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Ktedonoketone and 2'-oxosattabacin, benzenoid metabolites from a thermophilic bacterium *Thermosporothrix hazakensis* in the phylum *Chloroflexi*

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Abstract

A thermophilic bacterium *Thermosporothrix hazakensis* NBRC 105916 which belongs to the class *Ktedonobacteria* was investigated to explore its biosynthetic potential of secondary metabolites. UV-guided fractionation led to the identification of a new benzenoid metabolite designated ktedonoketone (**6**) and an α -diketone metabolite 2'-oxosattabacin (**7**) along with five known compounds. Compound **7** was previously described as a synthetic compound, but this is the first finding as a natural product. Compound **7** induced adipocyte differentiation at 10–20 μ M and autophagy at 1–10 μ M. Compound **6** showed weak inducing activity of adipocyte differentiation. The biosynthetic origin of hazakacin (**3**), an acyloin-type compound, was elucidated by ¹³C-labeled precursor-feeding experiments.

Introduction

After the intensive screening efforts on common mesophilic microorganisms, extreme environments such as deserts and deep sea are drawing attention as a remaining niche for unexplored or underexplored microbes for drug discovery [1, 2]. *Ktedonobacter racemifer* was first isolated from soil in Italy. It was Gram-positive and displayed morphological

similarity to actinomycetes, developing a multicellular filamentous structure with branched mycelia and grape-like spores [3]. However, its 16S rRNA gene sequence was nearest to the species in the phylum *Chloroflexi* (78–79% similarity to *Caldilinea*, *Roseiflexus*, and *Sphaerobacter*), phylogenetically apart from actinomycetes. Accordingly, a new taxon, class *Ktedonobacteria* was created in this phylum, currently including two additional genera *Thermosporothrix* and *Thermogemmatispora* [4, 5]. Except the first identified *K. racemifer* that grows around 30 °C, other *Ktedonobacteria* species display thermophilic characteristic with a preference to higher temperature ranging from 50 to 65 °C for optimal growth [6]. In addition to its actinomycete-like morphology, another notable feature of *Ktedonobacteria* is its relatively large genome size: the genome sizes are 13.7 Mbp for *K. racemifer* SOSP1-21^T, 7.3 Mbp for *T. hazakensis* SK20-1^T, and 5.6 Mbp for *Thermogemmatispora carboxidivorans* PM5^T [7–9]. Analysis of the genome sequence of *K. racemifer* SOSP1-21^T suggested that more than 200 genes are likely involved in secondary metabolism [7]. Meanwhile, only five metabolites are known from the class *Ktedonobacteria*: an unusual branched fatty acid (**1**) from *Thermogemmatispora* [10], and two acyloins, sattabacin (**2**) and hazakacin (**3**) [8], and two thiazole derivatives (**4** and **5**) from *T. hazakensis* (Fig. 1) [11].

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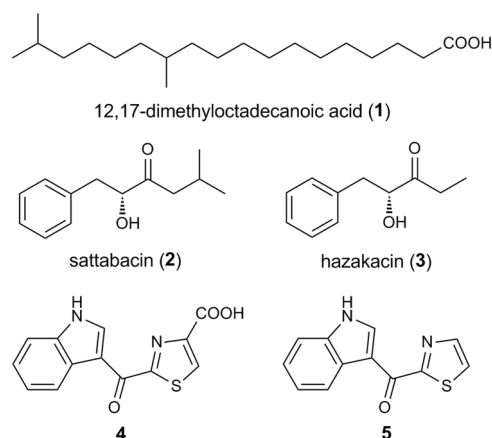


Fig. 1 Secondary metabolites identified from *Ktedonobacteria*

As a part of our continuing study on metabolic capability of thermophilic bacteria [12, 13], secondary metabolite production by *T. hazakensis* NBRC 105916 was investigated. In this paper, we report the isolation of ktedonoketone (6) and 2'-oxosattabacin (7) along with several known benzenoid metabolites (2, 3, 4, 8–11, Figs. 1, 2). Biosynthetic origin of an acyloin-type compound hazakacin (3) was also studied by ^{13}C -labeled precursor-feeding experiments.

Results and discussion

The producing strain *T. hazakensis* NBRC 105916 was precultured in the V22 medium at 50 °C and then cultured in the A-11M production medium at 50 °C for 6 days. The whole-culture broth was extracted with 1-butanol, and the concentrated extract was fractionated by silica gel and ODS column chromatographies. Final purification was achieved by preparative HPLC to give ktedonoketone (6, 2.9 mg) and 2'-oxosattabacin (7, 3.2 mg) from 6 l of main culture.

