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# Isolation of Streptonigrin and Its Novel Derivative from *Micromonospora* as Inducing Agents of p53-Dependent Cell Apoptosis

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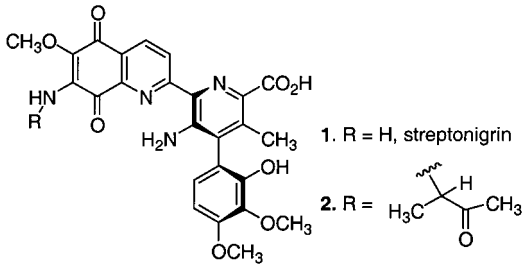
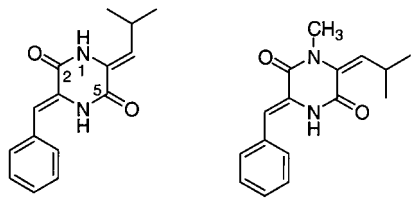
Streptonigrin (**1**) and its novel natural derivative 7-(1-methyl-2-oxopropyl)streptonigrin (**2**) were isolated from an actinomycete strain, *Micromonospora* sp. IM 2670. The inductions for **1** and **2** are more potent in the human neuroblastoma SH-SY5Y cells that contain wild-type p53 than in SH-SY5Y-5.6 cells that overexpress a dominant negative mutant of p53, thus suggesting that they induce apoptosis through a p53-dependent pathway.

The tumor suppressor protein p53 functions as a key component of a cellular emergency response mechanism. Activation of p53 can occur in response to a variety of stress signals including DNA damage, hypoxia, and activated oncogenes. Such activation then leads to the induction of a number of genes whose products trigger growth arrest or programmed cell death (apoptosis), thus eliminating damaged and potentially dangerous cells from the organism.[1] The importance of p53 has attracted considerable attention in the scientific field, as over half of all human cancers are linked with the loss of wild-type p53 function due to mutation of the p53 gene. However, a large number of cancers, such as human neuroblastoma, were found to carry the wild-type p53 gene. Neuroblastoma is one of the most common malignancies in childhood, and the cell lines have been widely employed in studies involving the function and regulation of wild-type p53 in tumor cells. Yu and colleagues reported that 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), at concentrations ranging from 20 to 100 $\mu$M, induced apoptosis in human neuroblastoma SH-SY5Y cells.[2] The cytotoxic effect of H-7 was correlated with its ability to induce nuclear accumulation of p53 in the cells. Furthermore, the apoptotic effect of H-7, but not other apoptotic insults, was effectively diminished in SH-SY5Y-5.6 cells that overproduced a dominant negative mutant of p53, thus suggesting that H-7 mediates its apoptotic effect through a p53-dependent mechanism. Encouraged by the findings, a high-throughput screen was established to identify novel natural products that can trigger wild-type p53 function using human neuroblastoma SH-SY5Y cells as the model system. Here we report the isolation and characterization of a known aminoquinone antibiotic streptonigrin (**1**) and its novel derivative (**2**) from a *Micromonospora* strain as inducing agents of p53-dependent apoptosis.




Six out of 12 000 actinomycete extracts that killed >70% of the SH-SY5Y cells and <35% of the SH-SY5Y-5.6 cells were identified from microtiter plate-based screening. One extract, 2670-1, exhibited reproducible selective cytotoxicity and caused an accumulation of p53 in the nucleus of SH-SY5Y cells (data not shown) and was selected for further purification.

The fermentation broth of *Micromonospora* sp. IM 2670 was freeze-dried and extracted with MeOH. The bioassay-active extracts were dissolved in MeOH−$H_2O$ (1:1) and extracted sequentially with hexane, $CH_2Cl_2$, EtOAc, and *n*-butanol. The bioassay-active $CH_2Cl_2$ extract was fractionated by reversed-phase HPLC ($C_{18}$, MeOH−$H_2O$ + 0.04% TFA, method A). The bioassay-active HPLC fraction was further purified by normal-phase preparative TLC (15% MeOH in $CHCl_3$) and reversed-phase HPLC ($C_{18}$, MeOH−$H_2O$ + 0.04% TFA, method B) to give compounds **1** and **2**. Compounds **3** and **4** were also isolated respectively from the fractions collected just before and after the bioactive HPLC fraction.



