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CONCISE SYNTHESIS OF KALASINAMIDE, MARCANINE A, AND GEOVANINE, AND ANTIPROLIFERATIVE ACTIVITY EVALUATION OF THEIR AZAANTHRACENONES

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We dedicate this paper to Professor Dr. Tohru Fukuyama on the celebration of his 70th birthday.

Abstract – The total syntheses of kalasinamide (**1**), marcanine A (**2**), and geovanine (**3**) have been conducted by the formation of a fused pyridone-ring azaanthracenone core based on a Curtius rearrangement that is followed by the microwave-assisted thermal electrocyclization of an isocyanato-containing 2-azahexatriene system. The antiproliferative activities of these synthetic compounds against the HCT-116 colon tumor cells were evaluated by the MTT assay.

INTRODUCTION

Alkaloids with an azaanthracenone core have attracted considerable biological and synthetic interest because of their intriguing structural features and extensive range of promising biological activities.[1] Several structural analogs of these alkaloids have been synthesized; further, some compounds have exhibited notable activity.[2]

The compounds, such as kalasinamide (**1**), marcanine A (**2**), and geovanine (**3**), are observed to belong to the azaanthracenone family of natural products (Figure 1).

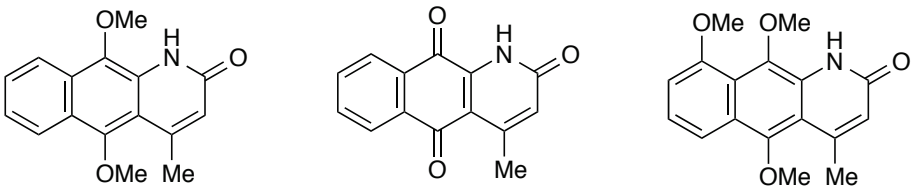


Figure 1. The structures of kalasinamide (**1**), marcanine A (**2**), and geovanine (**3**)

Kalasinamide (**1**) was initially discovered in Thailand in 2000 by Tuchinda and co-workers,[3a] initially from *Polyalthia suberosa* and later from various other plant species.[3b–f] The total synthesis of **1** has been achieved by two groups using a Knorr cyclization reaction to form a β-ketoamide from 2-aminonaphthalene[4a] and/or 2-aminonaphthoquinone[4b] as the key step.

Marcanine A (**2**) was reported to be cytotoxic to human cancer cells[5a] and was first isolated from the stem bark of *Goniothalamus marcanii* in 1999; it was subsequently found in various other plant species.[5b–h] The first total synthesis of **2** was achieved via the Diels–Alder reaction of quinolone-2,5,8-trione and 1-methoxybutadiene.[6a] Since then, the total synthesis of **2** has been reported by four groups.[4,6b,c]

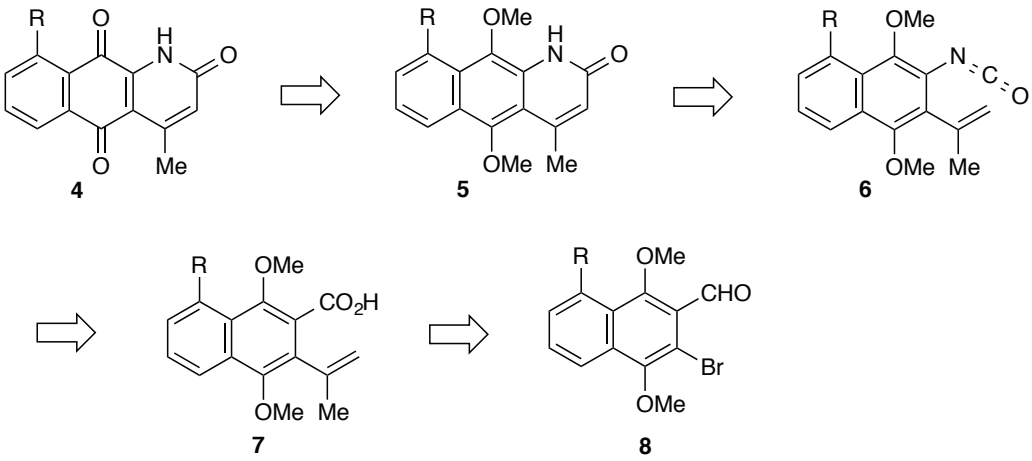
Geovanine (**3**) was first isolated from the trunk of *Annona ambotay* in 1987 by de Oliveira and co-workers[7a]; it was subsequently isolated by Dos Santos and co-workers from *Annona dioica* in Brazil.[7b] The first total synthesis of **3** was achieved by Ulrich and co-workers, who used an aryne derived from the cycloaddition of 7-bromoquinolone and furan as the diene in the reaction.[4a]

