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Discovery and Total Synthesis of Streptoaminals: Antimicrobial [5,5]-Spirohemiaminals from the Combined-Culture of *Streptomyces nigrescens* and *Tsukamurella pulmonis*

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Abstract: *A series of lipidic spirohemiaminals, designated streptoaminals, is reported. These were discovered by surveying the unique molecular signatures identified in the mass spectrometry data of the combined-culture broth of Streptomyces nigrescens HEK616 and Tsukamurella pulmonis TP-B0596. Mass spectrometry analysis showed that streptoaminals appeared as a cluster of ion peaks, which were separated by 14 mass unit intervals, implying the presence of alkyl chains of different lengths. The chemical structures of these compounds were elucidated by spectroscopic analysis and total synthesis. Streptoaminals with globular structures showed broad antimicrobial activities, whereas the planar structures of the 5-alkyl-1,2,3,4-tetrahydroquinolines found in the same combined-culture did not. This work shows the application of microbes as reservoirs for a range of chemical scaffolds.*

Natural product research has provided access to a wide range of novel chemical scaffolds with highly potent and selective biological activities.[1] Several natural product molecules have been used directly as medicines to treat various diseases, whilst others have been used as lead compounds in drug discovery or unique chemical tools for dissecting the molecular mechanisms underlying biological processes.[2] Disappointingly, however, the rate at which known metabolites are being rediscovered is currently increasing. Furthermore, the purification of the active substances responsible for biological effects of crude cell cultures or organism extracts can be time-consuming and labor-intensive. Several new approaches are currently being examined to overcome these issues and identify new scaffolds, including: 1) the isolation of new organisms with the aim of serendipitously discovering new chemical scaffolds;[3] 2) the whole-genome analysis of microbes to comprehensively catalogue their biosynthetic genes;[4] and 3) the introduction of deliberate challenges to chemical and biological elicitors with the aim of detecting cryptic metabolites.[5] Several effective approaches have also been developed for prioritizing metabolites with chemical and/or biological novelty, including: 1) the global analysis of biosynthetic gene clusters;[6] 2) the use of tandem mass spectrometry data to link microbial metabolites;[7] and 3) the integration of metabolomics data with phenotypic screening data.[8]

Actinomycetes contain dozens of biosynthetic gene clusters and are therefore considered to be one of the most promising sources of natural products, although a significant proportion of their gene clusters are transcriptionally silent.[4,9] It is noteworthy, however, that the co-culturing of actinomycetes and mycolic acid-containing bacteria, which we termed combined-culture, are a promising strategy for obtaining cryptic metabolites (that is, the products of silent genes).[10] We previously reported the isolation of a series of 5-alkyl-1,2,3,4-tetrahydroquinolines (5aTHQs) from a combined-culture of *Streptomyces nigrescens* HEK616 and *Tsukamurella pulmonis* TP-B0596 (Figure 1).[10f] Notably, these 5aTHQs were not detected in the culture broth of *S. nigrescens* or *T. pulmonis*, but were only produced when these two microbes were co-cultured. These metabolites consists of at least eight congeners based on the length of the alkyl chain substituted at position 5 of the 1,2,3,4-tetrahydroquinoline ring (Supporting Information, Figure S1). This structural feature gave 5aTHQs a unique molecular signature by LC-MS analysis, where the different compounds were observed as a cluster of ion peaks, which were separated by 14 mass unit intervals (Supporting Information, Figure S1). This finding let us examine the possibility that we could find out new metabolites by screening unique molecular signatures in LC-MS data. In the course of the screening, we identified a novel class of lipidic metabolites, which we subsequently designated streptoaminals. These compounds contained a spirohemiaminal moiety, which resulted in their concise three-dimensional structure. Herein, we report the isolation, structure elucidation, total synthesis, and biological evaluation of these streptoaminals.

