

Synthesis and Cytotoxic Evaluation of *N*-Alkyl-2-halophenazin-1-ones

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Cite This: *ACS Omega* 2020, 5, 27667–27674



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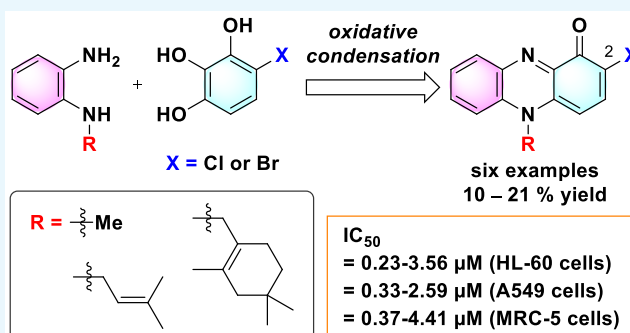


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Supporting Information

ABSTRACT: In this study, the synthesis of *N*-alkyl-2-halophenazin-1-ones has been established. Six *N*-alkyl-2-halophenazin-1-ones, including WS-9659 B and marinocyanins A and B, were synthesized by the direct oxidative condensation of 4-halo-1,2,3-benzenetriol with the corresponding *N*-alkylbenzene-1,2-diamines. One of the most significant features of the present method is that it can be successfully applied to the synthesis of *N*-alkyl-2-chlorophenazin-1-ones. The traditional chlorination of *N*-alkylphenazin-1-ones with *N*-chlorosuccinimide selectively occurs at the 4-position to afford the undesired *N*-alkyl-4-chlorophenazin-1-ones. Our synthetic route successfully circumvents this problem, culminating in the first chemical synthesis of WS-9659 B. The cytotoxicity of six *N*-alkyl-2-halophenazin-1-ones and three *N*-alkylphenazin-1-ones against human promyelocytic leukemia HL-60, human lung cancer A549, and normal MRC-5 cells was evaluated. Among the compounds tested in this study, 2-chloropyocyanin possesses significant selectivity toward A549 cells. The cytotoxic evaluation provides structural insights into the potency and selectivity of these compounds for cancer cells.



INTRODUCTION

Phenazines are a large group of nitrogen-containing heterocycles. To date, more than 100 phenazine derivatives have been identified in nature and over 6000 phenazine compounds synthesized.^{1–4} *N*-Alkylphenazin-1-ones are a minor group of the phenazines. To the best of our knowledge, only four *N*-alkylphenazin-1-ones [pyocyanin,^{5–10} lavanducyanin (WS-9659 A),^{11–15} phenazinomycin,^{16,17} and 1-hydroxy-7-oxolavanducyanin¹⁸ and seven *N*-alkyl-2-halophenazin-1-ones (WS-9659 B^{12–14} and marinocyanins A-F^{15,19}) have been identified as natural products. In the present study, we focused on *N*-alkylphenazin-1-ones and *N*-alkyl-2-halophenazin-1-ones, which are depicted in Figure 1. Among them, pyocyanin (1a), lavanducyanin (WS-9659 A) (2a), WS-9659 B (2c), and marinocyanins A (2b) and B (3b) are natural products, which were isolated from *Pseudomonas* and *Streptomyces* species (Figure 1).^{3,4} One of the simplest and best-known *N*-alkylphenazin-1-ones is pyocyanin (1a) isolated from *Pseudomonas aeruginosa*.^{5,6} Pyocyanin shows several biological activities including antibacterial, antifungal, and cytotoxic activities.⁵ This compound plays an important role in biofilm formation and quorum sensing in *P. aeruginosa*.^{6–10} Lavanducyanin (2a), isolated from the *Streptomyces* sp., shows cytotoxic activities against mouse leukemia P388 and mouse lymphoid leukemia L1210 cells with half-maximal inhibitory concentration (IC₅₀) values of 0.09 and 0.10 μg/mL, respectively.¹¹ The same compound and its 2-chlorinated derivative, respectively, named as WS-9659 A (2a) and WS-

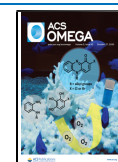
9659 B (2c), were isolated from the *Streptomyces* sp. as testosterone 5α-reductase inhibitors with IC₅₀ values of 0.5 and 10 μM, respectively.^{12–14} Marinocyanins A (2b) and B (3b), 2-brominated derivatives, were isolated from a marine-derived *Streptomyces* sp. and actinomycete strains CNS-284 and CNY-960.^{15,19} Compounds 2b and 3b inhibited TNF-α-induced NFκB activity (IC₅₀ values of 4.1 and 24.2 μM, respectively) and LPS-induced nitric oxide production (IC₅₀ values of >48.6 and 15.1 μM, respectively).¹⁵ They blocked PGE₂ production with greater efficacy (IC₅₀ values of 7.5 and 0.89 μM, respectively).¹⁵ Compounds 2b and 3b also showed strong cytotoxic activity against human colon carcinoma HCT116 cells with IC₅₀ values of 0.049 and 0.029 μM, respectively.¹⁹ In addition, both these compounds display modest antimicrobial activity against *Staphylococcus aureus* and the pathogenic yeast *Candida albicans*.¹⁹

Because of these diverse biological activities, *N*-alkylphenazin-1-ones are attractive synthetic targets.^{3,4,20–22} Our group recently reported the synthesis of 1a, 2a, 2b, 3a, and 3b.²² The oxidative condensation of pyrogallol with *N*-alkylbenzene-1,2-