During the course of our ongoing studies related to the synthetic usage of thermal electrocyclization to form azahexatrienes,[8] we reported the total synthesis of a 2-azaanthraquinone alkaloid scorpinone using the two type of electrocyclization methods that we have developed.[9] Additionally, we have also reported the construction of several fused heterocycles, such as isocryptolepine,[10] from the one-pot synthesis[11] of a fused pyridone-ring system based on a Curtius rearrangement, followed by the microwave (MW)-assisted thermal electrocyclization of an isocyanato-containing 2-azahexatriene system. Furthermore, we have searched for highly active compounds based on these naturally occurring compounds and their derivatives.[12]

In this study, we describe the total syntheses of the azaanthracenone alkaloids **1**, **2**, and **3** through the construction of fused pyridone-ring systems using the aforementioned one-pot synthesis of Curtius rearrangement and MW-assisted thermal electrocyclization processes. Additionally, the antiproliferative activities of these synthetic compounds against the HCT-116 colon tumor cells were evaluated by MTT assay.

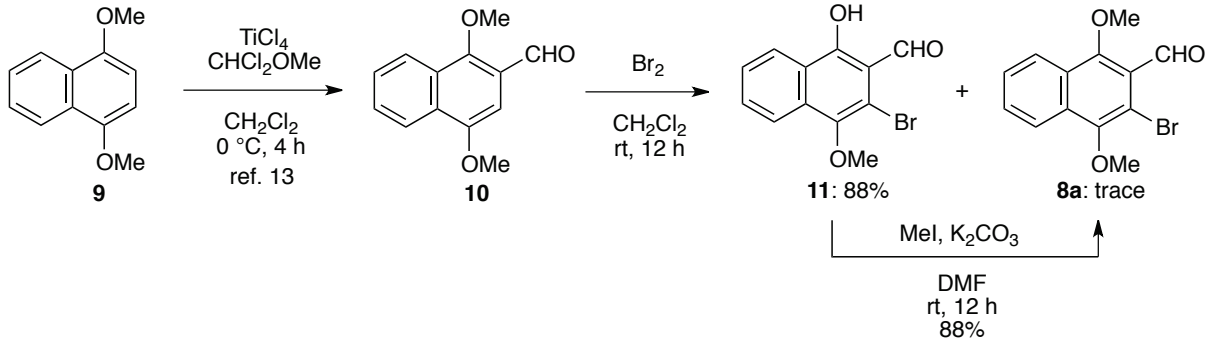
RESULTS AND DISCUSSION

Our retrosynthetic strategy for synthesizing azaanthracenones is illustrated in Scheme 1. We envisaged that the quinone moiety of azaanthracenone **4** could be achieved via the oxidation of 5,10-dimethoxyazaanthracenone **5**. We also considered that it may be possible to obtain azaanthracenone **5** via a one-pot synthesis of a fused pyridone-ring moiety based on a Curtius rearrangement, followed by the MW-assisted thermal electrocyclization of the isocyanato-containing 2-azahexatriene **6**. Furthermore, it was considered that it may be possible to synthesize naphthalene-2-carboxylic acid **7**, which is a precursor of **6**, from 3-bromonaphthalene-2-carbaldehyde **8**.



