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5-Alkyl-1,2,3,4-tetrahydroquinolines, New Membrane-Interacting Lipophilic Metabolites Produced by Combined Culture of *Streptomyces nigrescens* and *Tsukamurella pulmonis*

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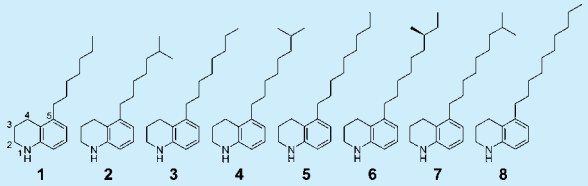
Supporting Information

ABSTRACT: Eight novel 5-alkyl-1,2,3,4-tetrahydroquinolines (5aTHQs) bearing different side chains have been isolated from a combined culture of *Streptomyces nigrescens* HEK616 and *Tsukamurella pulmonis* TP-B0596. The chemical structures including the absolute configuration were elucidated by spectroscopic analysis and total synthesis. 5aTHQs inhibited the growth of wild-type fission yeast while only weakly inhibiting the growth of several mutant strains synthesizing premature ergosterol. These results demonstrate that 5aTHQs are novel antifungals that may target cell membranes.



Membrane lipids are the major structural components of the cell membrane, where they are involved in numerous dynamic interactions with membrane proteins to orchestrate biological events, including signal transduction and cell polarity processes.[1,2] However, the roles played by lipids in these biological events are poorly understood because of their huge level of chemical diversity and the lack of suitable analytical tools.[3] A few small molecules have been used as research tools for dissecting the biological functions and the subcellular localization of membrane lipids.[4−6] Small molecules of this type can also be used as lead compounds in drug discovery; in fact, several membrane lipids have been targeted by antifungal and antibacterial drugs.[7,8] During the course of our screening program to identify novel membrane-targeting natural products, we isolated 5-alkyl-1,2,3,4-tetrahydroquinolines (5aTHQs), representing a novel series of THQ alkaloids, from the combined-culture of *Streptomyces nigrescens* HEK616 and *Tsukamurella pulmonis* TP-B0596 (Figure 1). Here, we report the isolation, structure determination, synthesis, and biological evaluation of these 5aTHQs.

Yeast mutant cells lacking ergosterol biosynthetic genes are known to be tolerant to lipid-targeting antifungals such as amphotericin B and syringomycin E.[4,5,9,10] Microbial culture extracts were screened using fission yeast wild-type and mutant cells, and it was found that the extract of a combined-culture broth of *S. nigrescens* HEK616 and *T. pulmonis* TP-B0596 showed less toxicity toward *erg2*Δ and *erg31*Δ *erg32*Δ mutant cells than it did toward the wild-type cells (Figure S1, Supporting Information). To isolate the constituents responsible for this selective antiyeast activity, the mycelium of this combined culture were harvested and extracted with a 1:1 (v/v) mixture of $CHCl_3$ and MeOH. The extract was concentrated to give a residue that was partitioned between *n*-hexane and 90% MeOH. Subsequent bioassay-guided fractionation of the active *n*-hexane layer using a combination of silica gel and ODS column chromatography afforded a mixture of lipophilic metabolites. Although it was not possible to separate all of these metabolites by HPLC over a conventional ODS column, it was possible to purify eight metabolites (**1**−**8**) using a reversed-phase HPLC column that had been functionalized with cholesterol (Figures 1b and Figure S2, Supporting Information).

We previously showed that production of metabolites in most strains of *Streptomyces* could be altered by coculturing the *Streptomyces* strains with mycolic acid-containing bacteria such as *T. pulmonis*, and we went on to describe this method as a "combined culture".[11] It is noteworthy that metabolites **1**−**8** were not detected in pure cultures of *S. nigrescens* HEK616 and *T. pulmonis* TP-B0596 (Figure 1b and Figure S3, Supporting Information). In contrast, the production of these metabolites was also observed when *S. nigrescens* HEK616 was cocultured with *Corynebacterium glutamicum*, which is another mycolic acid containing bacterium, although the metabolic effect of this bacterium was less potent than that of *T. pulmonis* (Figure S3, Supporting Information). These results therefore indicated that *S. nigrescens* HEK616 is responsible for the production of the metabolites.