Received: August 31, 2020

Accepted: October 2, 2020

Published: October 16, 2020



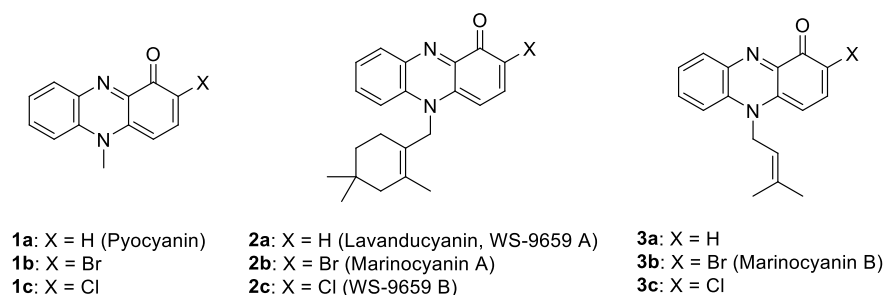
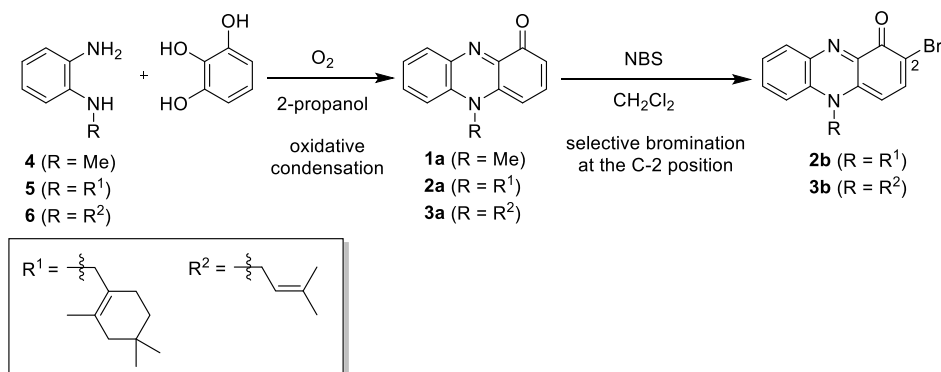


Figure 1. Structures of our target *N*-alkylphenazin-1-ones.

Scheme 1. Syntheses of Pyocyanin (1a), Lavanducyanin (2a), and Marinocyanins A (2b) and B (3b)



diamines (**4**, **5**, and **6**) under an oxygen atmosphere in 2-propanol afforded **1a**, **2a**, and **3a**, respectively (Scheme 1). Bromination of the resultant phenazin-1-ones (**2a** and **3a**) proceeded at the 2-position in a regioselective manner to afford **2b** and **3b**, respectively.

In the present study, we describe the synthesis of *N*-alkyl-2-halophenazin-1-ones by coupling 4-halo-1,2,3-benzenetriol with the corresponding *N*-alkylbenzene-1,2-diamines. The first synthesis of WS-9659 B (**2c**) was achieved by oxidative condensation of 4-chloro-1,2,3-benzenetriol with the corresponding *N*-alkylbenzene-1,2-diamine. Furthermore, the cytotoxic activities of synthetic *N*-alkylphenazin-1-ones and *N*-alkyl-2-halophenazin-1-ones were evaluated.

RESULTS AND DISCUSSION

Halogenation of Pyocyanin and Lavanducyanin. To obtain 2-chlorinated *N*-alkylphenazin-1-ones, halogenation of pyocyanin (**1a**) was examined. A drastic change in the regioselectivity between bromination and chlorination was observed (Scheme 2). Bromination of **1a** with *N*-bromosuccinimide (NBS) in CH₂Cl₂ afforded 2-bromopyocyanin (**1b**) in 51% yield. However, chlorination of **1a** with *N*-chlorosuccinimide (NCS) gave 4-chloropyocyanin (**7**) in 65% yield. The position of the chlorine atom in **7** was confirmed by nuclear Overhauser effect spectroscopy (NOESY) correlations. NOESY correlations between H-2 (δ_{H} 6.47) and H-3 (δ_{H} 7.44) and between the H-6 (δ_{H} 7.40) and *N*-methyl group (δ_{H} 4.07) are observed (Figures 2, S32 and S33 in the Supporting Information). The difference in outcome can presumably be attributed to steric hindrance between the *N*-methyl group in **1a** and the halogen atom. The van der Waals radius of a chlorine atom (1.75 Å) is smaller than that of a bromine atom (1.85 Å).^{23,24} Consequently, electrophilic substitution with the sterically large bromine atom at the 4-position is disfavored.

Scheme 2. Selective Halogenation of Pyocyanin with NBS and NCS

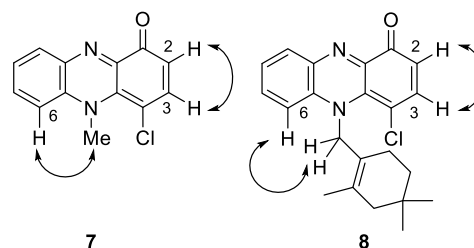
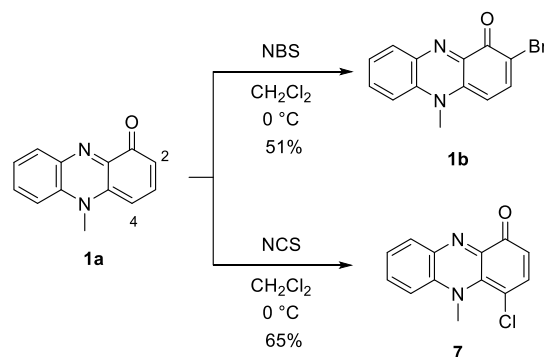
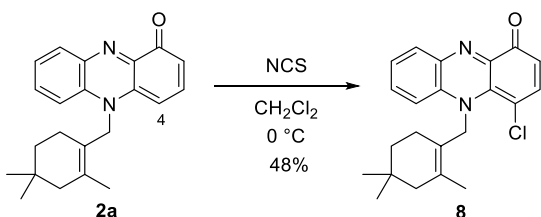


Figure 2. Selected NOESY correlations observed in **7** and **8**.