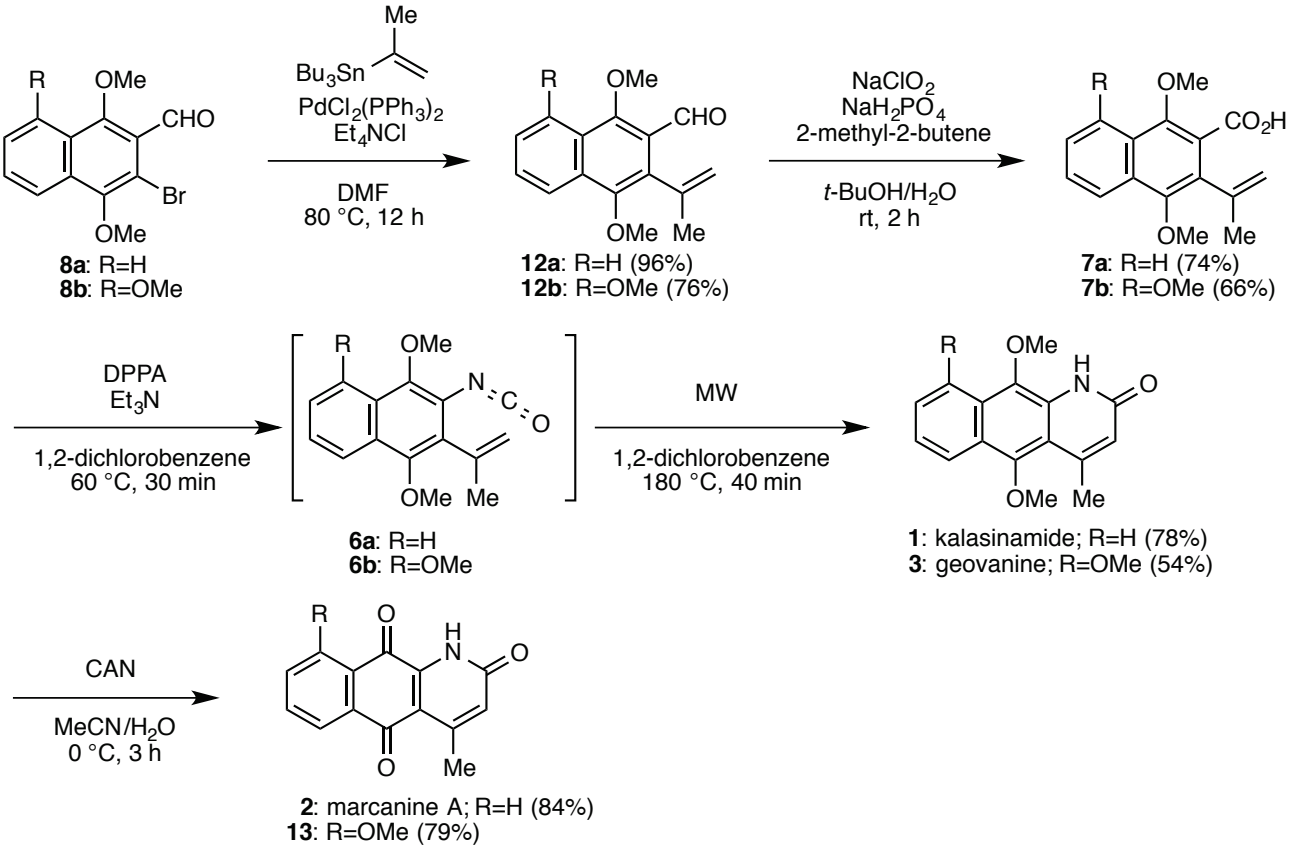
Scheme 1

To synthesize kalasinamide and marcanine A, we first prepared the 3-bromonaphthalene-2-carbaldehyde **8a** as a starting material. Based on the sequence illustrated in Scheme 2, naphthalene-2-carbaldehyde **10** was prepared via the reaction of 1,4-dimethoxynaphthalene (**9**) with α,α-dichloromethyl methyl ether in the presence of $TiCl_4$ in accordance with the Borch procedure.[13] Subsequently, a demethylated product **11** (88%) was obtained along with a trace amount of the desired product **8a** via the bromination of **10**. The resulting compound **11** was treated with methyl iodide (MeI) in the presence of potassium carbonate (K_2CO_3) to yield 3-bromonaphthalene-2-carbaldehyde **8a** in a 88% yield.



