Evaluating Indole-Related Derivatives as Precursors in the Directed Biosynthesis of Diazepinomicin Analogues[§]

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The effectiveness of precursor-directed biosynthesis to generate diazepinomicin (**1**) analogues with varied ring-A substitutents was investigated by feeding commercially available, potential ring-A precursors such as fluorinated tryptophans, halogenated anthranilates, and various substituted indoles into growing actinomycete culture DPJ15 (genus *Micromonospora*). Two new monofluorinated diazepinomicin analogues (**2** and **3**) were identified and characterized by spectroscopic methods. Both derivatives showed modest antibacterial activity against the Gram-positive coccus *Staphylococcus aureus* with MIC values in the range 8−32 μg/mL.

Previous activity-based screening conducted in our laboratory on fermentation extracts from the marine actinomycete cultures DPJ12 and DPJ15 (genus *Micromonospora*) led to the isolation of diazepinomicin (**1**), a farnesylated trihydroxy-dibenzodiazepinone.[1] Compound **1** was concurrently discovered by Ecopia BioSciences, Inc.[2] from a soil-derived *Micromonospora* sp. through their genomic scanning approach[3] and was named ECO-4601. Diazepinomicin-ECO-4601(**1**) has shown modest activity against Gram-positive bacteria, with a MIC value of 32 μg/mL,[1] and has been recognized for its anti-inflammatory activity, as well as in vivo antitumor activity against glioma, breast, and prostate cancers.[4] It is currently undergoing preclinical evaluation as an anticancer agent.[4] The structure of diazepinomicin is unique in that it possesses an atypical tricyclic (rings A−C) dibenzodiazepinone moiety as its core (Figure 1). Natural products with a dibenzodiazepinone core are exceptionally rare, and therefore, the biogenetic origin of this moiety is of great interest.[5]

A preliminary investigation of its biosynthesis based on the genomic analysis of the loci[5] encoding for diazepinomicin (**1**) suggested that rings A and B of the dibenzodiazepinone moiety in **1** are derived from different biosynthetic precursors despite their close resemblance. Accordingly, ring B may be formed from 2-amino-6-hydroxy[1,4]benzoquinone via 3-amino-5-hydroxybenzoic acid (AHBA), and ring A is derived from 3-hydroxyanthranilate via the shikimic acid pathway (Figure 1). Moreover, the biosynthetic origin of ring A was supported by precursor-feeding experiments with D-labeled 3-hydroxyanthranilate and ^{15}N- and D-labeled tryptophans.[5]

The observation that both 3-hydroxyanthranilate and tryptophan can act as efficient biosynthetic precursors of ring A prompted us to investigate the ability of *Micromonospora* sp. strain DPJ15 to accept non-native/chemically modified anthranilate and tryptophan derivatives, which could direct biosynthesis toward new ring-A-modified diazepinomicin analogues. Accordingly, 4-, 5-, or 6-monofluorinated-(±)-tryptophan derivatives (**1a**−**3a**) and mono-5-(F, Cl, Br, I)-substituted anthranilate derivatives (**1b**−**4b**) were evaluated as viable precursors in the biosynthetic pathway of **1** (Figure 2). The halogenated derivatives (**1**−**3a** and **1**−**4b**) were fed individually to growing cultures of *Micromonospora* sp. strain DPJ15 at a

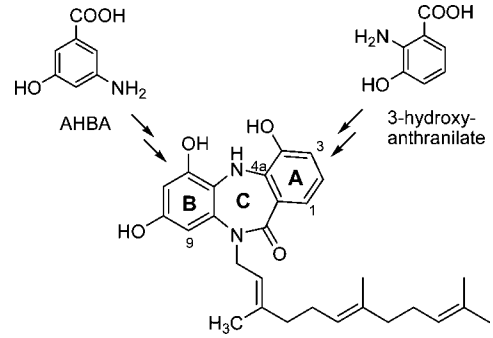

Figure 1. Diazepinomicin (**1**). Proposed biosynthetic precursors of ring A and B are shown.