The mycelium of the combined-culture of *S. nigrescens* HEK616 and *T. pulmonis* TP-B0596 was harvested, extracted, and concentrated to yield a crude extract, which was fractionated by silica gel column chromatography, followed

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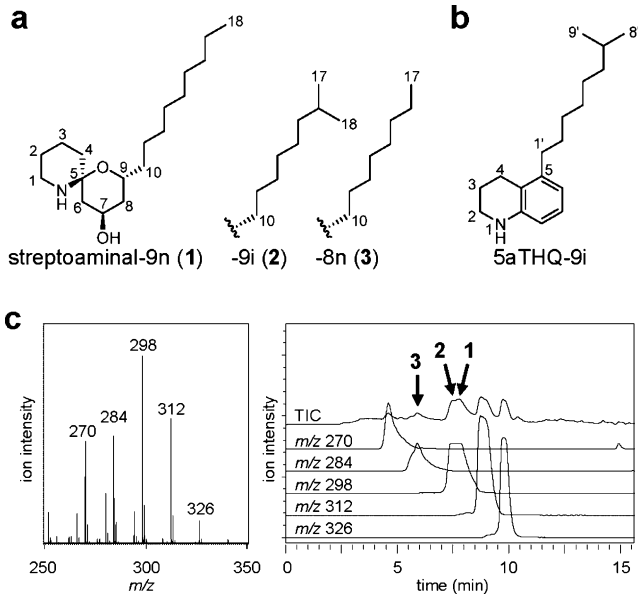

Figure 1. Chemical structures of streptoaminals and 5aTHQ-9i. a) Structures of streptoaminals-9n (**1**), −9i (**2**), and −8n (**3**). b) Structure of 5aTHQ-9i. c) A molecular signature in the mass spectrum and an LC-MS chromatogram of streptoaminals. Left: The mass spectrum of a fraction containing streptoaminals showed a cluster of ion peaks with 14 mass unit intervals. Right: LC-MS data. The total ion chromatogram (TIC) and mass chromatograms of streptoaminals (*m/z* 270, 284, 298, 312, and 326) are shown. The *m/z* values of metabolites (see arrows): **1** 298, **2** 298, **3** 284. A fraction containing streptoaminals **1**–**3** was analyzed by LC-MS on a COSMOSIL-5C_{18}-ARII column by gradient elution with 70–100% MeOH and 0.1% formic acid.

by LC-MS analysis. In this way, we identified a mixture of metabolites with mass ions in the range 270 to 326, which were separated by 14 mass unit intervals, suggesting that these metabolites contained an odd number of nitrogen atoms and had alkyl chains of different lengths (Supporting Information, Figure S1). The elution pattern of these congeners by LC-MS analysis was similar to that of the 5aTHQ congeners, which suggested that these new metabolites were chemically related to 5aTHQs (Figures 1; Supporting Information, Figure S1). It is noteworthy that these metabolites were produced in much larger quantities in the combined-culture system than they were in the presence of *S. nigrescens* alone (Supporting Information, Figure S1). Fractions containing streptoaminals were combined and further purified by column chromatography over silica gel, ODS and amino silica gel to afford a mixture of the metabolites containing different alkyl chains. Despite the broad and overlapping peak shapes obtained over the preparative HPLC columns, we successfully purified three congeners (**1**–**3**) by including TFA in the mobile phase.

The molecular formula of metabolite **1** was determined to be $C_{18}H_{34}NO_2$ by HR-ESI-MS (*m/z* 298.2731, calcd for 298.2741, $C_{18}H_{35}NO_2$ $[M+H]^+$). The ^1H, ^{13}C, DEPT, and HMQC NMR data for metabolite **1** revealed the presence of one CH_3, fourteen CH_2, and two CH (δ_C 63.9 and 67.8 ppm) groups, as well as one quaternary carbon (δ_C 86.9 ppm), all of which were determined to be sp^3-hybridized (Supporting Information, Table S1). Two degrees of unsaturation indicated that metabolite **1** has two ring systems. Although considerable overlap was observed between the proton signals in the upfield region of the ^1H NMR spectrum of metabolite **1** (δ_H 1.23–1.99 ppm), extensive analysis of its 2D NMR data, including COSY, TOCSY and HMQC-conjugated TOCSY spectra successfully revealed the presence of two spin systems, the first of which was H_2-1/H_2-2/H_2-3/H_2-4 (Figure 2). The downfield shift in the chemical shift values of CH_2-1 (δ_H 3.06, 3.13 ppm; δ_C 41.2 ppm) suggested that C-1 was attached to a nitrogen atom. HMBC analysis revealed correlations from H_2-1 and H_2-4 to C-5, which suggested the presence of a piperidine ring. We subsequently focused on the second spin system, H_2-6/H-7/H_2-8/H-9, where C-7 and C-9 were determined to be oxymethine carbons. HMBC analysis revealed correlations from H-7 and H_2-6 to C-5, as well as a correlation from H_2-6 to C-4, which suggested that C-4 and C-6 were connected through C-5. The chemical shift of the quaternary carbon C-5 (δ_C 86.9 ppm) implied the presence of a hemiaminal functionality, suggesting that C-9 was connected to C-5 though an oxygen atom to form 1-aza-9-oxaspiro-[5,5]undecan-7-ol. The remaining portion to be assigned was C_9H_{19}, which was deduced to be a nonyl group consisting of eight methylenes and one methyl group. COSY analysis revealed that this alkyl chain was connected to C-9. Taken together, these data suggested that metabolite **1** existed as the planar structure shown in Figure 1.