Ktedonoketone (6) was obtained as an optically active yellow oil. HRESITOFMS analysis gave a molecular formula of $\text{C}_{14}\text{H}_{16}\text{O}_2$, which was consistent with the ^1H and ^{13}C NMR data. The IR spectrum displayed absorption bands for hydroxy and carbonyl groups (3378 and 1703 cm^{-1}). Analysis of ^{13}C NMR and HSQC spectral data confirmed the presence of 11 carbons assignable to two downfield sp^2 carbons (δ_{C} 210.2 and 172.3), seven sp^2 carbons (five are proton-bearing), one oxygenated sp^3 carbon, two sp^3 methylenes, and two methyl carbons (Table 1). The ^1H NMR spectrum showed a typical resonance pattern for a phenyl group at δ_{H} 7.26 (2H, d), 7.37 (2H, t), and 7.32 (1H, t). The sp^2 carbon at δ_{C} 133.0 (C-6) was placed in this aromatic ring by HMBC correlations from the protons at δ_{H} 7.37 (H8 and H10) to this carbon. In addition, two methyl proton signals at δ_{H} 0.94 (3H, t) and 2.20 (3H, s), geminal methylene proton

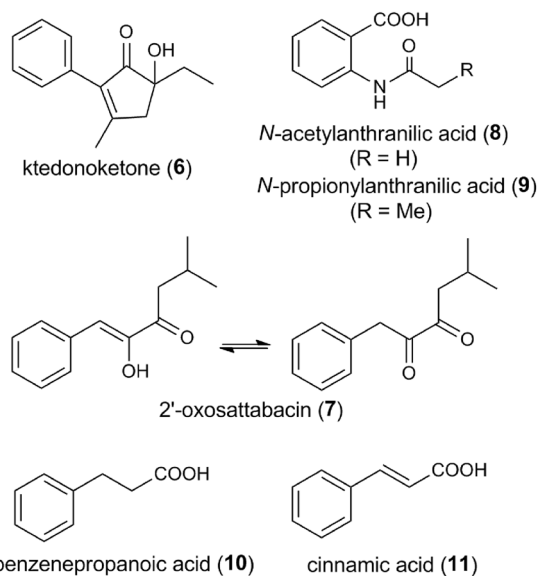


Fig. 2 Metabolites isolated from *Thermosporothrix hazakensis* NBRC 105916

Table 1 ^1H and ^{13}C NMR data for Ktedonoketone (6) in CD_3OD

position	$\delta_{\text{C}}^{\text{a}}$	δ_{H} mult (J in Hz) ^b	HMBC ^{b,c}
1	210.3, C		
2	139.1, C		
3	172.4, C		
4	46.7, CH_2	2.86, d (19.0) 2.66, d (19.0)	1, 2, 3, 5, 13 2, 3, 5
5	78.6, C		
6	133.1, C		
7, 11	130.3, CH	7.26, d (7.5)	2, 8 (10), 9
8, 10	129.4, CH	7.37, t (7.5)	6, 9
9	128.9, CH	7.32, t (7.5)	7 (11)
12	18.3, CH_3	2.20, s	2, 3, 4
13	31.7, CH_2	1.74, dd (13.5, 7.5) 1.67, dd (13.5, 7.5)	1, 4, 5, 14 1, 4, 5, 14
14	8.4, CH_3	0.94, t (7.5)	5, 13

^aRecorded at 100 MHz (reference δ_{C} 49.2)

^bRecorded at 500 MHz (reference δ_{H} 3.30)

^cHMBC correlations are from proton(s) stated to the indicated carbon

signals at δ_{H} 2.66 (1H, d) and 2.86 (1H, d), and another methylene proton signals at δ_{H} 1.73 (2H, m) were observed. COSY correlation of the triplet methyl (H14) to a multiplet methylene (H13) gave an ethyl group, which was in turn connected to C5 by HMBC correlations from H13 and H14 to this carbon. Connectivity for C6/C2/C3/C4 was established by HMBC correlations from a singlet methyl H12 to C2 (δ_{C} 139.0), C3 (δ_{C} 172.3) and C4, and H7 and H11 to C2. Finally, HMBC correlations from geminally coupled methylene protons H4 to C5, C1 (δ_{C} 210.2), and C2 allowed

the assembly of a cyclopentenone core. A hydroxy group was placed at C5 in consideration of the carbon chemical shift and the molecular formula to complete the planar structure of **6** (Fig. 3). Further exploration of the absolute configuration was hampered by the limited availability of the material and low reproducibility of production.