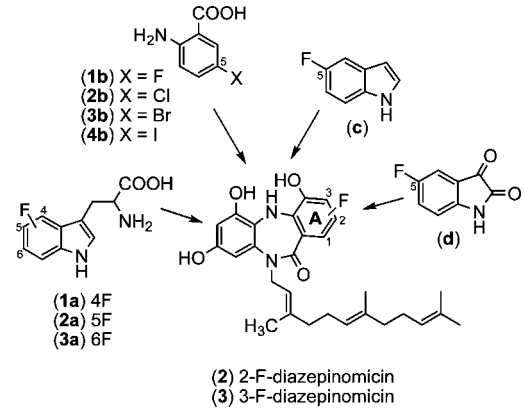

Figure 2. Precursor-directed biosynthesis of ring-A-modified diazepinomicin (**1**) analogues.

concentration of 0.15 g/L.[6] The incorporation efficiency of the precursors was followed by LC/UV/ESIMS over a period of 1−4 days.[7]

The LC-MS profiles recorded on day 4, following incubation with 5- and 6-fluorinated-(±)-tryptophans (**2a** and **3a**, respectively), indicated the formation of new fluorinated diazepinomicin analogues with substitution at ring A, 2-F-diazepinomicin (**2**) and 3-F-diazepinomicin (**3**), respectively. The relative incorporation rates of the fluorinated-(±)-tryptophans (**2a** and **3a**) were 5-F ≫ 6-F (based on HPLC-UV data; Figure 3). Surprisingly, 4-F-tryptophan (**1a**) was not incorporated into **1** and appeared to suppress cell growth significantly, in turn causing adverse effects on the biosynthesis of **1**. According to these results, the position of the

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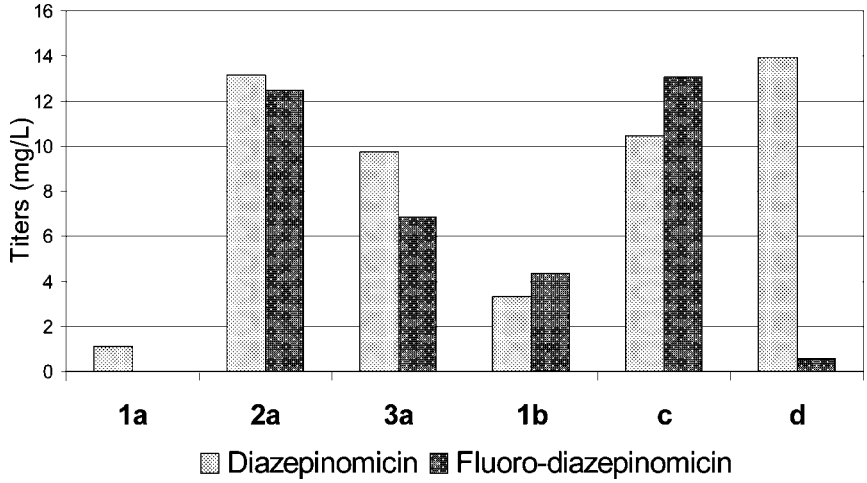

Figure 3. Relative rates of incorporation of the 5-F-precursors by the DPJ15 culture.

fluorine atom on the indole moiety seems to play a vital role in the organism's ability to accept these tryptophan analogues into the biosynthetic pathway of **1**. Furthermore, of the 5-(F, Cl, Br, I)-halogenated anthranilates (**1**−**4b**) that were evaluated as precursors, only 5-F-anthranilate (**1b**) yielded a diazepinomicin derivative. Therefore, it could be reasoned that halogen atoms larger than fluorine are not metabolized by the organism, probably due to unfavorable steric and/or electronic effects associated with these substituents.[8]

Since both 5-F-anthranilate (**1b**) and 5-F-tryptophan (**2a**) were shown to be viable precursors in the biosynthetic pathway of **1**, we decided to evaluate the feasibility of utilizing additional fluorinated indole compounds as precursors for the directed biosynthesis of fluorinated diazepinomicin. We chose 5-F-indole (**c**) and 5-F-isatin (**d**), based on the fact that indole and isatin are known to be associated with the biosynthetic/metabolic pathways to indole alkaloids.[9] Accordingly, culture DPJ15 was grown independently in the presence of 5-F-indole and 5-F-isatin (0.15 g/L each) and analyzed by LC-MS for the incorporation of these fluorinated precursors. Although fluorodiazepinomicin (**2**) was produced by the culture in the presence of 5-F-indole as well as 5-F-isatin, the former was preferentially incorporated over the latter.