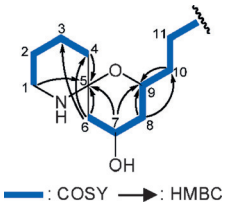

Figure 2. COSY and key HMBC correlations of the spirohemiaminal moiety of streptoaminal-9n (**1**).

The spirohemiaminal structure of metabolite **1** was shared by metabolites **2** and **3**, as evidenced by the similarities in their NMR spectra (Supporting Information, Table S1). Indeed, the only significant difference between the structures of metabolites **1**–**3** was the nature of the alkyl chain at their C-9 position. In a similar manner to 5aTHQs,[10f] metabolite **1** was designated as streptoaminal-9n, based on the molecular formula of its alkyl chain being C_9H_{19} with a normal-type terminal structure. Metabolite **2** had a C-9 alkyl chain with an *iso*-type branched methyl group, and was therefore designated as streptoaminal-9i. Metabolite **3** was designated as streptoaminal-8n because it had a normal-type octyl chain at its C-9 position. Although we were only able to purify three congeners in this study, LC-MS analysis of the crude extract suggested that there were several other streptoaminals with various alkyl chains, indicating the existence of a biosynthetic relationship to 5aTHQs (Supporting Information, Figure S1).

The relative stereochemistry of streptoaminal-9n (**1**) was deduced by the careful analysis of its NMR spectra and its chemical conversion to the corresponding isopropylidene derivative **4**. The conformation of the tetrahydropyran ring was initially analyzed by NOESY correlations and $^3J_{H/H}$ values (Figure 3a). A large coupling constant ($^3J_{H\text{-}8a/H\text{-}9}$ = 11.8 Hz) revealed that H-8a and H-9 were configured with an axial orientation. In contrast, the small coupling constants between H-7 and all of the vicinal protons (2.2–5.9 Hz)

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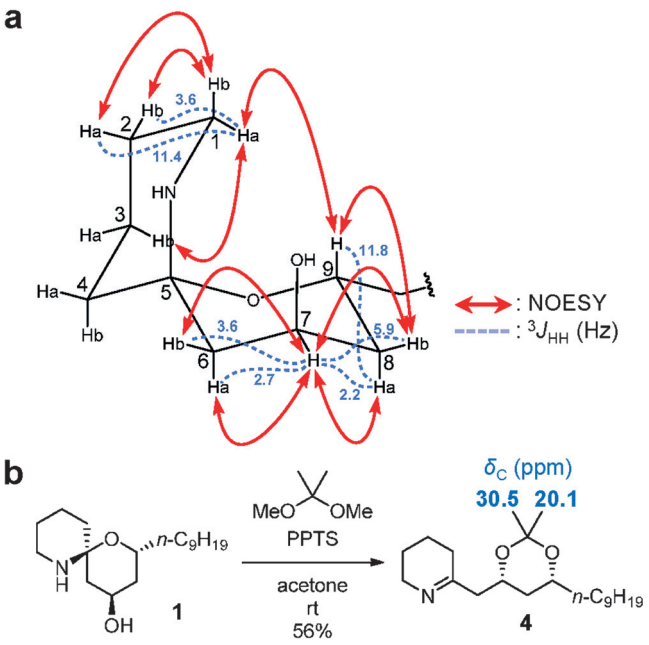
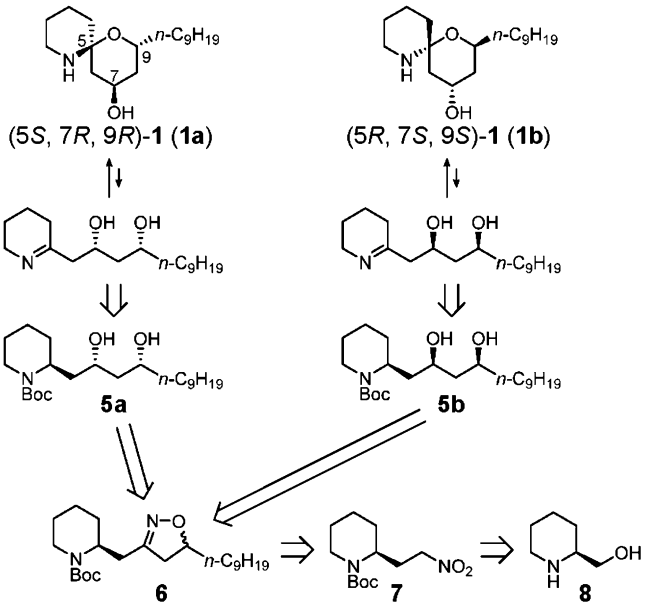


