% yield). Waxy substance (from CH_3OH): ^{1}H NMR (methanol-d_4, 400 MHz) δ 8.07 (1H, s), 8.05 (1H, s), 7.55 (1H, dd, J = 7.3, 1.6 Hz, 1), 7.48 (1H, s), 7.18−7.04 (2H, m), 7.00 (1H, s), 5.22 (2H, s), 5.18 (2H, s), 4.45 (4H, dd, J = 11.7, 5.9 Hz), 3.93 (2H, s), 3.12 (4H, br s), 2.74 (2H, q, J = 7.5 Hz), 2.39 (3H, s), 1.25 (3H, t, J = 7.5 Hz); ^{13}C NMR (methanol-d_4, 101 MHz) δ 149.89 (C), 149.17 (C), 146.73 (C), 145.14 (C), 144.92 (C), 140.63 (C), 130.18 (C), 129.13 (CH), 128.10 (C), 127.65 (C), 126.14 (CH), 126.10 (CH), 125.96 (CH), 122.39 (CH), 114.87 (CH), 112.14 (CH), 64.21 (CH_2), 63.73 (CH_2), 55.58 ($2CH_2$), 53.29 ($2CH_2$), 42.34 (CH_2), 41.70 (CH_3), 24.31 (CH_2), 15.51 (CH_3); HREIMS m/z 503.2759 (calcd for $C_{26}H_{33}N_9O_2$, 503.2757).

*N,N′-{[((4-Ethyl-5-methyl-5,6-dihydrophenanthridine-8,9-diyl)-bis(oxy))bis(methylene))bis(1H-1,2,3-triazole-4,1-diyl)]bis(ethane-2,1-diyl)}diacetamide (**14**).* Compound **12** (50 mg 0.1 mmol) was added to a solution of pyridine (3 mL) and Ac_2O (16 mg, 0.16 mmol). The resultant mixture was stirred at rt for 1 h under N_2 and poured into ice-cold H_2O (50 mL) with vigorous stirring. The mixture was extracted with EtOAc (2 × 30 mL), washed with saturated $NaHCO_3$ and brine, concentrated, and separated by silica gel column chromatography to afford compound **14** with a yield of 32%. Waxy substance (from $CHCl_3$): ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.57 (1H, s), 7.54 (1H, s), 7.48−7.44 (1H, m), 7.39 (1H, s), 7.16−7.07 (2H, m), 6.86 (1H, s), 5.10 (2H, s), 5.08 (2H, s), 4.42 (4H, dd, J = 10.8, 7.0 Hz), 3.95 (2H, s), 3.70 (4H, d, J = 4.1 Hz), 2.41 (3H, s), 1.93 (3H, s), 1.92 (3H, s), 1.32 (2H, t, J = 7.3 Hz), 1.24 (3H, t, J = 7.6 Hz); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.20 (2C), 148.84 (C), 148.29 (C), 145.72 (C), 144.07 (C), 143.81 (C), 139.73 (C), 128.63 (C), 128.23 (CH), 127.74 (C), 127.27 (C), 124.74 (CH), 124.20 (CH), 124.14 (CH), 121.07 (CH), 115.00 (CH), 112.55 (CH), 64.72 (CH_2), 64.10 (CH_2), 54.78 (CH_2), 49.81 (CH_2), 49.79 (CH_2), 41.42 (CH_3), 39.74 (CH_2), 39.68 (CH_2), 23.21 (CH_2), 23.03 ($2CH_3$), 14.87 (CH_3); HREIMS m/z 587.2960 [M]$^+$ (calcd for $C_{30}H_{37}N_9O_4$, 587.2969).

Bioactivity Assays. *Cell Culture.* HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum in a humidified 5% CO_2/95% air (v/v) atmosphere at 37 °C.

Reporter Gene Assay. HEK293T cells were transfected using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. For reporter gene assays, HEK293T cells were seeded in 24-well plates. Each well was transfected with 250 ng of plasmids in total, including 20 ng of TOPFlash and 25 ng of EGFP-C1. The LacZ plasmid was added to equalize the total amount of plasmid in the wells to 250 ng. Eighteen hours after transfection, the cells were treated with Wnt3a-conditioned medium (Wnt3a CM) or control medium (Ctr CM) for an additional 6 h and were lysed using a Boehringer Mannheim luciferase assay kit (200 μL/well) for luciferase assays. The fluorescence intensity emitted by green fluorescent protein in the resultant cell lysates was first determined in a Wallac multicounter capable of counting fluorescence and luminescence. Next, the luciferase substrate was added to the cell lysates, and the luciferase activities were determined by measuring the luminescence intensities using the same counter. The luminescence intensities were normalized against the fluorescence intensities.[20]

Target Gene Assay. Cells were treated with the synthesized compounds as indicated and control or Wnt3a CM for 6 h. Total RNA was extracted with TRIzol. Additionally, purified RNA was reverse transcribed using oligo(dT) priming and the Superscript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. The gene transcripts were quantified by quantitative real-time PCR using a Quantitative SYBR green PCR kit (Takara SYBR premix Ex Taq) and ABI Quant Studio 6. Gene expression was normalized by GAPDH. The following primer pairs were used for the target genes: *Axin2*: 5′-AGGCTAGCTGAGGTGT-3′ and 5′-AGGCTTGGATTGGAGAA-3′; *NKD1*: 5′-GTCAACCACTCCC-CAACATC-3′ and 5′-AATGGTGGTAGCAGCCAGAC-3′; *GAPDH*: 5′-AGGTCGGAGTCAACGGATTTG-3′ and 5′-TGTAAACCATGTAGTTGAGGTCA-3′.

Molecular Docking Protocol. Compounds **12** and HLY78 were docked with EPAC2 using the X-ray structure of axin (PDB code: 1WSR) and AutoDock 4.2. The H_2O molecules and ligand within the crystal structure were removed, and polar hydrogen moieties were added using AutoDockTools. In the structures of the analogues, all bonds were rotatable except the aromatic, amide, cyano, and double bonds; the protein was treated as a rigid structure. The docking runs were performed using the standard parameters of the program for interactive growth and subsequent scoring.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00825.

NMR spectra of the synthetic compounds (PDF)

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Notes

The authors declare no competing financial interest.

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