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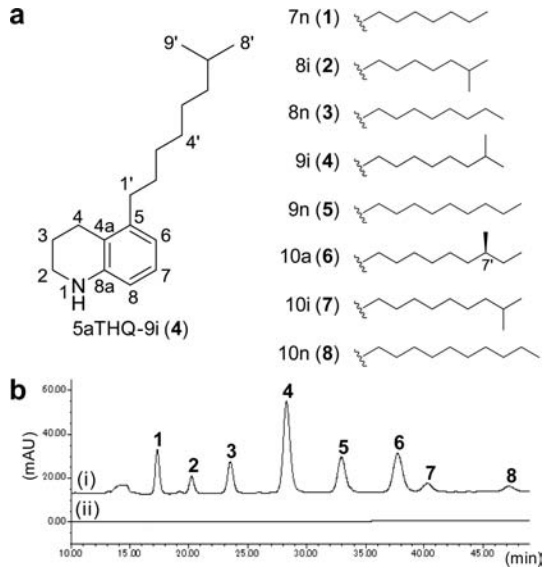


Figure 1. Structures and HPLC profiles of 5aTHQs. (a) Structures of the most abundant metabolite 5aTHQ-9i (**4**) and the alkyl chains of metabolites **1**−**8**. (b) HPLC chromatograms of the extract of (i) the combined-culture broth of *S. nigrescens* HEK616 and *T. pulmonis* TP-B0596 and (ii) the pure culture broth of *S. nigrescens* HEK616. Fractions containing 5aTHQs (**1**−**8**) were analyzed on a COSMOSIL Cholester column by isocratic elution of 90% MeOH. Chromatograms acquired at 220 nm are shown.

The LC−MS analysis revealed that the molecular weights of metabolites **1**−**8** were in the range of 231−273 with 14 mass unit intervals, which suggested that these molecules contained an alkyl chain. The most abundant metabolite was determined to be 5aTHQ-9i (**4**), which had a molecular formula of $C_{18}H_{29}N$, as determined by HR-ESI-MS (*m*/*z* 260.2374, calcd for 260.2373, $C_{18}H_{30}N$ $[M + H]^+$). The ^1H and ^{13}C NMR data of metabolite **4**, coupled with DEPT and HMQC experiments, revealed the presence of two equivalent CH_3, nine CH_2, four CH including three sp^2 carbons, and three quaternary sp^2 carbons (Table S1, Supporting Information). The COSY spectrum revealed the presence of an aromatic spin system H-6/H-7/H-8, which was found to be positioned on a 1,2,3-trisubstituted phenyl ring by HMBC analysis (Figure 2). The downfield shift of the chemical

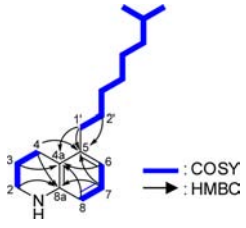


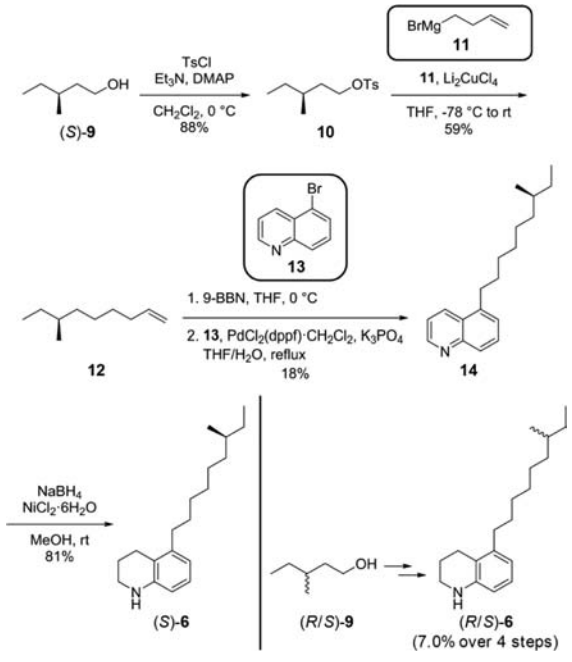
Figure 2. COSY and key HMBC correlations for 5aTHQ-9i (**4**).