The chlorination of lavanducyanin (**2a**) with NCS also proceeded at the 4-position in a regioselective manner to give **8** in 48% yield (Scheme 3). The position of the chlorine atom in **8** was also confirmed by NOESY correlations. NOESY correlations between H-2 (δ_{H} 6.49) and H-3 (δ_{H} 7.46) and between the H-6 (δ_{H} 7.27–7.25) and *N*-methylene group (δ_{H} 5.29) are observed (Figures 2 and S39 in the Supporting

Scheme 3. Chlorination of Lavanducyanin (2a)



Information). Thus, an alternative methodology for the synthesis of WS-9659 B (**2c**) is needed.

Oxidative Condensation of 4-Halo-1,2,3-benzenetriol with *N*-Alkylbenzene-1,2-diamines. To obtain *N*-alkyl-2-chlorophenazin-1-ones, reaction conditions for the oxidative condensation between the *N*-methylbenzene-1,2-diamine (**4**)²² and 4-chloro-1,2,3-benzenetriol (**9**)²⁵ were examined (Table 1). When **4** and **9** were reacted under an oxygen atmosphere,

Table 1. Oxidative Condensation of 4-Chloro-1,2,3-benzenetriol (9**) with *N*-Methylbenzene-1,2-diamine (**4**)^a**

entry	oxidant	additive	yield (%) ^b
1 ^c	O ₂		12
2 ^d	1,4-benzoquinone		10
3 ^d	<i>o</i> -chloranil		trace
4 ^e	O ₂	Na ₂ CO ₃	5
5 ^e	O ₂	K ₂ CO ₃	10
6 ^e	O ₂	Cs ₂ CO ₃	17

^aUnless otherwise noted, reactions were performed using **4** (0.246 mmol) and **9** (1.0 equiv) in 2-propanol (2.0 mL). ^bIsolated yield. ^cThe reaction was performed at room temperature (rt) under an oxygen atmosphere. ^dThe reaction was performed at 0 °C under an argon atmosphere using the oxidant (3.0 equiv) indicated. ^eThe reaction was performed at rt under an oxygen atmosphere using the additive (1.0 equiv) indicated.

the desired 2-chloropyocyanin (**1c**) was obtained in 12% yield (entry 1). NOESY correlations between the H-4 (δ_{H} 6.27) and *N*-methyl group (δ_{H} 4.04) and between the H-6 (δ_{H} 8.10) and *N*-methyl group in **1c** indicate the presence of the chlorine atom at the 2-position (Figures 3 and S11 in the Supporting

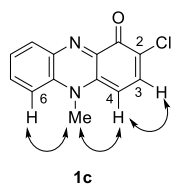


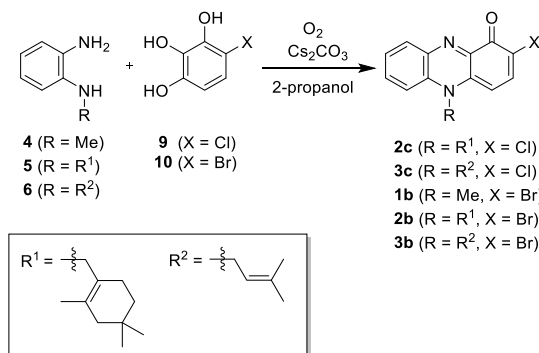
Figure 3. Selected NOESY correlations observed in **1c**.

Information). The use of 1,4-benzoquinone as an oxidant gave **1c** in 10% yield (entry 2). When *o*-chloranil was used in this reaction, only a trace amount of **1c** was obtained. These results indicate that molecular oxygen is the optimal oxidant. The addition of bases to the oxidative coupling with molecular oxygen was also examined (entries 4–6). The yield of **1c** was decreased by addition of Na₂CO₃ or K₂CO₃ (entries 4 and 5,

respectively) but slightly increased by addition of Cs₂CO₃ (entry 6). Other reaction conditions including the base, solvent, and temperature were also investigated but the yield of **1c** was not improved. Thus, the entry 6 gave the optimal conditions.

Using optimal conditions, several *N*-alkyl-2-halophenazin-1-ones were prepared (Table 2). The first synthesis of WS-5659

Table 2. Oxidative Condensation of 4-Halo-1,2,3-benzenetriols with *N*-Alkylbenzene-1,2-diamine^a



entry	1,2-diamine	4-halo-1,2,3-benzenetriols	product	yield (%) ^b
1 ^c	5	9	2c	20
2 ^d	6	9	3c	21
3 ^d	4	10	1b	18
4 ^c	5	10	2b	10
5 ^d	6	10	3b	15

^aUnless otherwise noted, reactions were performed at rt, with O₂ bubbling or under an oxygen atmosphere, using *N*-alkylbenzene-1,2-diamine (30.0 mg) and 4-halo-1,2,3-benzenetriols (1.0 equiv) in the presence of Cs₂CO₃ (1.0 equiv). ^bIsolated yield. ^cThe reaction was performed with oxygen bubbling. ^dThe reaction was performed under an oxygen atmosphere.