**3**, Albonoursin **4**, 1-*N*-Methylalbonoursin

Compound **1**, dark red or black solid, had retention times ($t_R$) of 27.3 and 15.1 min as determined by HPLC methods

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**Table 1.** $^1$H and $^{13}$C NMR Data for Compounds **1** and **2** ($\delta$ in ppm, $J$ in Hz) at 295 K

| | **1** | | | | | **2** | |
|---|---|---|---|---|---|---|---|
| | $^1$H NMR | | | $^{13}$C NMR | | $^1$H NMR | $^{13}$C NMR[d] |
| no. | $CDCl_3$ | dioxane-$d_8$ lit.[5] | DMSO-$d_6$ | dioxane-$d_8$ lit.[5] | DMSO-$d_6$[a] | $CDCl_3$ | $CDCl_3$ |
| 1 | | | | | | | |
| 2 | | | | 145.3 | 144.1 | | 143.6 |
| 3 | 8.48 (d, 8.4) | 8.93 (d, 9) | 9.01 (d) | 126.2 | 125.9 | 8.47 (8.4) | 125.4 |
| 4 | 8.69 (d, 8.4) | 8.38 (d, 9) | 8.36 (d) | 134.2 | 133.4 | 8.70 (8.4) | 134.4 |
| 4a | | | | 127.7 | 126.7 | | 130.1 |
| 5 | | | | 177.2 | 175.9 | | 177.8 |
| 6 | | | | 137.4 | 135.7 | | 136.0 |
| 6-OMe | 4.104 (s) | 3.96 (s) | 3.81 (s) | 60.2 | 59.7 | 4.001 (s) | 61.7 |
| 7 | | | | 141.0 | (141.6)[b] | | *e* |
| 7-$NH_2$ or 7-NH | 5.12 (br s) | 6.00 (br s) | 6.93 (br s) | | $\delta_N$ 122.4[c] | 5.97 (d, 6.9) | |
| 8 | | | | 181.0 | 180.3 | | *e* |
| 8a | | | | 160.8 | 159.8 | | *e* |
| 1′ | | | | | | | |
| 2′ | | | | 133.6 | 136.2 | | 139.5 |
| 2′-$CO_2H$ | 12.39 (br s) | 11.00 (br s) | 12.22 (br s) | 165.5 | 167.1 | | *e* |
| 3′ | | | | 138.9 | 134.8 | | 131.2 |
| 3′-Me | 2.50 (s) | 2.34 (s) | 2.17 (s) | 17.4 | 17.0 | 2.50 (s) | 17.5 |
| 4′ | | | | 130.3 | 133.9 | | 134.0 |
| 5′ | | | | 147.5 | (145.7)[b] | | *e* |
| 6′ | | | | 135.0 | 129.5 | | *e* |
| 7′ | | | | 115.7 | 114.8 | | *e* |
| 8′ | | | | 149.0 | 148.1 | | 146.3 |
| 8′-OH | 5.95 (br s) | 7.83 (br s) | 8.94 (br s) | | | 5.94 (br s) | |
| 9′ | | | | 137.9 | 136.9 | | 137.0 |
| 9′-OMe | 3.956 (s) | 3.84 (s) | 3.76 (s) | 60.7 | 60.3 | 3.945 (s) | 61.8 |
| 10′ | | | | 154.1 | 153.1 | | 153.5 |
| 10′-OMe | 3.998 (s) | 3.88 (s) | 3.85 (s) | 55.9 | 55.7 | 3.960 (s) | 55.7 |
| 11′ | 6.68 (d, 8.6) | 6.70 (d, 8.5) | 6.70 (d) | 105.2 | 104.4 | 6.69 (d, 8.6) | 105.2 |
| 12′ | 6.80 (d, 8.5) | 6.77 (d, 8.5) | 6.73 (d) | 125.6 | 124.6 | 6.81 (d, 8.6) | 125.6 |
| 1″ | | | | | | 4.74 (pent, 7.1) | 58.7 |
| 2″ | | | | | | | 205.8 |
| 3″ | | | | | | 2.18 (s) | 26.1 |
| 4″ | | | | | | 1.49 (d, 7.2) | 18.9 |