shift value of C-8a suggested that it was attached to a nitrogen atom. This nitrogen was conjugated to the other spin system H_2-2/H_2-3/H_2-4, judging from the chemical shift value of C-2 and an HMBC correlation from H_2-2 to C-8a. HMBC correlations from H_2-3 to C-4a, and from H_2-4 to C-5 and C-8a attached C-4 to C-4a, which allowed for the construction of the structure of the 1,2,3,4-tetrahydroquinoline (THQ) ring (Figure 2). Finally, HMBC correlations from H_2-1′ to C-5 and C-4a, and from H_2-2′ to C-5 revealed that an alkyl chain was positioned at C-5 of the THQ ring. The detection of two equivalent methyl groups indicated that the terminal structure of the alkyl chain was of the *iso*-type, and the structure of metabolite **4** was therefore designated as 5aTHQ-9i (i.e., *THQ* with a *5*-substituted *a*lkyl chain; the molecular formula of the alkyl chain is C_9H_{19} with *i*so-type terminal structure).

The 5aTHQ structure was found to be a common feature of metabolites **1**−**8** (Figure 1a). The alkyl chain at the C-5 position of each metabolite varied in terms of the number of carbon atoms and the branching pattern of the methyl groups. The number of carbons found in the side chains of metabolites **1**−**8** was in the range of 7−10, and the terminal structure of the side chains was normal- (without branched methyl), iso-, or anteiso-type, which could be easily determined by HR-ESI-MS and ^1H NMR analyses. The different compounds were given names according to these variations. For example, metabolite **5**, which had a C9 alkyl chain without a branched methyl group, was defined as 5aTHQ-9n, where the use of "n" refers to the normal-type branching. Metabolites **1**−**3** were also named in the same way as 5aTHQ-7n, 5aTHQ-8i and 5aTHQ-8n, respectively. Metabolites **6** (10a), **7** (10i), and **8** (10n) had a C10 alkyl chain with anteiso-, iso-, and normal-type terminal structures, respectively (Figure 1a).

5aTHQ-10a (**6**) was the only congener that contained a stereogenic center at C-7′. To elucidate the absolute configuration of 5aTHQ-10a (**6**), we synthesized racemic **6** as well as its (*S*)-enantiomer of **6** using a *B*-alkyl Suzuki−Miyaura coupling (Scheme 1).[12,13] Branched chiral alkene **12** was

Scheme 1. Synthesis of Racemic 5aTHQ-10a (6) and Its (*S*)-Enantiomer



prepared from commercially available (*S*)-3-methyl-1-pentanol (**9**) in two steps as previously reported.[14] Alkene **12** was reacted with 9-BBN to give an alkylborane, which was subjected to a Pd-catalyzed coupling reaction with 5-bromoquinoline (**13**) to yield alkylquinoline **14**. Finally, the quinoline ring in **14** was partially reduced with $NaBH_4$ in the presence of $NiCl_2$ to complete the synthesis of (*S*)-5aTHQ-10a (**6**) in 7.5% yield over four steps from (*S*)-3-methyl-1-pentanol (**9**). Racemic **6** was prepared in

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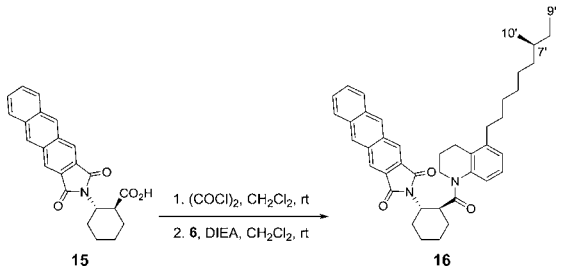
the same manner from racemic 3-methyl-1-pentanol (**9**). The NMR and MS data of the (*S*)-enantiomer and racemic **6** prepared in the current study were identical to those of the natural material, thus confirming the planar structure of metabolite **6**. The optical rotation of natural **6** was +6.7 (*c* 0.50, 20 °C in MeOH), which was comparable to that of synthetic (*S*)-**6** (+6.9 under the same conditions). This result indicated that the absolute configuration of natural **6** was *S*.