B (**2c**) was achieved by coupling between **5** and **9** (entry 1). The ¹H and ¹³C NMR spectroscopic data were identical to those reported for natural **2c**.¹² Oxidative coupling of **9** with **6** gave **3c** in 21% yield (entry 2). The desired oxidative coupling also proceeded using 4-bromo-1,2,3-benzenetriol (**10**)²⁶ as a coupling partner to give *N*-alkyl-2-bromophenazin-1-ones. The coupling between **4** and **10** afforded 2-bromopyocyanin (**1b**) in 18% yield (entry 3). Marinocyanins A (**2b**) and B (**3b**) were synthesized by oxidative coupling of **10** with **5** and **6**, respectively (entries 4 and 5).

Evaluation of Cytotoxic Activity against Human Cancer Cells and Normal Cells. Initially, the cytotoxic activity of **1a**, **1b**, and **1c** against human promyelocytic leukemia HL-60 cells, human lung adenocarcinoma A549 cells, and human normal lung fibroblast MRC-5 cells was evaluated using the WST-8 method.²⁷ The concentrations of the compound required to reduce cell viability by 50% (IC₅₀) are summarized in Table 3. Table 3 also shows the values of the selectivity index (SI),^{28–30} which is defined as the ratio of the IC₅₀ value against normal cells to the corresponding IC₅₀ value against cancer cells of similar tissue. Because both A549 and MRC-5 are derived from the lung, the SI value in Table 3 is defined as the IC₅₀ value against normal MRC-5 cells divided by the IC₅₀ value against A549 cells. Generally, the compounds with a SI value higher than 2 are considered to give selective cytotoxicity against cancer cells.²⁸ SN-38, the active metabolite of irinotecan, was used as a positive control.³¹ The cytotoxicity

Table 3. Cytotoxic Activity of 1a–1c against Human Cancer Cells and Normal Cells (IC₅₀, μM) and Their Selective Indexes^a

compounds	IC ₅₀ (μM)			SI ^c
	HL-60	A549	MRC-5	
1a	5.82 ± 0.133	5.52 ± 0.280	21.8 ± 2.550	3.95
1b	3.07 ± 0.080	2.59 ± 0.056	3.36 ± 0.204	1.30
1c	3.56 ± 0.052	0.76 ± 0.065	4.41 ± 0.069	5.80
SN-38 ^b	<0.05	0.038 ± 0.003	<0.1	

^aThe concentration of the compound needed to reduce cell viability by 50% (IC₅₀) was determined using the WST-8 assay. IC₅₀ values are expressed as the mean ± SD of triplicate experiments. ^bSN-38 was used as a positive control. ^cThe SI is defined as the IC₅₀ value against normal MRC-5 cells divided by the IC₅₀ value against A549 cells.

against normal MRC-5 cells of **1a** was less-potent than that against leukemia HL-60 cells and cancer A549 cells. The SI value of **1a** (SI = 3.95) indicates a significant selectivity toward cancer cells. However, the cytotoxicity of **1b** against normal MRC-5 cells was almost the same as that against leukemia HL-60 cells and cancer A549 cells. Among compounds **1a–c**, the compound **1c** exhibited the most potent and selective cytotoxicity against cancer A549 cells. At least 5.8-fold and 4.6-fold higher concentrations of **1c** are required to induce cytotoxicity against MRC-5 cells, as compared to those required against A549 and HL-60 cells, respectively. The differential cytotoxicity of **1c** was highly significant (SI = 5.80), suggesting that **1c** is considered as a candidate for the development of chemotherapeutic agents. Pyocyanin (**1a**) has been reported to diffuse through the cell membrane and increase the intracellular ROS and Ca²⁺ levels.³² The presence of the chlorine atom at the 2-position in **1a** may enhance the generation of ROSs or increase the chemical reactivity or binding affinity with the target molecules in A549 cells.

Next, the cytotoxic activity of **2a–c** and **3a–c** against HL-60, A549, and MRC-5 cells was evaluated (Table 4). Although

Table 4. Cytotoxic Activity of 2 and 3 against Human Cancer Cells and Normal Cells (IC₅₀, μM) and Their Selective Indexes^a

compounds	IC ₅₀ (μM)			SI ^c
	HL-60	A549	MRC-5	
2a	0.36 ± 0.023	0.38 ± 0.003	0.68 ± 0.013	1.79
2b	0.27 ± 0.017	0.73 ± 0.015	0.60 ± 0.008	0.82
2c	0.23 ± 0.013	0.75 ± 0.006	0.37 ± 0.019	0.49
3a	2.59 ± 0.195	0.88 ± 0.023	0.87 ± 0.063	0.99
3b	0.67 ± 0.038	0.33 ± 0.010	0.54 ± 0.009	1.64
3c	2.20 ± 0.070	0.66 ± 0.016	0.66 ± 0.041	1.00
SN-38 ^b	0.056 ± 0.002	0.052 ± 0.003	0.092 ± 0.001	1.77

^aThe concentration of the compound needed to reduce cell viability by 50% (IC₅₀) was determined using the WST-8 assay. IC₅₀ values are expressed as the mean ± SD of triplicate experiments. ^bSN-38 was used as a positive control. ^cThe SI is defined as the IC₅₀ value against normal MRC-5 cells divided by the IC₅₀ value against A549 cells.

these compounds showed cytotoxicity against HL-60 cells, compounds **3a** and **3c** displayed weaker activity than the other compounds. Compounds **2a–c** and **3a–c** exhibited cytotoxicity against A549 and MRC-5 cells at concentrations lower than 1 μM. Unfortunately, no significant difference in the IC₅₀ values between these compounds was observed. The SI values

of **2a–c** and **3a–c** are lower than 2. Selectivity against A549 cells was not observed. The displacement of the methyl group with the longer alkyl groups at the nitrogen atom in **1a** and **1c** decreased the selective cytotoxicity toward A549 cells.