Subsequently, the incorporation rate of the 5-F-precursors (tryptophan, anthranilate, indole, and isatin) by DPJ15 was quantitated by comparing the amount (mg/L) of 2-F-diazepinomicin (**2**) produced from each of these substrates (Figure 3). According to LC-UV analysis, the relative rates of incorporation of the 5-F-precursors were indole (**c**) > tryptophan (**2a**) > anthranilate (**1b**) > isatin (**d**).

On the basis of HRFTMS analysis, the new fluorodiazepinomicin positional isomers **2** and **3** shared the same molecular formula of $C_{28}H_{33}FN_2O_4$, featuring 13 degrees of unsaturation, and showed a predominant protonated molecular ion $[M + H]^+$ at *m*/*z* 481. Monofluorination on the dibenzodiazepinone moiety was confirmed on the basis of FTMS/MS experiments. Accordingly, fragment ion spectra generated using ESI-FTMS SORI-CID[10] on the protonated molecular ion $[M + H]^+$ of **2** (Figure 4; Table 1) were compared with the fragment ions observed for diazepinomicin (**1**).[1] For compound **2**, all of the four structurally characteristic fragment ions (***a***−***d***) showed an addition of +18 amu (when compared to diazepinomicin **1**[1]), which was consistent with the incorporation of a fluorine atom onto the tricyclic moiety.

Next, the location of the fluorine atom on the tricyclic moiety of compounds **2** and **3** needed to be established by NMR spectroscopy. In order to isolate and purify sufficient quantities of **2** and **3** for NMR analysis, scale-up fermentations were carried out (1 L of growth medium, 2.8 L Fernbach flask)[7] using 5-F-indole and 6-F-tryptophan[11] as precursors, respectively. The ^{1}H NMR

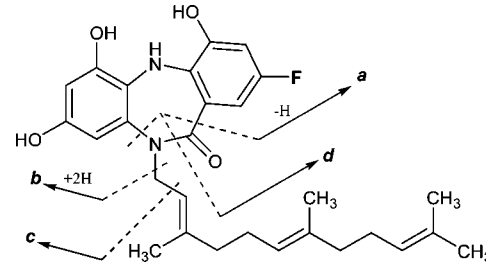

Figure 4. Proposed fragmentation pathway for the $[M + H]^+$ ions observed in the nanoelectrospray FTMS SORI-CID mass spectrum of **2**.

Table 1. MS Fragmentation of **2** Observed in the FTMS SORI-CID (Sustained Off-Resonance Irradiation with Collision-Induced Dissociation) Mass Spectrum

ion	relative (%)	*m*/*z*	composition	Δ mmu
a	4	232.0407	$C_{12}H_7FNO_3^+$	0.31
b	47	277.0620	$C_{13}H_{10}FN_2O_4^+$	0.16
c	100	289.0621	$C_{14}H_{10}FN_2O_4^+$	0.24
d	47	261.0435	$C_{13}H_8FNO_4^+$	0.31

spectrum of **2**, recorded in DMSO-d_6 (600 MHz), was very similar to that of **1** (DMSO-d_6, 400 MHz), but showed relevant differences in the δ_H 6.00−7.10 region of the aromatic resonances (Table 2), suggesting that compounds **1** and **2** are identical with regard to the farnesyl side chain but varied in the tricyclic portion. Close inspection of the NMR spectra for the dibenzodiazepinone core of **1**[1] and **2** revealed clear differences in chemical shift ($\Delta\delta$) and $^3J_{H/H}$ coupling constant values for ring A, whereas their corresponding ^{1}H and ^{13}C NMR assignments for ring B were essentially identical.