Figure 3. Relative configurations of streptoaminal-9n (**1**). a) Key NOESY correlations and $^3J_{H/H}$ values of streptoaminal-9n (**1**). b) The *syn* relationship of the 1,3-diol function was deduced by the characteristic ^{13}C NMR chemical shifts observed in the corresponding isopropylidene derivative **4**.



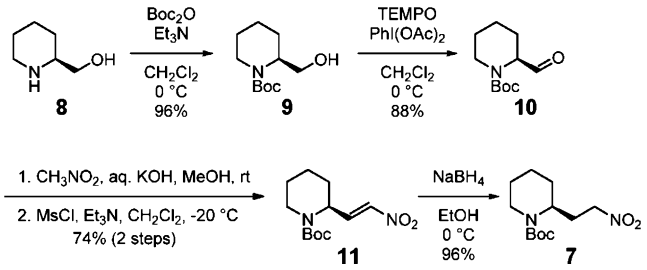
Scheme 1. Retrosynthetic analysis of streptoaminal-9n (**1**).

indicated that H-7 was equatorial. A correlation was also observed between H-1a and H-9 in the NOESY spectrum of **1**, which suggested that these protons were positioned on the same face of the tetrahydropyran ring. It is noteworthy that this configuration would require the nitrogen atom at C-5 to be placed in an axial position. Furthermore, this configuration would require H-1a to be placed in an axial position, which was confirmed by the large coupling constant observed between H-1a and H-2a, as well as the NOESY correlation between H-1a and H-3b.

We subsequently applied the isopropylidene method to confirm the relative stereochemistry of the 1,3-diol. Because hemiaminals exist in equilibrium, it was envisaged that the 1,3-diol moiety in the ring-opened imine form could be reacted with 2,2-dimethoxypropane under acidic conditions to give the corresponding isopropylidene derivative. As expected, the reaction of streptoaminal-9n (**1**) with 2,2-dimethoxypropane in the presence of PPTS gave the isopropylidene derivative **4** (Figure 3b). NMR analysis revealed a large difference between the chemical shifts of the two methyl carbons of the isopropylidene moiety (δ_C 30.5 and 20.1 ppm), indicating the *syn* relationship of the two hydroxy groups.[11] This result was consistent with the configuration deduced by NMR analysis (Figure 3a). Taken together, these data indicated that relative stereochemistry of streptoaminal-9n (**1**) was 5*S**, 7*R**, 9*R**.

Given that streptoaminals **1**–**3** were determined to be optically active, we conducted total synthesis of (+)-**1** and (−)-**1** to determine the absolute stereochemistry of these compounds (Scheme 1). It was envisaged that enantiomers **1a** and **1b** could be prepared from the corresponding imine tautomers, which could be synthesized from diastereomers **5a** and **5b**, respectively, via sequential *N*-chlorination and dehydrochlorination reactions. The *syn*-1,3-diols **5a** and **5b** could both be synthesized from isoxazoline **6** by the reductive cleavage of its N−O bond, followed by sequential hydrolysis and stereoselective reduction steps. A diastereomeric mixture of isoxazoline **6** could be prepared by an intermolecular cycloaddition reaction between the chiral nitro compound **7** and 1-undecene. Lastly, compound **7** could be prepared from the commercially available chiral amino alcohol **8**.