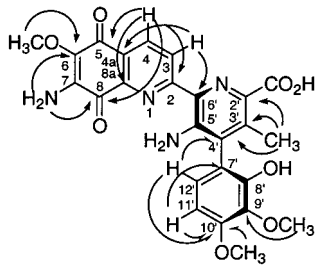
[a] The signals were assigned by COSY, $^1$H−$^{13}$C HMQC, and HMBC (optimal $J$ = 10, 8, 6, 4, and 2 Hz). [b] Not confirmed. [c] Obtained from $^1$H−$^{15}$N HSQC. [d] The chemical shifts obtained and assigned from COSY, $^1$H−$^{13}$C HMQC, and HMBC (optimal $J$ = 8 and 4 Hz). [e] Unable to determine or assign.

A and B, respectively. Its UV (MeOH−$H_2O$ + 0.04% TFA, $\lambda_{max}$ 245, 292−302 (sh), and 374 nm) suggested that it contained a highly conjugated system, such as benzoquinone. The $^1$H NMR spectrum of **1** was relatively simple (Table 1), although its $^{13}$C NMR indicated that it had 25 carbons. In addition, $^1$H−$^1$H COSY, $^1$H−$^{13}$C HMQC, and HMBC experiments showed the presence of three $CH_3O$, one $CH_3$, and four protons (in two coupled systems) attached to aromatic rings. From its HRMS (electrospray, $m/z$ 507.1499, $[M + H]^+$) and NMR data, the formula of **1** was deduced as $C_{25}H_{22}N_4O_8$ (calcd for $C_{25}H_{22}N_4O_8$ + H, 507.1516), which best matched that of the known aminoquinone antibiotic streptonigrin.[3] The $^1$H NMR ($CDCl_3$) data of the isolated **1** were identical to those reported for **1**[4] and to those obtained from an authentic sample (Sigma, S 1014). The $^{13}$C NMR (DMSO-$d_6$) data were also in accord with those recorded in dioxane-$d_8$ (Table 1).[5] The 7-$NH_2$ was assigned by $^1$H−$^{13}$C HMBC ($^3J$ to C-6 and C-8) and $^1$H−$^5$N HSQC ($\delta_N$ 122.4). The isolated **1** and streptonigrin (Sigma) had identical HPLC and UV profiles. Both compounds induced apoptosis in SH-SY5Y cells through a p53-dependent pathway.