Scheme 2

To synthesize **1** and **2**, a Stille reaction of 3-bromonaphthalene **8a** with isopropenyltributyltin in the presence of $PdCl_2(PPh_3)_2$ generated 3-isopropenylnaphthalene **12a** in a 96% yield. Subsequently, **12a** was oxidized with $NaClO_2$ in *t*-BuOH/H_2O at rt for 2 h to produce carboxylic acid **7a** as a key precursor in a 74% yield. Further, the treatment of **7a** with diphenylphosphoryl azide (DPPA)[14] and triethylamine (Et_3N) in 1,2-dichlorobenzene at 60 °C for 30 min generated isocyanate **6a** *in situ* (by monitoring the disappearance of **7a** by thin layer chromatography), which was further heated at 180 °C for 40 min under MW irradiation to produce **1** in a 78% yield. Finally, **1** was oxidized using the conditions described by Groth[4a] for constructing a quinone moiety to synthesize **2**. Using the aforementioned synthetic procedures, we were able to develop a new route for constructing the azaanthracenone frameworks.



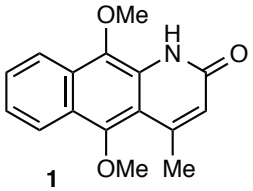
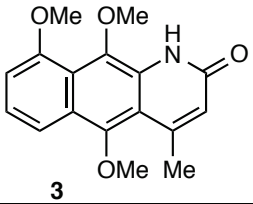
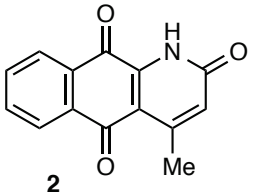
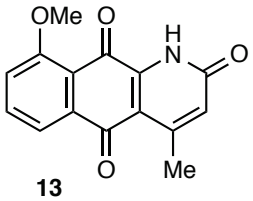
Scheme 3

Based on the aforementioned results, we attempted to synthesize **3**. The starting material, 3-bromo-1,4,8-trimethoxynaphthalene-2-carbaldehyde (**8b**), was prepared according to the procedure described by Yamamoto.[15] As illustrated in Scheme 3, the synthesis of **3** could be achieved in four steps from **8b** in an overall yield of 27.1% using the same synthetic method as that used for kalasinamide (**1**). Furthermore, the oxidation of **3** with CAN resulted in the formation of quinone analog **13** in 79% yield. The structures of all of the synthetic compounds were confirmed using ^1H and ^{13}C-NMR and mass

spectrometry. All the aspects of physical and spectroscopic data for the synthetic compounds **1**, **2**, and **3** were observed to be consistent with those of the natural products.

Further, the antiproliferative activities of the synthesized azaanthracenones (**1**, **2**, **3**, and quinone analog **13**) against the HCT-116 cells were assessed by the MTT assay (Table 1). At a dosage of 100 μM, compounds **1** and **3** did not exhibit any antiproliferative activity against the HTC-116 cells, whereas compounds **2** and **13**, which possessed quinone moieties, were observed to inhibit the tumor cell viability by 7.6% and 48.3%, respectively. Furthermore, at a dosage of 10 μM, **2** was observed to inhibit the tumor cell viability by 37.9%. Therefore, it is observed that a quinone moiety is necessary to observe the antiproliferative activity of the azaanthracenone derivatives.

Table 1. Evaluation of the cell growth inhibitory activity against the HCT-116 cell lines

	Cell viability (%)			Cell viability (%)	
	10 μM	100 μM		10 μM	100 μM
 **1**	101.1	101.5	 **3**	105.3	97.9
 **2**	37.9	7.6	 **13**	103.6	48.3

CONCLUSIONS

To summarize, the total syntheses of the azaanthracenone alkaloids, including kalasinamide, marcanine A, and geovanine, were achieved by the one-pot synthesis of a fused pyridone-ring system based on a Curtius rearrangement, followed by the microwave-assisted thermal electrocyclization of an isocyanato-containing 2-azahexatriene system as a key preparation method. Additionally, four compounds, including the three synthetically produced natural products, were evaluated to determine their antiproliferative activities against the HCT-116 cells. The quinone analogs were observed to exhibit more potent activity than that exhibited by their corresponding hydroquinone analogs; further, marcanine A was observed to exhibit the optimal activity from among all the compounds.