The Ohrui–Akasaka method was applied to natural and synthetic **6** to further confirm the absolute stereochemistry of the material. The Ohrui–Akasaka method has been used to discriminate the chirality of linear primary alcohols with an anteiso-type terminal structure following their condensation with (1*S*,2*S*)-2-(anthracene-2,3-dicarboximido)cyclohexane-carboxylic acid (**15**). This bulky chiral acid generates a strong anisotropic effect, which can be used to distinguish between a pair of diastereomers by HPLC or NMR analysis (Scheme 2).[15−17] Chiral-labeling carboxylic acid **15** was reacted with

Scheme 2. Condensation of Natural or Synthesized 5aTHQ-10a (6) with the Chiral-Labeling Carboxylic Acid 15



oxalyl chloride to give the corresponding acid chloride, which was reacted with synthesized racemic **6** to yield a mixture of two diastereomers (**16a**) (Scheme 2). The synthetic (*S*)-enantiomer and natural **6** were also subjected to the same amidation reaction to give the corresponding amides **16b** and **16c**, respectively. ^{1}H NMR analysis of amides **16a−c** at room temperature resulted in a series of broad peaks (Figure S4, Supporting Information). However, the signal of H_3-9′ protons in amide **16a** appeared as two sets of triplets (δ_H 0.829 and 0.837 ppm with a 1:1 ratio) in CD_3OD at −20 °C, which allowed for the differentiation of the 7′*R* and 7′*S* diastereomers (Figure 3). In contrast, only one of these triplet signals (δ_H 0.837 ppm) was observed in the spectra of amides **16b** and **16c** (Figure 3). On the basis of these results, the absolute configuration of natural 5aTHQ-10a (**6**) was concluded to be 7′*S*. To date, the Ohrui–Akasaka method has

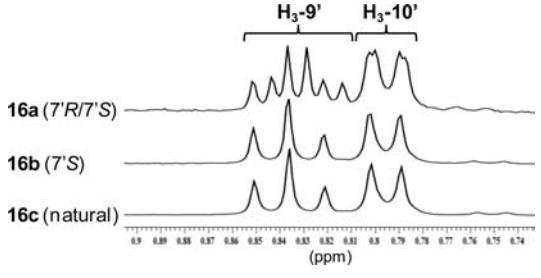


Figure 3. Application of the Ohrui–Akasaka method to 5aTHQ-10a (**6**). ^{1}H NMR signals of the two methyl groups, H_3-9′ and H_3-10′, in amide **16** are shown. Compounds **16a−c** were derived from synthesized racemic **6**, synthesized (*S*)-**6**, and natural **6**, respectively. The NMR spectra were measured in CD_3OD at −20 °C.

been successfully used to determine the configuration of challenging stereogenic centers in carboxylic acids,[18] aldehydes,[19,20] and alcohols.[21−24] To the best of our knowledge, however, the work described in the current study represents the first reported application of the Ohrui–Akasaka method to determine the configuration of a chiral amine.

Growth of the wild-type fission yeast cells was found to be moderately inhibited by 5aTHQ-9i (**4**). In contrast, mutant cells lacking genes involved in ergosterol biosynthesis (i.e., *erg2*Δ, *erg31*Δ *erg32*Δ, *sts1/erg4*Δ, and *erg5*Δ cells) were tolerant to this metabolite, with cell growth being unaffected even in the presence of 200 μM of 5aTHQ-9i (**4**) (Figure 4a). In an

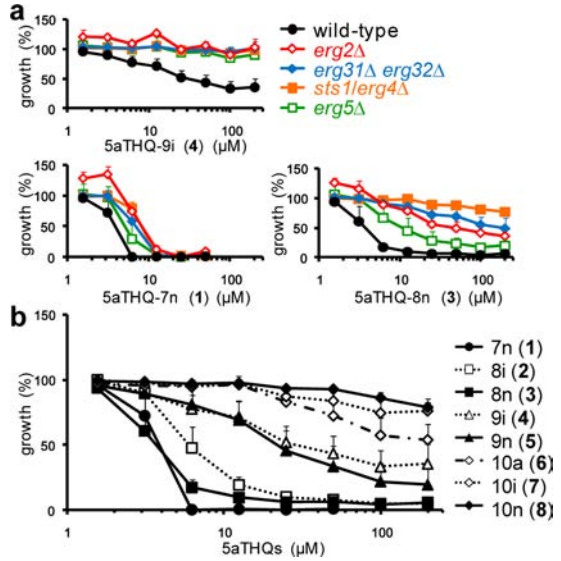


Figure 4. Growth inhibitory activities of 5aTHQs against fission yeast cells. (a) The effects of 5aTHQ-9i (**4**), 5aTHQ-7n (**1**), and 5aTHQ-8n (**3**) were examined against the wild-type (black), *erg2*Δ (red), *erg31*Δ *erg32*Δ (blue), *sts1/erg4*Δ (orange), and *erg5*Δ (green) cells. (b) SAR of the 5aTHQ compounds. The effects against the wild-type cells are shown. Cells were treated with compounds **1−8** for 24 h. Data represent the mean values of three independent experiments. Error bars indicate the SD.