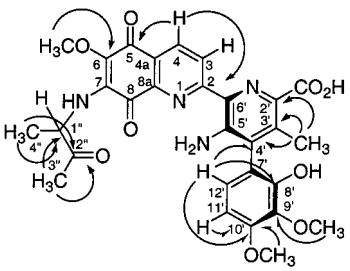
Compound **2**, a red solid, is less polar than **1** ($t_R$ = 28.1 and 21.1 min as determined by HPLC methods A and B, respectively) but has a similar UV spectrum: $\lambda_{max}$ 250, 295−305 (sh), 377 nm. Although the $^1$H NMR spectrum of **2** is similar to that of **1**, it contains two additional $CH_3$ at $\delta_H$ 1.49 (d, $J$ = 7.2 Hz) and 2.18 (s) and two additional protons at $\delta_H$ 4.74 (pent, $J$ = 7.1 Hz) and 5.97 (d, $J$ = 6.9 Hz). The 2D NMR experiments ($^1$H−$^1$H COSY, $^1$H−$^{13}$C HMQC, and HMBC) revealed that the proton at $\delta_H$ 4.74 ($\delta_C$ 58.7) is coupled to the protons at $\delta_H$ 1.49 and $\delta_H$ 5.97, with the latter connected to an electronegative atom. Both protons at $\delta_H$ 2.18 and $\delta_H$ 1.49 correlate with carbons at $\delta_C$ 58.7 and $\delta_C$ 205.8 (C=O or imine C=N−). The formula of **2** was deduced from its HRESIMS data ($m/z$ 577.1935, $[M + H]^+$) as $C_{29}H_{28}N_4O_9$ (calcd for $C_{29}H_{28}N_4O_9$ + H, 577.1968). The difference between the formula of **1** and **2** was $C_4H_6O$; that is, compound **2** was most probably a derivative of **1** with structure resembling either I or II (Figure 2) and was derivatized at either 7-$NH_2$ or 5′-$NH_2$. Since derivatization could cause a change in the chemical shifts of the neighboring protons, a thorough study of the chemical shift changes between **1** and **2** ($\Delta\delta_H$ = −0.10 (6-OMe), −0.04 (10′-OMe), −0.01 (for 9′-OMe, 11′-H, and 12′-H) and +0.01 (8′-OH)) was carried out. The biggest change occurred at the 6-$CH_3O$ group, thus suggesting that 7-$NH_2$ had been altered. In addition, the disappearance of the broad singlet of 7-$NH_2$ at 5.12 ppm (2H) and the appearance of a new doublet at 5.97 ppm (1H) in the $^1$H NMR spectrum of **2** provided an important piece of evidence that derivatization has indeed occurred at 7-$NH_2$.

To further support that the structure was I and not II, syntheses of imines **6**, **8**, and **9** were carried out (Scheme 1). The chemical shifts ($\delta_C$ and $\delta_N$, in $CDCl_3$) of imine **6** were deduced from the NMR data of the reaction mixture of **5** and acetone. Reaction of **5** with acetoin **7** provided the imine **9** (31%) and the acetamide **10** (51%) but not imine **8**. As expected, the chemical shifts of the four-carbon unit of compound **2** are very similar to that of **9**, thus confirming that compound **2** is a derivative of compound **1** at 7-$NH_2$ and possesses a structure containing I.

Compound **3** was obtained as a white solid (HPLC $t_R$ = 26.4 min by method A, UV $\lambda_{max}$ 233, 318 nm). $^1$H and $^{13}$C NMR (including DEPT) show that it has 15 protons and 15 carbons. The molecular formula of **3** was determined



Streptonigrin **1**



Compound **2**

**Figure 1.** $^{1}H-^{13}C$ HMBC correlations (arrows) for **1** (optimal $J = 10$, 8, 6, 4, and 2 Hz, in DMSO-$d_6$) and **2** (optimal $J = 8$ and 4 Hz, $CDCl_3$).

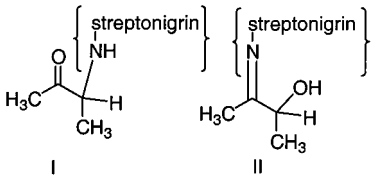
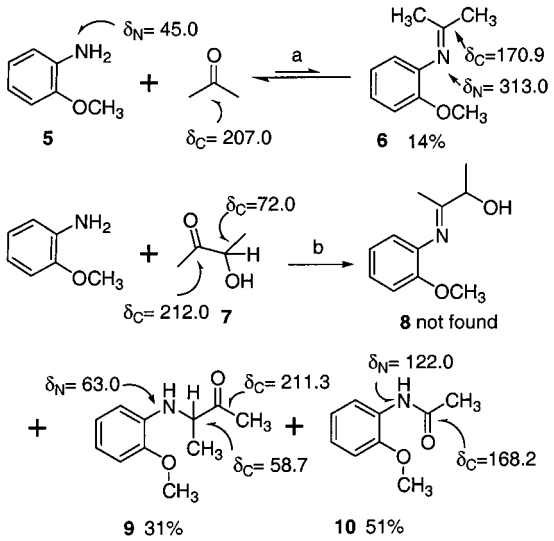