CONCLUSIONS

In this study, the synthesis of *N*-alkyl-2-halophenazin-1-one was established. Six *N*-alkyl-2-halophenazin-1-ones, including WS-9659 B (**2c**) and marinocyanins A (**2b**) and B (**3b**), were synthesized by the oxidative condensation of 4-halo-1,2,3-benzenetriols with *N*-alkylbenzene-1,2-diamines. The present strategy enables the highly convergent and selective synthesis of *N*-alkyl-2-halophenazin-1-ones. In addition, the cytotoxicity of compounds **1a–c**, **2a–c**, and **3a–c** against human leukemia HL-60 cells, cancer A549 cells, and normal MRC-5 cells was evaluated. Among the compounds tested in this study, 2-chloropyocyanin (**1c**) exhibited the most selective cytotoxicity against A549 cells. The high SI value of **1c** (SI = 5.80) suggests that **1c** is considered as a potential candidate for the development of chemotherapeutic agents. Halogenated phenazines have recently attracted increasing attention as a promising class of biofilm-eradicating agents that effectively target multiple persistent bacterial phenotypes.^{33,34} Thus, halogenation of phenazines and phenazinones is one of the valuable approaches for improving their biological activities.³⁵ The present study will be invaluable not only for analyzing the molecular and cellular biological activities of *N*-alkylphenazin-1-ones and their halogenated derivatives but also in the design of more potent and selective cytotoxic derivatives against cancer cells. Additional biological studies to investigate the mechanism of action of these compounds are currently underway.

EXPERIMENTAL SECTION

General Information. All reactions sensitive to air or moisture were carried out under an argon atmosphere under anhydrous conditions, unless otherwise noted. Solvents and reagents were used without further purification unless otherwise noted. Analytical thin-layer chromatography (TLC) was performed using silica gel 60 F₂₅₄ plates (0.25 mm, normal phase, Merck) and silica gel 60 RP-18 F_{254S} plates (0.25 mm, reversed phase, Merck). Normal-phase flash column chromatography was performed using silica gel 60 (particle size 40–63 μm; 230–400 mesh ASTM; SilicaFlash F60, SiliCycle Inc.). Reversed-phase flash column chromatography was performed using octadecylsilyl silica gel (C18, particle size 15–30 μm, FUJIFILM Wako Pure Chemical Co.). Melting point (*M_p*) data were determined using a Shimadzu MM-2 instrument and were uncorrected. IR spectra were recorded on a Horiba FT-720 spectrometer, using KBr pellets (solid). ¹H and proton-decoupled ¹³C (¹³C{¹H}) NMR spectra were recorded on a Bruker Avance 400 spectrometer (400 and 100 MHz, respectively), using chloroform-*d* (CDCl₃), dimethyl sulfoxide-*d*₆ (DMSO-*d*₆), and methanol-*d*₄ (CD₃OD) as the solvent. Chemical shift values are expressed in δ (ppm) relative to tetramethylsilane (TMS, δ 0.00 ppm) or the residual solvent resonance (CDCl₃, δ 77.0 ppm for ¹³C NMR; DMSO-*d*₆, δ 2.49 ppm for ¹H NMR and δ 39.7 ppm for ¹³C NMR; and CD₃OD, δ 3.30 ppm for ¹H NMR and δ 49.0 ppm for ¹³C NMR). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, and m = multiplet), coupling constants (*J*; Hz), and

integration. Mass spectra were obtained using a Sciex XS500R quadrupole time-of-flight (QTOF) high-resolution mass spectrometer using electrospray ionization (ESI). High purity levels of all synthetic compounds were verified using ^1H NMR spectra (see the Supporting Information). A549 cells were purchased from the Riken Cell Bank. HL-60 and MRC-5 cells were purchased from the National Institute of Biomedical Innovation, Health and Nutrition.

2-Bromo-5-methylphenazin-1(5H)-one (1b). NBS (9.3 mg, 52.3 μmol) was added to a solution of **1a**²² (10.6 mg, 50.4 μmol) in CH_2Cl_2 (5.0 mL). The mixture was stirred at 0 °C for 1 h. The reaction was quenched by the addition of a saturated aqueous NaHCO_3 solution. The mixture was diluted with CHCl_3 . After the layers were separated, the organic layer was washed with water and brine, dried over Na_2SO_4 , and concentrated to give a residue. The residue was purified by reversed-phase flash chromatography ($\text{MeOH}/\text{H}_2\text{O} = 4/1$) to give **1b** (7.5 mg, 51%) as a dark-blue solid. Reversed-phase TLC ($\text{MeOH}/\text{H}_2\text{O} = 4/1$) Rf 0.48; mp 129–130 °C; IR (KBr) ν_{max} : 2958, 2914, 2848, 1724, 1620, 1560, 1493 cm^{-1} ; ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 8.24 (d, $J = 8.1$ Hz, 1H), 8.13–8.10 (m, 2H), 8.00 (t, $J = 7.6$ Hz, 1H), 7.67 (t, $J = 7.6$ Hz, 1H), 6.25 (d, $J = 8.6$ Hz, 1H), 4.04 (s, 3H); $^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, $\text{DMSO}-d_6$): δ 171.0, 145.4, 144.5, 136.4, 136.0, 135.2, 133.0, 132.7, 125.8, 116.0, 108.3, 91.9, 35.7; HRMS (ESI/QTOF) m/z : $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{13}\text{H}_{10}^{79}\text{BrN}_2\text{O}$, 288.9971; found, 288.9969.