2'-Oxosattabacin (**7**) was obtained as a pale yellow amorphous solid. The high-resolution ESITOFMS analysis gave an $[M-H]^-$ ion peak at m/z 203.1081, appropriate for a molecular formula of $C_{13}H_{16}O_2$ (Δ -0.3 mmu, calcd for $C_{13}H_{15}O_2$). The ^{13}C NMR spectrum showed only ten carbon signals, suggesting the presence of symmetrical structure(s) in this molecule. In the 1H NMR spectrum, proton signals for a doublet methyl (δ_H 1.01, 6H), a methine (δ_H 2.27, 1H), and a methylene (δ_H 2.71, 2H) group were observed, and COSY-correlations connected these protons to give an isobutyl group (Fig. 4). Aromatic protons at δ_H 7.39 (1H), 7.40 (2H), and 7.84 (2H) were assigned to a phenyl group along with the carbons (δ_C 127.5, 128.6, and 130.2) directly bonding to these protons and an sp^2 carbon (δ_C 134.2). The remaining proton signal at δ_H 6.45 was directly connected to a carbon at δ_C 113.2. These three fragments and two downfield oxygenated sp^2 carbons (δ_C 197.6 and 147.1) were assembled into a single structure on the basis of HMBC correlations. A long-range correlation from the methylene H4' to C3' established the connectivity between C3' and C4'. HMBC correlations from the olefinic proton H1' to C2, C2', and C3' confirmed the carbon connectivity from C1 to C3', thereby establishing the α,β -unsaturated ketone substituted by an isobutyl and a phenyl group. Finally, HMBC correlations from an exchangeable proton at δ_H 7.38 to C1', C2', and C3' established the enol functionality, completing the structure of an enol form of **7** (Fig. 4). 1H and ^{13}C NMR resonances and 2D NMR cross-peaks derived from the keto-form of **7** were also observed (Table 2, Figs. SI6–SI10). The ratio of two tautomeric isomers was ~4:1 in $CDCl_3$. We next analyzed the configuration of the enol double bond. A NOESY correlation observed between H1' and H4' was suggestive of *Z*-configuration for C1'–C2' double bond. In addition, the $^3J_{CH}$ coupling constant determined by the J-HMBC spectrum of **7** was 4.1 Hz for a long-range coupling between H1' and C3', but this value was equivocal to undoubtedly assign the geometrical isomer (see Fig. S20 for typical examples of $^3J_{CH}$ values of a set of *E*- and *Z*-olefins). We thus synthesized two models (*Z*)-**12** and (*E*)-**12**, in which the double-bond geometry was fixed as a methyl vinyl ether to compare the $^3J_{CH}$ values of both *E*- and *Z*-isomers (Scheme 1).

To obtain (*Z*)-**12** and (*E*)-**12** with defined double-bond geometry, we utilized the well-established *syn*-elimination of selenoxides (Scheme 1). The synthesis of (*Z*)-**12** began with the *syn*-selective Evans aldol reaction of known oxazolidinone **13** [14, 15] and benzaldehyde to give an