**Figure 2.**

**Scheme 1**[a]

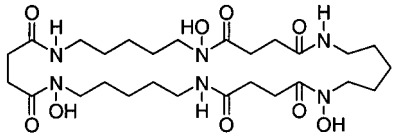



[a] Reagents and conditions: (a) **5** (0.35 mmol) in acetone (ca. 10−15 mL) stirred at room temperature for 23 h; (b) **5** (0.49 mmol) and **7** (2.3 mmol) in $CH_2ClCH_2Cl$, stirred at room temperature for 73 h.

as $C_{15}H_{16}N_2O_2$ by HRESIMS. The structure of **3** was determined by 2D NMR experiments (COSY, HMQC, and HMBC). Compound **3** was confirmed to be the known diketopiperazine alkaloid, albonoursin, with a 3*Z*,6*Z*-configuration.[6] The $^{1}H$ NMR data of **3** were identical to those reported.[7]

Compound **4** has a UV spectrum similar to that of **3**, but is less polar ($t_R$ = 28.9 min by HPLC method A). The molecular formula of **4** was determined as $C_{16}H_{18}N_2O_2$ by HRESIMS. It was found to be the known alkaloid 1-*N*-methylalbonoursin with 3*Z*,6*E*-configuration as confirmed by 1D and 2D NMR experiments. Its $^{1}H$ NMR data were identical to those reported.[8]

Compound **11**, a red compound isolated from the HPLC fraction (16.5−17.0 min, a peak with $t_R$ = 16.8 min, method A), shows broad UV absorption from 380 to 470 nm ($\lambda_{max}$ = 422 nm) and only very broad lines in the $^{1}H$ NMR spectrum, thus indicating that it is not an analogue of **1**. The formula of **11** was deduced as $C_{27}H_{46}N_6O_9Fe$ by HRESIMS, suggesting that it is the ferric salt of desferrioxamine E or norcardamine (**12**).[9] The presence of iron is



desferrioxamine E or norcardamine **12**

consistent with the line broadening effect seen in the $^{1}H$ NMR spectrum. Furthermore, the presence of chelating compound desferrioxamine E in *Micromonospora* is not surprising, as it was found to protect isolated DNA and bacterial cells against streptonigrin-induced toxic effects.[10] When the MeOH solution of **11** was treated with Dowex 50W ($H^+$) resin, a new peak showing M + $H^+$ at *m/z* 601.403 ($C_{27}H_{48}N_4O_9$ + H) appeared in the MS. The ratio of peak intensity of *m/z* 654/601 was less than 3% after three treatments, thus indicating that the ferric cation has undergone almost complete exchange with the proton.

Bioassay demonstrated that both **1** and **2** exhibit very potent and selective cytotoxicity. Streptonigrin **1** is 92× more toxic toward the SH-SY5Y ($IC_{50}$ = 0.05 $\mu$M) than toward the SH-SY5Y-5.6 ($IC_{50}$ = 4.6 $\mu$M) cell lines, suggesting that apoptosis occurs through a p53-dependent pathway. Compound **2** is ca. 18× less potent ($IC_{50}$ = 0.9 and 75 $\mu$M for SH-SY5Y and SH-SY5Y-5.6 cell lines, respectively) than its parent **1**. This agrees with previous reports indicating that cell toxicity of streptonigrin is mediated by its aminoquinone domain since the antitumor actions of streptonigrin are lost when the aminoquinone moiety is blocked.[11,12] Compounds **3** and **4** are not active at up to 100 $\mu$M concentration. Compound **11** (tested as crude) is also inactive. 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine (H-7) is less potent and less selective when compared with compounds **1** and **2** ($IC_{50}$ = 40 and 80 $\mu$M for SH-SY5Y and SH-SY5Y-5.6 cell lines, respectively).