4-Chloro-5-methylphenazin-1(5H)-one (7). NCS (7.1 mg, 53.2 μmol) was added to a solution of **1a**²² (10.6 mg, 50.4 μmol) in CH_2Cl_2 (5.0 mL). The mixture was stirred at 0 °C for 2 h. The reaction was quenched by the addition of a saturated aqueous NaHCO_3 solution. The mixture was diluted with CHCl_3 . After the layers were separated, the organic layer was washed with water and brine, dried over Na_2SO_4 , and concentrated to give a residue. The residue was purified by reversed-phase flash chromatography ($\text{MeOH}/\text{H}_2\text{O} = 4/1$) to give **7** (8.0 mg, 65%) as a dark-blue solid. TLC ($\text{CHCl}_3/\text{MeOH} = 9/1$) Rf 0.66; mp 125–127 °C; IR (KBr) ν_{max} : 3035, 2920, 1728, 1637, 1574, 1514, 1493 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3 , TMS): δ 8.17 (dd, $J = 8.1, 1.1$ Hz, 1H), 7.74–7.70 (m, 1H), 7.44 (d, $J = 10.0$ Hz, 1H), 7.44–7.40 (m, 1H), 7.40 (d, $J = 8.6$ Hz, 1H), 6.47 (d, $J = 10.0$ Hz, 1H), 4.07 (s, 3H); $^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, CDCl_3): δ 178.7, 146.9, 145.9, 136.0, 135.5, 134.6, 132.8, 132.7, 124.6, 119.1, 113.6, 100.3, 41.3; HRMS (ESI/QTOF) m/z : $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{13}\text{H}_{10}^{35}\text{ClN}_2\text{O}$, 245.0476; found, 245.0478.

4-Chloro-5-((2,4,4-trimethylcyclohex-1-en-1-yl)methyl)phenazin-1(5H)-one (8). NCS (4.5 mg, 33.7 μmol) was added to a solution of **2a**²² (10.6 mg, 31.9 μmol) in CH_2Cl_2 (3.0 mL). The mixture was stirred at 0 °C for 1.5 h. The reaction was quenched by the addition of a saturated aqueous NaHCO_3 solution. The mixture was diluted with CHCl_3 . After the layers were separated, the organic layer was washed with water and brine, dried over Na_2SO_4 , and concentrated to give a residue. The residue was purified by reversed-phase flash chromatography ($\text{MeOH}/\text{H}_2\text{O} = 4/1$) to give **8** (5.6 mg, 48%) as a dark-blue solid. TLC ($\text{CHCl}_3/\text{MeOH} = 9/1$) Rf 0.47; mp 114–116 °C; IR (KBr) ν_{max} : 2954, 2920, 2850, 1724, 1716, 1643, 1603, 1514, 1469 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3): δ 8.18 (dd, $J = 8.0, 1.4$ Hz, 1H), 7.65–7.61 (m, 1H), 7.46 (d, $J = 10.1$ Hz, 1H), 7.40 (t, $J = 8.0$ Hz, 1H), 7.27–7.25* (m, 1H), 6.49 (d, $J = 10.1$ Hz, 1H), 5.29 (s, 2H), 1.81 (br s, 5H), 1.48 (br s, 2H), 1.16 (t, $J = 6.4$ Hz, 2H), 0.73 (s, 6H), (*this proton signal was

assigned based on the HMQC spectrum.); $^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, CDCl_3): δ 178.8, 146.9, 146.4, 136.1, 134.7, 134.0, 133.0 (2C), 130.3, 124.6, 123.2, 118.9, 114.5, 100.4, 54.2, 46.2, 35.0, 28.9, 27.9 (2C), 23.2, 19.3; HRMS (ESI/QTOF) m/z : $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{22}\text{H}_{24}^{35}\text{ClN}_2\text{O}$, 367.1572; found, 367.1579.

General Procedure for the Coupling between 4 and 9 under an Oxygen Atmosphere (Entries 1, 4, 5, and 6 in Table 1). A solution of **4** (30.0 mg, 245.6 μmol) and **9** (1.0 equiv) in the absence or presence of the additive (1.0 equiv) indicated in Table 1 in 2-propanol (2.0 mL) was stirred under an O_2 atmosphere at rt in the dark until no further TLC changes were observed. The reaction was quenched by the addition of 1 M aqueous HCl solution. The mixture was diluted with CHCl_3 . After the layers were separated, the aqueous layer was neutralized with 4 M aqueous NaOH solution. The aqueous layer was extracted with CHCl_3 twice. The combined organic layer was dried over Na_2SO_4 and concentrated to give a residue. The residue was purified by silica gel column chromatography and reversed-phase flash chromatography.

Following the general procedure, the reaction was performed using **4** (30.0 mg, 245.6 μmol) and **9** (39.8 mg, 247.9 μmol) in the presence of Cs_2CO_3 (81.0 mg, 248.6 μmol) for 4 days to give **1c** (10.3 mg, 17%) as a dark-blue solid (entry 6). Reversed-phase TLC ($\text{MeOH}/\text{H}_2\text{O} = 4/1$) Rf 0.39; mp 124–125 °C; IR (KBr) ν_{max} : 3043, 2924, 1716, 1622, 1560 cm^{-1} ; ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 8.24 (dd, $J = 8.2, 1.2$ Hz, 1H), 8.10 (d, $J = 8.7$ Hz, 1H), 8.02–7.98 (m, 1H), 7.96 (d, $J = 8.7$ Hz, 1H), 7.66 (td, $J = 8.2, 1.2$ Hz, 1H), 6.27 (d, $J = 8.7$ Hz, 1H), 4.04 (s, 3H); $^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, $\text{DMSO}-d_6$): δ 170.8, 145.9, 141.2, 136.2, 136.0, 134.6, 133.0, 132.7, 125.8, 118.0, 115.9, 90.7, 35.6; HRMS (ESI/QTOF) m/z : $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{13}\text{H}_{10}^{35}\text{ClN}_2\text{O}$, 245.0476; found, 245.0476.