Placement of the fluorine atom on C-2 of ring A was consistent with its ^{13}C NMR chemical shift (δ_C 156.3) and the splitting pattern of the neighboring protons H-1 and H-3 ($^3J_{H/F}$ = brd, 9.2 Hz). The upfield shifts of the ^{13}C NMR resonances of C-1 (δ_C 104.3) and C-3 (δ_C 104.4) were also in agreement with the C-2 (*ortho*) fluorination. The assignment of C-4a (δ_C 138.8) was guided by the $^3J_{C/H}$ correlation from both H-1 (δ_H 6.63) and H-3 (δ_H 6.59) to C-4a (Table 2). Additionally, the arrangement of H-1 and H-3 on ring A was confirmed by a HMBC cross-peak from H-1 (δ_H 6.63) to the amide carbonyl C-11 (δ_C 167.4) on ring C. The connectivity between adjoining rings of the tricyclic moiety was established by means of distinct $^3J_{C/H}$ correlations between the exchangeable proton at δ_H 6.72 and ring-junction quaternary carbons at C-9a (δ_C 135.0) and C-11a (δ_C 124.3). The rest of the chemical shift assignments and coupling constant values of 2-F-diazepinomicin (**2**) were comparable to those reported for **1**.[1]

Table 2. ^{1}H and ^{13}C NMR (600/150 MHz) Data for 2-F-Diazepinomicin (**2**) in DMSO-d_6

position	δ_C	δ_H (mult., J = Hz)	COSY	HMBC
1	104.3	6.63 (brd, 9.2)		C-2, C-4a, C-11
2	156.3			
3	104.4	6.59 (brd, 9.2)		C-2, C-4a
4	149.1			
4a	138.8			
5		6.72		C-4, C-9a, C-11a
5a	125.8			
6	148.3			
7	99.9	6.19 (d, 2.3)	H-9	C-5a, C-6, C-8
8	153.3			
9	100.5	6.14 (d, 2.3)	H-7	C-7, C-8, C-5a
9a	135.0			
11	167.4			
11a	124.3			
12	48.7	4.39 (d, 6.0)	H-13	C-11
13	121.9	5.25 (brdd, 5.7, 5.7)	H-12, H-15, H-23	C-15, C-23
14	136.9			
15	39.4	1.97 (m)	H-16	C-13, C-23
16	26.3	2.03 (m)	H-15, H-17	C-17
17	124.1	5.07 (m)	H-16, H-24	C-16, C-24
18	134.9			
19	39.6	1.92 (m)	H-20	C-18, C-20, C-24
20	26.1	1.99 (m)	H-19, H-21	C-18, C-19, C-21
21	124.5	5.04 (m)	H-20, H-25, H-26	C-20, C-25
22	131.0			
23	16.6	1.65 (brs)	H-13	C-13, C-15
24	16.2	1.55 (brs)	H-17	C-17, C-19
25	17.9	1.52 (brs)	H-21	C-21, C-22, C-26
26	25.9	1.61 (brs)	H-21	C-21, C-22, C-25

The substitution pattern on the tricyclic moiety of 3-F-diazepinomicin (**3**) was established in a similar manner. Comparison of the NMR data for **1** (DMSO-d_6, 400 MHz) and **3** (DMSO-d_6, 600 MHz) indicated that the ^{1}H and ^{13}C chemical shift differences ($\Delta\delta$) were localized in the ring-A portion of the molecule (Figure 5). Even though the chemical shift of C-3 could not be extracted from the NMR data due to poor signal-to-noise ratio, the location of the fluorine atom on C-3 of ring A was evident by the upfield shift of the *ortho* proton (H-2; δ_H 6.64). Similarly, due to C-3 fluorination, changes in chemical shifts were observed for the carbon resonances of ring A; carbon C-2 (δ_C 108.4) showed an upfield shift of ~12 ppm, and the carbon *meta* to fluorine (C-1, δ_C 122.1) displayed a downfield shift of ~6 ppm. The tricyclic ring assembly was established on the basis of HMBC correlations from the exchangeable proton at δ_H 6.78 to the neighboring carbons $\delta_{C\text{-}4}$ 146.5, $\delta_{C\text{-}5a}$ 125.2, and $\delta_{C\text{-}6}$ 148.4. Due to the broadening of ring-A proton resonances, *ortho* and *meta* H−F spin coupling constants ($^3J_{H\text{-}2/F}$ and $^3J_{H\text{-}1/F}$, respectively) could not be extracted.