Many anticancer agents, such as cisplatin,[13] bis-Pt(III) complex,[14] mitomycin C, and actinomycin D,[15] which cause DNA damage, are strong inducers of apoptosis. Many of them have also been observed to induce nuclear accumulation of p53 in cells expressing wild-type p53 protein.[16] Streptonigrin has long been known to exert its genotoxic effects by causing DNA and chromosome damage through generation of free radicals. This, however, is the first report demonstrating that streptonigrin, as well as its novel derivative **2**, can induce apoptosis through a p53-dependent pathway in human neuroblastoma cells. Compound **1** also caused nuclear accumulation of p53 and induced DNA ladders in SH-SY5Y cells. Further studies on the effect of streptonigrin in mediating p53-dependent apoptosis will be published elsewhere.

## Experimental Section

**General Experimental Procedures**.[17] All the 1D and 2D NMR experiments for $^{1}H$ (400.13 MHz), $^{13}C$ (100.61 MHz), and $^{15}N$ (40.55 MHz) nuclei were obtained on a Bruker AVANCE-400 digital NMR spectrometer. $^{1}H-^{13}C$ and $^{1}H-^{15}N$ 2D experiments (HMQC, HSQC, and HMBC) were run with Z-gradient

selection. HRMS spectra were determined using a PerSeptive Biosystems Mariner TOF spectrometer.

**Isolation and Taxonomy of the Actinomycete Strain IM 2670**. The procedures for the isolation and taxonomic characterization of the strain IM 2670 were as described by Wang et al.[18] The actinomycete strain IM 2670, from which streptonigrin and its derivative were purified, was isolated from a soil sample collected in the Singapore Botanic Garden. Its colony exhibits properties characteristic of *Micromonospora* on an ISP 4 medium plate. The colonies do not grow aerial mycelium. Dark-colored spore accumulation forms on the colony surface. Single spores are born on short sporephores on the substrate mycelium. The color of the colony is orange. No diffusible pigment was produced on ISP 2, ISP 3, ISP 4, and Bennett medium plates. The cell wall peptidoglycans contained *meso*-diaminopimelic acid. The complete nucleotide sequence of the 16S rRNA gene of IM 2670 was determined for phylogenetic analysis. IM 2670 was placed within the *Micromonospora* clade on the phylogenetic tree, and the 16S rRNA gene sequences are higher than 97% identical between IM 2670 and other *Micromonospora* species. On the basis of morphological, chemotaxonomic, and phylogenetic evidences, the actinomycete strain IM 2670 was assigned to the genus *Micromonospora*.

**High-Throughput Screens (HTS)**. HTS was performed in transparent 96-well plates (Nunclon). Neuroblastoma cell lines SH-SY5Y and SH-SY5Y-5.6 containing HA-tagged 91 amino acid mini p53 protein were employed in the primary screens.[2] Cell cytotoxicity was measured by using the Kit CellTiter 96 $AQ_{ueous}$ Non-Radioactive Proliferation Assay (Promega, http://www.promega.com). A fixed number of cells ($10^5$ cells/mL) in 100 $\mu$L of RPMI media containing hygromycin (0.1 mg/mL) was plated out the day before. The cells were incubated with 1 $\mu$L of crude extracts for 24 h. At the 20th hour, MTS (Owen's reagent, or 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner slat), prepared according to manufacturer's direction, was added to a final concentration of 0.04 mg/well and incubated for 4 h at 37 °C. The plates were read at $OD_{490}$ in a multilabel counter (Wallac 1420 Victor). Reference control wells containing cells and blank wells containing media only were treated with the appropriate amount of methanol. Extracts, which were found to kill at least 70% of SH-SY5Y cells but less than 35% of SH-5Y-5.6, were repeated in duplicates in 96-well plates. Samples that exhibited reproducible inhibition were subjected to dose−response experiments. Those with distinctive dose−response curves were selected for western blotting.