General Procedure for the Coupling between 4 and 9 Using the Oxidant (Entries 2 and 3 in Table 1). A solution of **4** (245.6 μmol) and **9** (1.0 equiv) in the presence of the oxidant (3.0 equiv) indicated in Table 1 in 2-propanol (2.0 mL) was stirred at 0 °C until no further TLC changes were observed. The reaction was quenched by the addition of 1 M aqueous HCl solution. The mixture was diluted with CHCl_3 . After the layers were separated, the aqueous layer was neutralized with 4 M aqueous NaOH solution. The aqueous layer was extracted with CHCl_3 twice. The combined organic layer was dried over Na_2SO_4 and concentrated to give a residue. The residue was purified by silica gel column chromatography and reversed-phase flash chromatography to yield **1c**.

Following the general procedure, the reaction was performed using **4** (30.0 mg, 245.6 μmol), **9** (40.0 mg, 249.1 μmol), and 1,4-benzoquinone (79.6 mg, 736.4 μmol) for 1 h to give **1c** (6.1 mg, 10%) (entry 2).

General Procedure of *N*-((2,4,4-Trimethylcyclohex-1-en-1-yl)methyl)benzene-1,2-diamine (5) with 4-Halo-1,2,3-benzenetriols (General Procedure A; Entries 1, 4 in Table 2). A solution of *N*-alkylbenzene-1,2-diamine (30.0 mg) and 4-halo-1,2,3-benzenetriols (1.0 equiv) in the presence of the Cs_2CO_3 (1.0 equiv) in 2-propanol (1.0 mL) was stirred and bubbled with O_2 from an O_2 balloon at rt in the dark until no further TLC changes were observed. The reaction was quenched by the addition of water. The mixture was diluted with CHCl_3 . After the layers were separated, the organic layer was washed with water and brine, dried over Na_2SO_4 , and

concentrated to give a residue. The residue was purified by silica gel column chromatography and reversed-phase flash chromatography.

Marinocyanin A (2b). Following the general procedure A, the reaction was performed using **5** (30.0 mg, 122.8 μmol) and **10** (25.2 mg, 122.9 μmol) in the presence of Cs_2CO_3 (40.0 mg, 122.8 μmol) in 2-propanol with O_2 bubbling for 2 days. The residue was purified by silica gel column chromatography ($\text{CHCl}_3/\text{MeOH} = 50/1$) and reversed-phase flash chromatography ($\text{MeOH}/\text{H}_2\text{O} = 4/1$) to give **2b** (5.0 mg, 10%) as a dark-blue solid. The structure of **2b** was confirmed by comparison of the ^1H and $^{13}\text{C}\{^1\text{H}\}$ NMR spectra with those reported for **2b**.²²

WS-9659 B (2c). Following the general procedure A, the reaction was performed using **5** (30.0 mg, 122.8 μmol) and **9** (19.9 mg, 123.9 μmol) in the presence of Cs_2CO_3 (40.2 mg, 123.4 μmol) in 2-propanol with O_2 bubbling for 2 days. The residue was purified by silica gel column chromatography ($\text{CHCl}_3/\text{MeOH} = 50/1$) and reversed-phase flash chromatography ($\text{MeOH}/\text{H}_2\text{O} = 4/1$) to give **2c** (8.9 mg, 20%) as a dark-blue solid. Reversed-phase TLC ($\text{MeOH}/\text{H}_2\text{O} = 4/1$) Rf 0.43; mp 154–157 $^\circ\text{C}$ (lit. 152–153 $^\circ\text{C}$);¹² IR (KBr) ν_{max} : 2918, 2848, 2362, 1699, 1626, 1604, 1562, 1495 cm^{-1} ; ^1H NMR (400 MHz, CDCl_3): δ 8.40 (d, $J = 7.8$ Hz, 1H), 7.82 (t, $J = 7.8$ Hz, 1H), 7.76 (d, $J = 7.8$ Hz, 1H), 7.57–7.52 (m, 2H), 5.89 (d, $J = 8.4$ Hz, 1H), 5.10 (br s, 2H), 1.91 (s, 3H), 1.88 (br s, 2H), 1.53–1.45 (br s 2H), 1.20 (t, $J = 6.3$ Hz, 2H), 0.83 (s, 6H); $^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, CDCl_3): δ 172.7, 146.5, 140.9, 136.2, 135.4, 134.1, 133.8, 133.2, 131.5, 125.1, 120.9, 120.3, 113.6, 90.4, 50.1, 46.2, 34.8, 28.9, 28.0 (2C), 22.7, 19.5; HRMS (ESI/QTOF) m/z : $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{22}\text{H}_{24}^{35}\text{ClN}_2\text{O}$, 367.1572; found, 367.1568.