Subsequently, we decided to further evaluate the ability of *Micromonospora* sp. DPJ15 to incorporate selected 5- and 7-substituted indole derivatives into ring A of diazepinomicin (**1**). A number of commercially available indole derivatives were chosen on the basis of their chemical diversity (Figure 6). Each of these compounds (**1**−**11e** and **1**−**3f**) was added at two different concentrations (0.075 and 0.15 g/L) into the fermentation medium. After

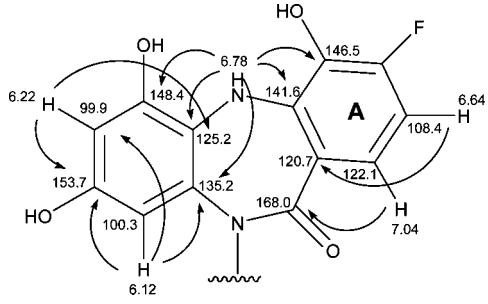

Figure 5. Key HMBC correlations and ^{1}H and ^{13}C assignments for the dibenzodiazepinone core of 3-F-diazepinomicin (**3**).

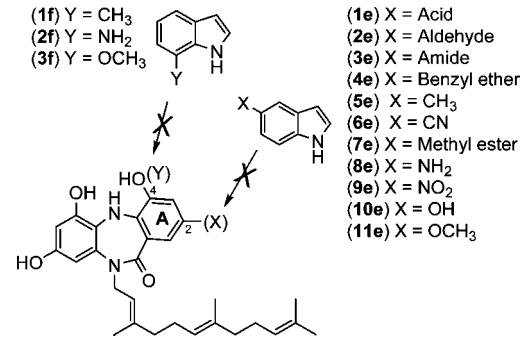

Figure 6. Attempted precursor-directed biosynthesis of ring-A-substituted diazepinomicin analogues.

Table 3. MIC Values (μg/mL) of Compounds **2** and **3** Compared to **1**

test organism	**1**	**2**	**3**
Staphylococcus aureus 375	16	16	32
Staphylococcus aureus 310	8	8	16
Enterococcus faecium 379	32	16	32
Enterococcus faecium 436	64	32	32
Escherichia coli 389	>128	>128	>128
Escherichia coli 442	>128	>128	>128
Candida albicans 54	>128	>128	>128

4 days, the fermentation extracts were analyzed by LC/UV/MS for the presence of new 2- or 4-substituted diazepinomicin analogues. Unfortunately, there was no evidence of incorporation of any of the 5- or 7-substituted indole analogues into diazepinomicin (**1**). In general, feeding of these indole derivatives appeared to have little or no effect on the production of **1**; however, compounds **2f**, **1e**, **8e**, and **9e** caused a dramatic decrease in the production titers of **1** at the two concentrations (0.075 and 0.15 g/L) tested.

In summary, our work has demonstrated that when one of the protons on the indole ring is replaced by a substituent larger than a fluorine atom (5- or 7-monosubstituted indole analogues), the compound is no longer incorporated into **1**. The sole preference for fluorinated analogues over other substituents could be attributed to the near isosteric nature of hydrogen and fluorine atoms. Furthermore, the inability to accept these substituted indole analogues into the biosynthetic pathway of **1** may reflect high specificity of the biosynthetic enzymes toward the native substrates.

The two new fluorinated diazepinomicin analogues (**2** and **3**) showed weak (>128 μg/mL) to modest (8−32 μg/mL) antibacterial activity against a selected group of Gram-positive and Gram-negative bacteria (Table 3) and failed to show any significant antifungal properties against *Candida albicans*.

Experimental Section

General Experimental Procedures. UV data were acquired using a HP1100 HPLC system equipped with a photodiode array detector. IR spectra were recorded on a Nicolet NEXUS-470 FT-IR spectrometer. NMR spectra were recorded on a Bruker AVANCE spectrometer operating at 600/150 MHz at 300 K with 1.7 mm TCI indirect detection Cryo-probe equipped with Z gradient. Each sample was dissolved in DMSO-d_6 and placed in a 1.7 mm o.d. NMR tube. Chemical shifts are reported in ppm and were referenced to TMS. High-resolution ESI-FTMS analyses were performed on a Bruker (Billerica, MA) APEXII FTICR mass spectrometer equipped with an actively shielded 9.4 T superconducting magnet (Magnex Scientific Ltd., Oxford UK), an external Bruker APOLLO ESI source, and a Synrad 50W CO_2 CW laser.[12] Mass spectra were internally/externally calibrated using HP tuning mix. In the FTMS-MS experiments, the abundant protonated molecular ion ($[M + H]^+$, m/z 481) was isolated using multi-CHEF (correlation harmonic excitation fields) and selectively activated and fragmented using SORI-CID (sustained off-resonance irradiation with collision-induced dissociation). Semipreparative HPLC was carried out on an Agilent 1100 series instrument (diode array detector) using a