We initially synthesized intermediate **7** from (*S*)-(+)-2-hydroxymethylpiperidine (**8**; Scheme 2). Compound **8** was



Scheme 2. Synthesis of intermediate **7**.

Boc-protected under conventional conditions to give **9**, which was oxidized with TEMPO to yield aldehyde **10**.[12] Aldehyde **10** was then subjected to a Henry reaction,[13] followed by a dehydration step to give the nitrovinyl compound **11**. The subsequent reduction of the olefin group with $NaBH_4$ furnished **7** in a good yield.

Next, we synthesized the *syn*-1,3-diols **5a** and **5b** (Scheme 3). The nitro group in compound **7** was activated to a nitrile oxide and coupled with 1-undecene by a [3+2] cycloaddition to afford compound **6** as a mixture of diaste-

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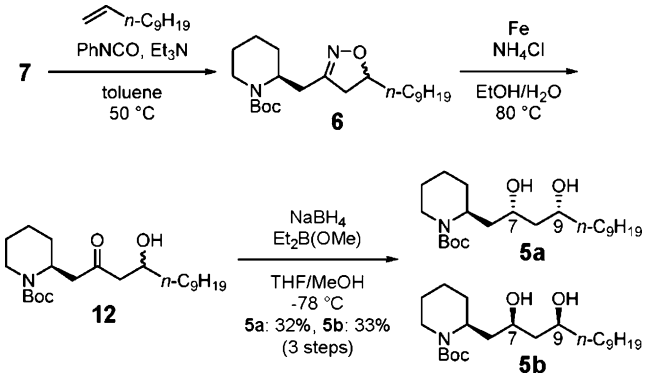
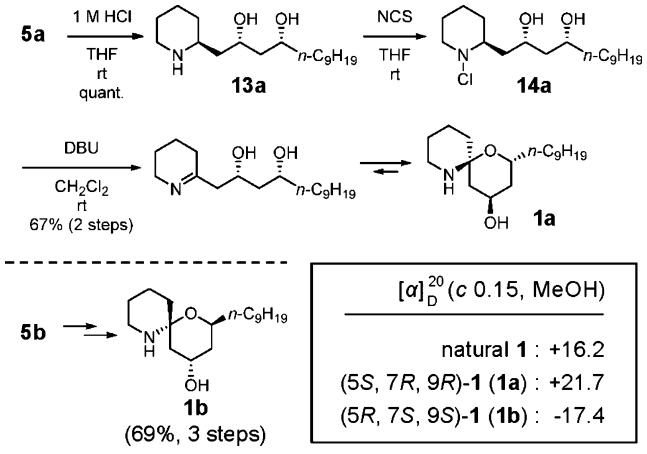


Scheme 3. Synthesis of diols **5a** and **5b**.

reomers.[14] The isoxazoline ring was opened by reducing the N–O bond,[15] followed by the hydrolysis of the resulting imine to yield a diastereomeric mixture of β-hydroxy ketones **12**. The ketone group in **12** was reduced using $NaBH_4$ in the presence of $Et_2B(OMe)$ to afford **5a** and **5b**, both of which contained a *syn*-1,3-diol moiety.[16] Diastereomers **5a** and **5b** were readily separated by silica gel column chromatography. The absolute configurations of the *syn*-diol groups in **5a** and **5b** were determined to be (7*S*,9*R*) and (7*R*,9*S*), respectively, based on the NMR analyses of their isopropylidene derivatives and bis-(*R*)- or (*S*)-MTPA esters (Supporting Information, Figure S2).[11a,17]

Finally, the synthesis of (+)-/(−)-streptoaminal-9n (**1a**/**1b**) was accomplished by the oxidation of the secondary amines in **5a** and **5b**, respectively (Scheme 4). The *N*-Boc



Scheme 4. Synthesis of (+)- and (−)-streptoaminal-9n (**1**).