**Isolation and Purification Procedures**. The bioassay-active fermentation broth (4 L) of *Micromonospora* sp. IM 2670 was freeze-dried. The solid residue was extracted with MeOH (3.5 L) and 10% $H_2O$ in MeOH (2 L × 2) at room temperature. The bioassay-active extracts were combined and concentrated under reduced pressure below 35 °C. The wet residue was diluted with MeOH (250 mL) and $H_2O$ (250 mL), and then the solution was extracted with hexane (300 mL × 3), $CH_2Cl_2$ (300 mL × 3), ethyl acetate (300 mL × 3), and *n*-butanol (300 mL × 3). The bioassay data showed that the $CH_2Cl_2$ extract (11 g) was most active. This extract was purified (separately in two equal portions) by a preparative HPLC system (Waters Delta Prep 4000) equipped with a 996 photodiode array detector, using Prep Nova-Pak $HRC_{18}$ column segments (6 $\mu$m, 40 mm × 210 mm; flow rate, 30 mL/min; solvent A = $H_2O$ + 0.04% TFA, B = MeOH + 0.04% TFA; 5% to 100% B in 30 min, linear gradient, then 100% B for additional 5 min, method A). Fractions corresponding to different peaks were collected. The most active fraction (27.0−28.5 min, containing two major peaks at $t_R$ = 27.3 and 28.1 min, 370 nm) was further purified by preparative TLC (silica, 15% MeOH in $CHCl_3$) to give two bands. They were separately subjected to another HPLC purification (Novapak, $C_{18}$, 19 × 300 mm, flow 10 mL/min, 65% B for 30 min, then 65% to 100% B in 5 min, and maintained at 100% B for an additional 5 min, method B). Compound **1** (dark red or black solid, 1.0 mg, $t_R$ = 15.1 min) and compound **2** (red solid, 1.0 mg, $t_R$ = 21.1 min) were obtained.

Two HPLC fractions (method A, 25.5−27.0 min, major peak $t_R$ = 26.4 min and 28.5−29.5 min, major peak $t_R$ = 28.9 min) were purified by preparative TLC (5% MeOH in $CHCl_3$) to give a white solid **3** (2.5 mg) and a pale yellow solid **4** (1.7 mg), respectively. In a scaled-up fermentation (10 L), the $CH_2Cl_2$ extract was first purified by Si flash chromatography (gradient $CH_2Cl_2$−MeOH) and then followed by HPLC purification. Compound **1** (8.4 mg) and a red solid **11** (20 mg, HPLC fraction, 16.5−17.0 min, major peak $t_R$ = 16.8 min at 450 nm, method A) were obtained.

**Streptonigrin (1)**: red or black solid; UV (65% MeOH in $H_2O$ with 0.04% TFA) $\lambda_{max}$ (relative absorption) 245 (1.00), 292−302 (sh, 0.46), 374 (0.44) nm; NMR data, see Table 1; HRESIMS *m*/*z* 507.1499 (calcd for $C_{25}H_{23}N_4O_8$ + H, 507.1516).

**5-Amino-4-(2-hydroxy-3,4-dimethoxyphenyl)-6-[6-methoxy-7-(1-methyl-2-oxopropylamino)-5,8-dioxo-5,8-dihydroquinolin-2-yl]-3-methylpyridine-2-carboxylic acid or 7-(1-methyl-2-oxopropyl)streptonigrin (2)**: red solid; UV (65% MeOH in $H_2O$ with 0.04% TFA) $\lambda_{max}$ (relative absorption) 250 (1.00), 295−305 (sh, 0.40), 377 (0.42) nm; NMR data, see Table 1; HRESIMS *m*/*z* 577.1935 (calcd for $C_{29}H_{28}N_4O_9$ + H, 577.1968).

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**Supporting Information Available:** NMR, UV, and MS data for compounds **3** and **4**; preparative procedures and NMR data for compounds **6**, **9**, and **10**. This material is available free of charge via the Internet at http://pubs.acs.org.

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