EXPERIMENTAL

General Methods: All non-aqueous reactions were carried out under an atmosphere of nitrogen in dried glassware unless otherwise noted. Solvents were dried and distilled according to standard protocols. Analytical thin layer chromatography was performed with Silica gel $60PF_{254}$ (Merck). Silica gel column chromatography was performed with Silica gel 60 (70–230 mesh, Kanto Chemical Co. Lit.). All melting points were determined on Yanagimoto micro melting point apparatus and are uncorrected. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a JEOL AL-300. Infrared spectra were recorded with ATR method using a Horiba FT-720 spectrophotometer and Technologies DuraScop. Low and high-resolution mass spectra were recorded on JEOL JMS-700 spectrometers by direct inlet system. The reaction of microwave (MW) irradiation was carried out by Discover of CEM Co. Ltd. with 2450 MHz.

3-Bromo-1-hydroxy-4-methoxynaphthalene-2-carbaldehyde (11): Br_2 (0.95 mL, 18.5 mmol) was added slowly to a solution of 1,4-dimethoxy-2-naphthaldehyde **10** (2.0 g, 9.25 mmol) in CH_2Cl_2 (30 mL) at 0 °C. After stirring at rt for 12 h, 10% $Na_2S_2O_4$ aq. was added and extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated *in vacuo*. The residue was purified by column chromatography using EtOAc-hexane (1:9, v/v) as an eluent to give 3-bromo-1-hydroxynaphthalene **11** (2.27 g, 88%) as yellow solid and trace amount of 1,4-dimethoxynaphthalene **8a**. mp 94–95 °C (EtOAc). IR (ATR) ν: 1620 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 13.76 (1H, s), 10.38 (1H, s), 8.44 (1H, dd, *J* = 8.4, 1.3 Hz), 8.05 (1H, dd, *J* = 8.4, 1.3 Hz), 7.75 (1H, dt, *J* = 8.4, 1.3 Hz), 7.60 (1H, dt, *J* = 8.4, 1.3 Hz), 3.96 (3H, s). ^{13}C-NMR ($CDCl_3$) δ: 197.6, 161.4, 145.8, 132.6, 131.7, 126.9, 125.2, 125.0, 122.2, 112.2, 111.2, 61.4. MS *m*/*z*: 280 (M^+), 282 (M^++2). HRMS (EI) calcd for $C_{12}H_9BrO_3$ 279.9735; found 279.9749.

3-Bromo-1,4-dimethoxynaphthalene-2-carbaldehyde (8a): MeI (1.1 mL, 17.8 mmol) was added to a mixture of 3-bromo-1-hydroxynaphthalene **11** (1.0 g, 3.56 mmol) and K_2CO_3 (2.46 g, 17.8 mmol) in DMF (30 mL) at 0 °C. After stirring at rt for 12 h, the reaction mixture was quenched with water and extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated *in vacuo*. The residue was purified by column chromatography using EtOAc-hexane (1:9, v/v) as an eluent to give 1,4-dimethoxynaphthalene **8a** (920 mg, 88%) as white solid. mp 98–99 °C (Et_2O). IR (ATR) ν: 1693 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 10.55 (1H, s), 8.25 (1H, dd, *J* = 8.3, 1.5 Hz), 8.14 (1H, dd, *J* = 8.3, 1.5 Hz), 7.71 (1H, dt, *J* = 8.3, 1.5 Hz), 7.64 (1H, dt, *J* = 8.3, 1.5 Hz), 4.07 (3H, s), 4.01 (3H, s). ^{13}C-NMR($CDCl_3$) δ: 189.6, 157.1, 152.3, 130.3, 128.9, 128.5, 127.3, 124.7, 123.0, 122.9, 98.3, 65.8, 55.8. MS *m*/*z*: 294 (M^+), 296 (M^++2). HRMS (EI) calcd for $C_{13}H_{11}BrO_3$ 293.9892; found 293.9888.