Phenomenex Luna 5 μm C_{18} (250 × 10 mm) column. LC-MS data were obtained on a Thermo-Finnigan LCQ Deca instrument equipped with an Agilent 1100 LC system (diode array detector) and a YMC ODS-A 3 μm, 120 A (100 × 2.0 mm) column. All of the standard and substituted precursor analogues were purchased from Sigma-Aldrich. HPLC grade solvents were used, purchased from either J. T. Baker, Inc. or EMD (OmniSolv).

Fermentations. All fermentations were performed in Bennett's medium (1% dextrose, 0.1% yeast extract, 0.077% desiccated beef extract, 0.2% NZ-amine type A) made in deionized water. Initial precursor incorporation studies were carried out at concentrations of both 0.075 and 0.15 g/L. After each precursor was added separately to 15 mL of Bennett's, the medium was first sonicated and then warmed to 50 °C in a water bath to facilitate solubilization. Following maximum solubilization,[13] the pH was adjusted to 7.3 for each condition. The media were then filter sterilized (Acrodisc 25 mm syringe filter, 0.2 μm HT Tuffryn membrane sterile, Pall Life Sciences), and 10 mL of each medium was transferred into individual 50 mL sterile Erlenmeyer flasks. Activated preautoclaved HP20 resin in water was added to each flask at 5% v/v. The fermentations were inoculated at 2% v/v with first-stage seed and incubated at 28 °C and 200 rpm for 5 days.

Large-scale fermentations were performed in 1 L of Bennett's per 2.8 L Fernbach flask with precursors at 0.15 g/L. Activated HP20 resin was added to each flask at 5% w/v, and the media were sterilized by autoclaving. The fermentations were inoculated at 3% v/v with second-stage seed and incubated at 28 °C and 200 rpm for 7 days.

Analysis of the Relative Efficiency of Uptake of 5-F-Tryptophan, 5-F-Anthranilate, 5-F-Indole, and 5-F-Isatin. Feeding experiments were set up as described above (10 mL of Bennett's per 50 mL Erlenmeyer flask, 0.15 g/L precursor, 5% HP20 resin v/v). Fermentations were conducted in triplicate for each precursor feeding and inoculated with 4% v/v second-stage seed. After incubation for 5 days at 28 °C and 200 rpm, two mL of each fermentation condition was sampled. The cells and resin were pelleted by centrifugation at 14 000 rpm. The supernatants were decanted, and the cell/resin pellets were extracted with methanol (1 mL × 2). The extracts were evaporated to dryness in vacuo and resolubilized in 200 μL of methanol (10-fold concentration). Standards of diazepinomicin (**1**) were made at 5, 20, and 50 μg/mL in methanol. The standards and extracts were analyzed by HPLC/UV/MS. A standard curve was created using the measurements of the UV peak areas for the diazepinomicin standards. Titers of diazepinomicin and fluorodiazepinomicin analogues were calculated for each fermentation extract according to the standard curve.

Extraction and Isolation. The harvested culture broth (1 L) was transferred into plastic bottles and centrifuged at 4000 rpm for 40 min. The aqueous supernatant was decanted, and the resin together with the cell mass was sonicated with methanol (250 mL × 3) in a room-temperature water bath and recentrifuged at 4000 rpm for 30 min. The combined methanol extracts were concentrated to dryness in vacuo.