empirical sense, these chemical genetic interactions suggested that 5aTHQ-9i (**4**) could be physically interacting with the cell membrane. Antifungals that target membrane lipids have been shown to be less effective toward yeast *erg* mutant cells, although the molecular mechanisms linking these chemical genetic and physical interactions largely remain to be clarified.[4,5,9,10] In contrast, the efficacy of small molecules that target intracellular proteins is often higher in *erg* mutant cells.[10] The structure–activity relationships (SARs) of the eight natural 5aTHQs (**1−8**) were evaluated in terms of their inhibitory activity toward the growth of wild-type fission yeast cells. The inhibitory activity of the different compounds was found to be dependent on the number of carbon atoms in the alkyl chain. Among congeners, 5aTHQ-7n (**1**) exhibited the most potent growth inhibition with an MIC value of 6.3 μM while the MIC value of amphotericin B was 0.25 μM (Figure S5, Supporting Information). The activity decreased as the number of carbon atoms in the side chain increased (Figures 4b). Metabolites **6−8** all had C10 alkyl chains and showed very weak inhibitory activities. However, these compounds exhibited significant differences in terms of their selectivity profiles. For example, the *erg* mutant cells were more tolerant toward 5aTHQ-9i (**4**) than they were toward 5aTHQ-

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7n (**1**) (Figure 4a). Furthermore, 5aTHQ-8i (**2**) and 5aTHQ-8n (**3**) were less potent but more selective than the smaller congener 5aTHQ-7n (**1**) and more potent but less selective than the larger congener 5aTHQ-9i (**4**) (Figure 4 and Figure S5, Supporting Information). We previously reported the occurrence of a similar tendency in heronamide C and its congener, polyene macrolactams that target membrane lipids: heronamide C potently inhibited growth of both wild-type and *erg* mutant cells, while 8-deoxyheronamide C, a less potent congener, inhibited growth of wild-type cells but showed apparently lower effect to *erg* mutant cells.[5] This fact implied that these 5aTHQ compounds could also be targeting membrane lipids. Interestingly, the addition of a methyl group at the ω-2 position had a significant impact on the potency and the selectivity, e.g., compare 5aTHQ-7n (**1**) with 5aTHQ-8i (**2**), 5aTHQ-8n (**3**) with 5aTHQ-9i (**4**), or 5aTHQ-9n (**5**) with 5aTHQ-10i (**7**) (Figure S5, Supporting Information). The molecular processes underlying these chemical genetic interactions are of considerable interest, and further research is therefore required to develop a deeper understanding of the driving forces behind these interactions.

The 1,2,3,4-THQ structure can be found in several natural products, as well as a wide range of pharmacologically important reagents.[25−27] Several natural products belonging to this structural class have been isolated from a variety of different sources, including actinomycetes, fungi, plants and several other organisms. Among them, 5aTHQs represent one of the simplest structures. Angustureine and several related metabolites are another group of simple THQ compounds isolated from plants where the substitution is at C-2 rather than C-5 both with and without *N*-methylation.[28,29]

In conclusion, we have isolated a series of novel 5aTHQ alkaloids from the combined culture of *S. nigrescens* HEK616 and *T. pulmonis* TP-B0596. These 5aTHQ compounds exhibited inhibitory activity toward the growth of yeast cells, most likely by targeting the membrane lipids. The length and methylation pattern of the side chain at the C5-position of these compounds had a critical effect on their potency and selectivity toward the growth of yeast cells. These 5aTHQ compounds could be useful chemical tools for studying the structure and function of cell membranes. Further SAR study would be helpful for the mode of action study and drug development. The biosynthetic mechanisms and physiological functions of 5aTHQs in the producing organism are also of considerable interest because these metabolites were only produced under coculture conditions. Research aimed at addressing these issues is currently underway in our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

Detailed experimental procedures, compound characterization data, supplementary figures and tables, and copies of the NMR spectra of compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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