3-Isopropenyl-1,4-dimethoxynaphthalene-2-carbaldehyde (12a): A solution of isopropenyltributyltin (2.25 g, 6.78 mmol) in DMF (10 mL) was added to a mixture of 1,4-dimethoxynaphthalene **8a** (1.0 g, 3.39 mmol), $PdCl_2(PPh_3)_2$ (119 mg, 0.170 mmol) and Et_4NCl (841 mg, 1.02 mmol) in DMF (20 mL).

After being stirred at 80 °C for 12 h, the reaction mixture was quenched with an aqueous KF solution (30%), and then the mixture was stirred at rt for 30 min. The mixture was filtered through a Celite pad, and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated *in vacuo*. The residue was purified by column chromatography using EtOAc-hexane (1:9, v/v) as an eluent to give 3-isopropenylnaphthalene **12a** (833 mg, 96%) as yellow oil. IR (ATR) ν: 1689, 1346 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 10.43 (1H, s), 8.25 (1H, dd, *J* = 8.3, 1.2 Hz), 8.11 (1H, dd, *J* = 8.3, 1.2 Hz), 7.66 (1H, dt, *J* = 8.3, 1.2 Hz), 7.58 (1H, dt, *J* = 8.3, 1.2 Hz), 5.42–5.43 (1H, m), 4.93–4.94 (1H, m), 4.07 (3H, s), 3.91 (3H, s), 2.21 (3H, s). ^{13}C-NMR ($CDCl_3$) δ: 191.1, 156.7, 149.2, 140.9, 132.7, 131.7, 129.2, 128.2, 126.8, 124.1, 123.8, 122.8, 116.9, 64.8, 62.6, 24.6. MS *m*/*z*: 256 (M^+). HRMS (EI) calcd for $C_{16}H_{16}O_3$ 256.1099; found 256.1083.

3-Isopropenyl-1,4,8-trimethoxynaphthalene-2-carbaldehyde (12b): The same procedure as above was carried out using aldehyde **8b** (100 mg, 0.92 mmol) to give 3-isopropenylnaphthalene **12b** (67 mg, 76%) as yellow oil. IR (ATR) ν: 1685 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 10.52 (1H, s), 7.72 (1H, dd, *J* = 8.3, 1.5 Hz), 7.56 (1H, dt, *J* = 8.3, 1.5 Hz), 6.95 (1H, dd, *J* = 8.3, 1.5 Hz), 5.32–5.33 (1H, m), 4.85–4.86 (1H, m), 4.05 (3H, s), 3.94 (3H, s), 3.86 (3H, s), 2.18 (3H, s). ^{13}C-NMR ($CDCl_3$) δ: 191.5, 158.5, 157.5, 149.0, 141.4, 134.2, 132.8, 129.5, 125.6, 119.7, 115.3, 107.6, 106.8, 64.9, 62.5, 56.2, 24.4. MS *m*/*z*: 286 (M^+). HRMS (EI) calcd for $C_{17}H_{18}O_4$ 286.1205; found 286.1212.

3-Isopropenyl-1,4-dimethoxynaphthalene-2-carboxylic acid (7a): A mixture of the 3-isopropenylnaphthalene **12a** (850 mg, 3,32 mmol), NaH_2PO_4 (777 mg, 4.98 mmol), $NaClO_2$ (1.20 g, 13.3 mmol) and 2-methyl-2-butene (1.76 mL, 16.6 mmol) in *t*-BuOH/H_2O (15 mL/3 mL) was stirred at rt for 2 h. After removal of solvent, the reaction mixture was extracted with CH_2Cl_2. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated *in vacuo* to give carboxylic acid **7a** (670 mg, 74%) as orange oil. **7a** was used for next reaction without purification. IR (ATR) ν: 2935, 1701, 1439 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 8.14–8.15 (1H, m), 8.12–8.13 (1H, m), 7.53–7.63 (2H, m), 5.51 (1H, br s), 5.33–5.34 (1H, m), 5.08–5.09 (1H, m), 4.07 (3H, s), 3.92 (3H, s), 2.23 (3H, s). ^{13}C-NMR ($CDCl_3$) δ: 172.4, 149.5, 149.0, 142.2, 130.1, 129.5, 127.6, 127.4, 126.5, 124.3, 122.8, 122.5, 116.7, 63.6, 62.1, 23.6. MS *m*/*z*: 272 (M^+). HRMS (EI) calcd for $C_{16}H_{16}O_4$ 272.1049; found 272.1063.