group of diol **5a** was removed under acidic conditions and the resulting amine was chlorinated with *N*-chlorosuccimide to give **14a**. The dehydrochlorination of *N*-chloropiperidine **14a** under basic conditions afforded the cyclic imine, which tautomerized to give the hemiaminal form **1a**. Compound **1b** was synthesized from diol **5b** in the same manner. Compounds **1a** and **1b** were purified by preparative HPLC as the corresponding TFA salts under the same conditions as those used to isolate natural streptoaminal-9n (**1**). The physicochemical properties of compounds **1a** and **1b**, as well as their MS and NMR data, were consistent with those of the natural product, indicating that we had successfully synthesized (+)- and (−)-streptoaminal-9n from the common starting material **8** in 11 steps. The optical rotations of natural **1** and synthetic **1a** and **1b** were determined to be +16.2, +21.7, and −17.4, respectively (*c* 0.15, 20°C in MeOH). Taken together, these results indicated that the absolute configuration of natural streptoaminal-9n (**1**) was 5*S*, 7*R*, 9*R*. Streptoaminals-9i (**2**) and −8n (**3**) also gave positive optical rotations under the same conditions (+14.9 and +14.3, respectively), suggesting that the 5*S*, 7*R*, 9*R* stereochemistry was conserved in these compounds.

We examined the antibacterial activities of these streptoaminals against several bacteria, including *Bacillus subtilis*, *Staphylococcus aureus*, and *Escherichia coli* using a disk diffusion assay. The results of this assay revealed that naturally occurring **1** exhibited significant growth inhibition to all of these microbes (Table 1; Supporting Information, Figure S3). We also found that synthetic (+)- and (−)-streptoaminal-9n (**1a** and **1b**, respectively) showed antibacterial activities, being comparable to that of natural **1**. In contrast, 5aTHQ-9i did not show any antibacterial activity (Table 1; Supporting Information, Figure S3). We next checked the chemical genetic interactions between streptoaminals and fission yeast lipid mutant cells (Supporting Information, Figure S4). Yeast ergosterol mutants have been reported to be tolerant to membrane-targeting compounds, but they are often susceptible to bioactive compounds that have intracellular target molecules when compared to wild-type cells.[18] We reported that ergosterol mutants were significantly tolerant to 5aTHQs, suggesting that they might target cell membrane.[10f] On the other hand, ergosterol mutant cells were more sensitive to all streptoaminals (natural **1**–**3**, and synthetic (+)- and (−)-**1**) than wild-type cells, implying that streptoaminals have intracellular targets. These results therefore suggest that streptoaminals and 5aTHQs have different mechanisms of action against microbes.

Table 1: Antibacterial activity of natural and synthetic streptoaminal-9n, and 5aTHQ-9i.[a]

	B. subtilis[b]	*S. aureus*[b]	*E. coli*[c]
natural **1**	12	10	10
synthetic (+)-1 (**1a**)	12	10	10
synthetic (−)-1 (**1a**)	12	10	10
5aTHQ-9i	n.a.[d]	n.a.	n.a.

[a] Diameter of the inhibitory zone [mm] is shown. In the disc diffusion assay, 6 mm diameter paper disks were used. Three independent experiments were carried out, and the representative results are shown. [b] 30 μg of streptoaminals or 100 μg of 5aTHQ was loaded on the disc. [c] 100 μg of the compound was loaded on the disc. [d] Not active. Halo was not observed.

In summary, we have discovered and fully characterized a series of antimicrobial streptoaminals, which possess a [5,5]-spirohemiaminal ring system. These streptoaminals were

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detected as novel lipidic metabolites in the combined-culture broth of *S. nigrescens* and *T. pulmonis* by LC-MS analysis, where they were separated by 14 mass unit intervals. The serendipitous discovery of new chemical scaffolds from unique natural resources could be accelerated by utilizing their characteristic molecular signatures, such as their ion peak patterns. Spirohemiaminal is rarely found in natural products, but have been shown to be useful for the construction of complex chemical scaffolds (Supporting Information, Figure S5).[19] Notably, spirohemiaminal is the only function to construct the bulky 3D architecture of streptoaminals, which might contribute to the broad spectrum antimicrobial activity. In contrast, the planar 5aTHQs show no antibacterial activity. The molecular mechanisms underlying the biological activities of streptoaminals and the relationship between the biosynthetic pathways responsible for the synthesis of streptoaminals and 5aTHQs are currently under investigation.

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