The crude residue was dissolved in CH_3CN/H_2O (1:1, v/v) and chromatographed on a YMC-Pack-ODS-A column (250 × 20 mm, flow rate 10 mL/min, detection 320 nm) using a gradient of 50%−100% CH_3CN in H_2O (0.01% TFA) over 20 min to yield five fractions. Fraction 4 was further purified on a semipreparative C_{18}-HPLC column (YMC-Pack-ODS-A column, 5 μm, 250 × 10 mm, flow rate 2.5 mL/min, detection 254 and 230 nm, solvent gradient 50%−95% CH_3CN in H_2O over 10 min, then held for 10 min at 95% CH_3CN) to obtain purified 2-F-diazepinomicin (**2**).

2-F-Diazepinomicin (2): UV (MeOH/H_2O gradient with 0.025% formic acid, HP 1100 photodiode array detector) λ_{max} (relative absorption) 206 (1), 230 (0.5), 300 (0.3) nm; IR (film) ν_{max} 3680, 2972, 1055, 1033, 1012, 903, 726; ^{1}H and ^{13}C NMR data, see Table 2; HRESIMS *m/z* 481.24892 $[M + H]^+$ (calcd for $C_{28}H_{34}FN_2O_4^+$, 481.24972).

The crude residue was dissolved in MeOH and chromatographed on a SunFire-Waters-C18 column (10 μm, 250 × 19 mm, flow rate 10 mL/min, detection 220 and 280 nm) using a gradient of 50%−100% MeOH in H_2O over 20 min, then held for 5 min at 100% MeOH to yield 24 fractions. Fraction 13 was further purified on a semipreparative C18-HPLC column (YMC-Pack-ODS-A column, 5 μm, 250 × 10 mm, flow rate 2.0 mL/min, detection 254 and 230 nm, solvent gradient 60%−95% MeOH in H_2O over 10 min, then held for 10 min at 95% MeOH) to obtain purified 3-F-diazepinomicin (**3**).

3-F-Diazepinomicin (3): UV (MeOH/H_2O gradient with 0.025% formic acid, HP 1100 photodiode array detector) λ_{max} (relative absorption) 208 (1), 235 (0.5), 300 (0.2) nm; IR (film) ν_{max} 3680, 2980, 1055, 1033, 1012, 903, 723; ^{1}H and ^{13}C NMR data for the tricyclic core, see Figure 5; HRESIMS *m/z* 481.24972 $[M + H]^+$ (calcd for $C_{28}H_{34}FN_2O_4^+$, 481.24972).

Determination of Minimum Inhibitory Concentration. The minimum inhibitory concentration (MIC) was determined by the broth dilution method using Mueller-Hinton II agar (Baltimore Biological Laboratories) following the recommendations of the National Committee for Clinical Laboratory Standards. An inoculum level of 5 × 10^5 cfu/mL and a range of antibiotic concentrations (128−0.0625 μg/mL) were used. The MIC was determined after the microtiter plates were incubated for 18 h at 35 °C in an ambient air incubator. The test organisms were the following: *Staphylococcus aureus* 375 (ATCC3538p), *Staphylococcus aureus* 310 (SSC8224, methicillin-resistant), *Enterococcus faecium* 379 (NC12204, vancomycin-resistant), *Enterococcus faecium* 436 (ID3151), *Escherichia coli* 389 (GC4560 imp), *Escherichia coli* 442 (GC4559), *Candida albicans* 54 (CA300).

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Supporting Information Available: ^{1}H NMR spectra and selected 2D spectra of 2-F-diazepinomicin (**2**) and 3-F-diazepinomicin (**3**). This material is available free of charge via the Internet at http://pubs.acs.org.

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(6) A preliminary study was conducted with three different precursor concentrations (0.5, 0.15, 0.075 g/L) to determine the ideal feeding dosage of the precursors with the least amount of feedback inhibition.
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(11) Even though initial analytical scale (10 mL growth medium) experiments were conducted with 6-F-indole as the precursor, subsequently we discovered that a relatively higher production of 3-F-diazepinomicin (**3**) could be achieved in the presence of 6-F-tryptophan (**3a**). Therefore, scale-up fermentations were carried out with 6-F-tryptophan as the precursor.
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(13) The solubilization of the precursors was closely monitored visually. Heating and sonication were performed until no particulates remained in the medium. Efficient solubilization was confirmed by HPLC-UV-MS analysis of each sample at time zero (before inoculation).

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