3-Isopropenyl-1,4,8-trimethoxynaphthalene-2-carboxylic acid (7b): The same procedure as above was carried out using 3-isopropenylnaphthalene **12b** (65 mg, 0.23 mmol) to give carboxylic acid **7b** (45 mg, 66%) as yellow oil. **7b** was used for next reaction without purification. IR (ATR) ν: 2939, 1701, 1439 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 8.44 (1H, br s), 7.73 (1H, dd, *J* = 8.5, 1.0 Hz), 7.47 (1H, dd, *J* = 8.5, 1.0 Hz), 6.93 (1H, dd, *J* = 8.5, 1.0 Hz), 5.31–5.33 (1H, m), 5.08–5.09 (1H, m), 4.01 (3H, s), 3.94 (3H, s), 3.88 (3H, s), 2.20 (3H, s). ^{13}C-NMR ($CDCl_3$) δ: 172.4, 156.6, 149.6, 148.9, 141.9, 132.0, 130.8, 127.6, 125.8, 119.7,

116.9, 115.0, 106.6, 64.1, 62.0, 56.2, 23.6. MS *m/z*: 302 (M^+). HRMS (EI) calcd for $C_{17}H_{18}O_5$ 302.1154; found 302.1168.

Kalasinamide (1): A solution of carboxylic acid **7a** (25 mg, 0.09 mmol), DPPA (58 μL, 0.27 mmol), and Et_3N (38 μL, 0.27 mmol) in 1,2-dichlorobenzene (1 mL) was stirred at 60 °C for 30 min to generate isocyanate **6a** *in situ* (by monitoring the disappearance of **7a** by thin layer chromatography), which was further were heated under MW irradiation at 180 °C for 40 min. After cooling to an ambient temperature, the reaction mixture was concentrated in *vacuo*. The residue was purified by column chromatography using EtOAc-hexane (1:3, v/v) as an eluent to give kalasinamide (**1**) (20 mg, 78%) as yellow solid. mp 232–234 °C (EtOH) (Lit.[3a] mp 234–235.5 °C). IR (ATR) ν: 1650, 1365 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 9.04 (1H, br s), 8.19 (1H, dd, *J* = 8.5, 1.2 Hz), 8.06 (1H, dd, *J* = 8.5, 1.2 Hz), 7.59 (1H, dt, *J* = 8.5, 1.2 Hz), 7.47 (1H, dt, *J* = 8.5, 1.2 Hz), 6.46 (1H, q, *J* = 0.7 Hz), 3.99 (3H, s), 3.98 (3H, s), 2.80 (3H, d, *J* = 0.7 Hz). ^{13}C-NMR ($CDCl_3$) δ: 161.6, 151.7, 148.7, 135.9, 128.2, 128.1, 127.9, 124.7, 124.0, 123.5, 123.1, 121.1, 114.0, 64.1, 61.9, 23.3. MS *m/z*: 269 (M^+). HRMS (EI) calcd for $C_{16}H_{15}NO_3$ 269.1052; found 269.1067.

Geovanine (3): The same procedure as above was carried out using carboxylic acid **7b** (43 mg, 0.14 mmol) to give geovanine (**3**) (23 mg, 54%) as yellow solid. mp 188–190 °C (EtOAc) (Lit.[7a] mp 190–192 °C). IR (ATR) ν: 1651 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 9.13 (1H, br s), 7.78 (1H, dd, *J* = 8.4, 1.1 Hz), 7.36 (1H, dt, *J* = 8.4, 1.1 Hz), 6.92 (1H, dd, *J* = 8.4, 1.1 Hz), 6.48 (1H, q, *J* = 0.7 Hz), 4.05 (3H, s), 3.93 (3H, s), 3.91 (3H, s), 2.78 (3H, d, *J* = 0.7 Hz). ^{13}C-NMR ($CDCl_3$) δ: 161.7, 155.2, 151.1, 148.4, 136.4, 129.0, 126.1, 124.6, 123.6, 120.8, 115.7, 114.3, 106.7, 63.8, 62.7, 56.2, 23.2. MS *m/z*: 269 (M^+). HRMS (EI) calcd for $C_{17}H_{17}NO_4$ 269.1052; found 269.1057.