General Procedure of N-Methylbenzene-1,2-diamine (4) or N-(3-Methylbut-2-en-1-yl)benzene-1,2-diamine (6) with 4-Halo-1,2,3-benzenetriols (General Procedure B; Entries 2, 3, and 5 in Table 2). A solution of N-alkylbenzene-1,2-diamine (30.0 mg) and 4-halo-1,2,3-benzenetriols (1.0 equiv) in the presence of Cs_2CO_3 (1.0 equiv) in 2-propanol was stirred under an O_2 atmosphere at rt in the dark until no further TLC changes were observed. The reaction was quenched by the addition of 1 M aqueous HCl solution. The mixture was diluted with CHCl_3 . After the layers were separated, the aqueous layer was neutralized with 4 M aqueous NaOH solution. The aqueous layer was extracted with CHCl_3 twice. The combined organic layer was dried over Na_2SO_4 and concentrated to give a residue. The residue was purified by silica gel column chromatography or reversed-phase flash chromatography.

2-Bromo-5-methylphenazin-1(5H)-one (1b). Following the general procedure B, the reaction was performed using **4** (30.0 mg, 245.6 μmol) and **10** (50.7 mg, 247.3 μmol) in the presence of Cs_2CO_3 (80.5 mg, 247.1 μmol) in 2-propanol (2.0 mL) under an O_2 atmosphere for 3 days. The residue was purified by reversed-phase flash chromatography ($\text{MeOH}/\text{H}_2\text{O} = 2/1$) to give **1b** (12.7 mg, 18%) as a dark-blue solid.

Marinocyanin B (3b). Following the general procedure B, the reaction was performed using **6** (30.0 mg, 170.2 μmol) and **10** (35.8 mg, 174.6 μmol) in the presence of Cs_2CO_3 (53.5 mg, 164.2 μmol) in 2-propanol (1.0 mL) under an O_2 atmosphere for 2 days. The residue was purified by silica gel column chromatography ($\text{CHCl}_3/\text{MeOH} = 50/1$) and reversed-phase flash chromatography ($\text{MeOH}/\text{H}_2\text{O} = 4/1$) to give **3b** (8.5 mg, 15%) as a dark-blue solid. The structure of

3b was confirmed by comparison of the ^1H and $^{13}\text{C}\{^1\text{H}\}$ NMR spectra with those reported for **3b**.²²

2-Chloro-5-(3-methylbut-2-en-1-yl)phenazin-1(5H)-one (3c). Following the general procedure B, the reaction was performed using **6** (30.0 mg, 170.2 μmol) and **9** (27.3 mg, 170.0 μmol) in the presence of Cs_2CO_3 (55.6 mg, 170.6 μmol) in 2-propanol (1.5 mL) under an O_2 atmosphere for 2 days. The residue was purified by reversed-phase flash chromatography ($\text{MeOH}/\text{H}_2\text{O} = 2/1$) to give **3c** (10.6 mg, 21%) as a dark-blue solid. Reversed-phase TLC ($\text{MeOH}/\text{H}_2\text{O} = 4/1$) Rf 0.38; mp 145–149 $^\circ\text{C}$; IR (KBr) ν_{max} : 3035, 2958, 2918, 2848, 1724, 1624, 1562, 1496 cm^{-1} ; ^1H NMR (400 MHz, CD_3OD): δ 8.31 (d, $J = 7.8$ Hz, 1H), 8.07–8.03 (brm, 1H), 7.99–7.93 (m, 2H), 7.72 (t, $J = 7.8$ Hz, 1H), 6.41 (d, $J = 8.7$ Hz, 1H), 5.37 (br s, 2H), 5.21–5.18 (br s, 1H), 2.03 (s, 3H), 1.80 (d, $J = 0.9$ Hz, 3H); $^{13}\text{C}\{^1\text{H}\}$ NMR (100 MHz, CD_3OD): δ 171.4, 146.4, 144.3, 140.4, 138.9, 138.1, 134.5, 134.0, 133.5, 128.0, 119.5, 117.3, 116.8, 93.8, 48.7*, 25.6, 18.7 (*this carbon signal was assigned based on the HMQC spectrum.); HRMS (ESI/QTOF) m/z : $[\text{M} + \text{H}]^+$ calcd for $\text{C}_{17}\text{H}_{16}^{35}\text{ClN}_2\text{O}$, 299.0946; found, 299.0942.

Cell Lines and Culturing. A549 cells were cultured at 37 $^\circ\text{C}$ with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HL-60 cells were cultured at 37 $^\circ\text{C}$ with 5% CO_2 in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS. MRC-5 cells were cultured in Minimum Essential Medium Eagle-alpha modification (α -MEM) without ribonucleosides and deoxyribonucleosides supplemented with 10% FBS.

Cytotoxicity Evaluation. Cell growth was evaluated using cell counting kit-8 according to the manufacturer's instructions based on the WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay.²⁷ For the assay, the adherent A549 and MRC-5 cells were cultured in a 96-well plate with each well containing 2000 cells in a total volume of 100 μL , and HL-60 cells were cultured in a 96-well plate with each well containing 5000 cells in a total volume of 100 μL . The concentration of DMSO in the cell cultures was 0.1–0.2% (v/v). The plates also included blank wells (0 cells) and control wells (2000 cells/100 μL for the adherent cells and 5000 cells/100 μL for HL-60 cells). A549 and MRC-5 cells were preincubated for 24 h before exposure to the test compounds. The plates were incubated with various concentrations of each compound for 72 h. At the end of incubation, 10 μL of the solution provided with the cell counting kit was then added, and the resulting mixture was incubated for 2 h at 37 $^\circ\text{C}$. The absorbance values were then measured at 450 nm with a 96-well plate reader. Cell growth inhibition was evaluated as the ratio of the absorbance of the sample to that of the control.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsomega.0c04253>.

^1H , $^{13}\text{C}\{^1\text{H}\}$, and 2D NMR spectra for synthetic compounds (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This study was partly supported by JSPS KAKENHI (no. 20K05868) to K.K.

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