Marcanine A (2): A solution of CAN (407 mg, 0.74 mmol) in MeCN/H_2O (0.5 mL/1.0 mL) was added to a solution of kalasinamide (**1**) (40 mg, 0.15 mmol) in MeCN (1 mL) at 0 °C for 3 h. The reaction mixture was quenched with water, and then was extracted with EtOAc. The organic layer was washed with water and brine, dried over Na_2SO_4, and evaporated *in vacuo*. The residue was recrystallized from MeOH to give malcanine A (**2**) (30 mg, 84%) as yellow solid. mp 292 °C dec (Lit.[6a] mp > 300 °C). IR (ATR) ν: 1651, 1639, 1589 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 9.74 (1H, br s), 8.24 (1H, dd, *J* = 7.5, 1.3 Hz), 8.19 (1H, t, *J* = 7.5, 1.3 Hz), 7.87 (1H, dt, *J* = 7.5, 1.3 Hz), 7.78 (1H, dt, *J* = 7.5, 1.3 Hz), 6.69 (1H, q, *J* = 1.0 Hz), 2.72 (3H, d, *J* = 1.0 Hz). ^{13}C-NMR ($CDCl_3$) δ: 181.4, 177.9, 160.3, 152.2, 139.8, 135.8, 133.7, 133.3, 129.9, 127.7, 127.5, 126.7, 116.1, 22.8. MS *m/z*: 239 (M^+). HRMS (EI) calcd for $C_{17}H_{17}NO_4$ 239.0582; found 239.0585.

9-Methoxy-4-methylbenzo[*g*]quinoline-2,5,10(1*H*)-trione (13): The same procedure as above was carried out using geovanine **3** (10 mg, 0.033 mmol) to give azaanthracenone **13** (7 mg, 79%) as yellow solid. mp > 300 °C dec. IR (ATR) ν: 1658, 1643, 1601 cm^{-1}. ^{1}H-NMR ($CDCl_3$) δ: 9.71 (1H, br s), 7.89 (1H, dd, *J* = 8.0, 1.2 Hz), 7.80 (1H, dt, *J* = 8.0, 1.2 Hz), 7.33 (1H, dd, *J* = 8.0, 1.2 Hz), 6.63 (1H, q, *J* = 1.0

Hz), 4.07 (3H, s), 2.69 (3H, d, J = 1.0 Hz). ^{13}C-NMR ($CDCl_3$) δ: 181.1, 175.8, 160.5, 152.0, 140.6, 137.0, 135.4, 129.4, 126.7, 120.7, 120.2, 117.3, 114.9, 56.7, 22.6. MS *m/z*: 239 (M^+). HRMS (EI) calcd for $C_{15}H_{11}NO_4$ 269.0688; found 269.0695.

Cell viability assays

The HCT-116 cells' viability assay was conducted using the MTT method based on the procedure described by Mosmann.[16] Briefly, cells were placed in 96-well flat bottomed tissue culture plates with 3.0 × 10^3 cells per well in a 100 μL culture medium. This was followed by incubation at 37 °C in an atmosphere of 5% CO_2 for 24 h to allow the cells to attach onto the wells. The cells were treated with the indicated concentrations of test agents in a culture medium without FBS. Following a further 48 h incubation, 10 μL of MTT (5 mg/mL in phosphate-buffered saline) were added per well, and the plate was incubated for 4 h to allow the MTT to metabolize by cellular mitochondrial dehydrogenases. The excess MTT was aspirated and the produced formazan crystals were dissolved by adding 100 μL dimethyl sulfoxide. The absorbance of the purple formazan was read at 570 nm using a microplate reader. The results following the test agents' exposure were calculated as a percentage relative to untreated controls.

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