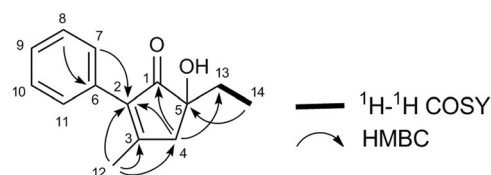


Fig. 3 1H - 1H COSY and key HMBC correlations for **6**

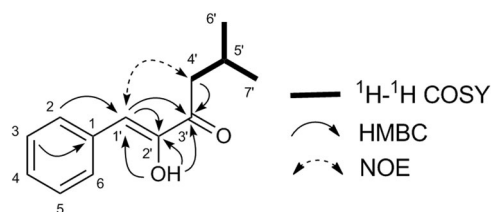


Fig. 4 1H - 1H COSY and key HMBC correlations for **7**

Table 2 1H and ^{13}C NMR data for 2'-Oxosattabacin (**7**) in $CDCl_3$

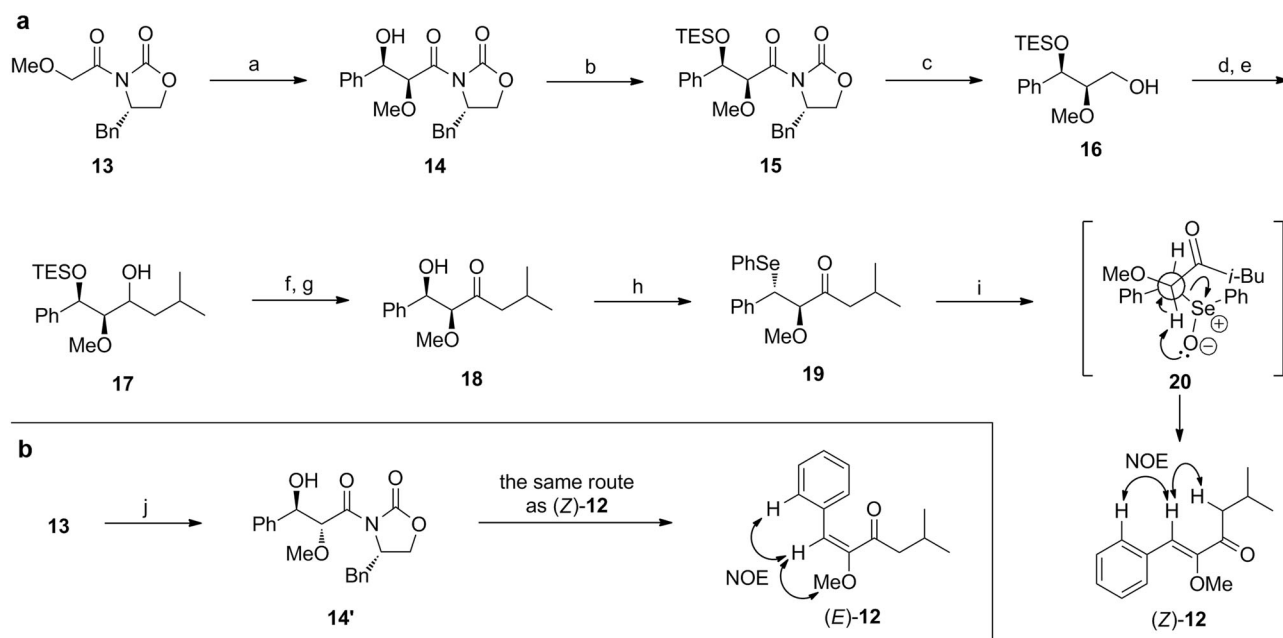
position	δ_C^a , type	δ_H mult (J in Hz) ^b	HMBC ^{b,c}
1	enol 134.2, C keto 132.3, C		
2, 6	enol 130.2, CH keto 129.8, CH	7.84, d (8.0) 7.98, d (8.0)	3 (5), 4, 1'
3, 5	128.6, CH	7.40, m	1
4	127.5, CH	7.39, m	
1'	enol 113.2, CH keto 42.8, CH ₂	6.45, s 4.04, s	2 (6), 2', 3' 1, 2 (6), 2'
2'	enol 147.1, C keto 196.5, C		
3'	enol 197.6, C keto 199.7, C		
4'	enol 43.9, CH ₂ keto 45.0, CH ₂	2.71, d (7.0) 2.58, d (7.0)	3', 5', 6' (7') 3', 5', 6' (7')
5'	enol 26.9, CH keto 24.3, CH	2.27, m 2.05, m	4', 6' (7')
6', 7'	enol 22.46, CH ₃ keto 22.37, CH ₃	1.01, d (6.7) 0.86, d (6.7)	4', 5' 4', 5'
2'-OH	enol	7.38, s	1', 2', 3'

^aRecorded at 125 MHz (reference δ_C 77.0)

^bRecorded at 500 MHz (reference δ_H 7.27)

^cHMBC correlations are from proton(s) stated to the indicated carbon

inseparable 3.6:1 mixture of **14** and its diastereomers. Silylation of the mixture afforded **15** and the corresponding diastereomers, the latter of which were removed by SiO_2 column chromatography. The oxazolidinone **15** was converted straightforwardly into **17** in three steps (reduction to form **16**, its oxidation to an aldehyde, and the Grignard reaction). Oxidation of **17** [16] followed by TES deprotection afforded **18**, which was treated with PhSeH under



Scheme 1 Synthesis of model compounds (*Z*- and (*E*)-12. Reagents and conditions: (a) *i*-Pr₂NEt, PhCHO, *n*-Bu₂BOTf, CH₂Cl₂ -78 to 0 °C, **14**/other diastereomers = 3.6:1; (b) TESOTf, 2,6-lutidine, CH₂Cl₂, 0 °C to rt, 36% from **13**; (c) LiBH₄, Et₂O, MeOH, 0 °C to rt, 91%.; (d) TEMPO, KBr, aq. NaOCl, NaHCO₃, CH₂Cl₂ 0 °C;

(e) isobutylmagnesium chloride, THF, 0 °C to rt, dr = 20:1; (f) AZADOL, KBr, aq. NaOCl, NaHCO₃; (g) TBAF/AcOH, THF, 0 °C to rt, 49% from **16**; (h) PhSeH, ADPP, *n*-Bu₃P, THF, rt; (i) aq. H₂O₂, NaHCO₃, THF, 0 °C, 54% from **18**; (j) MgCl₂, Et₃N, PhCHO, TMSCl, THF, rt, then TFA, MeOH, rt, **14**'/other diastereomers = 1.8:1

the Mitsunobu conditions [17] to give stereochemically inverted selenide **19** as a single diastereomer. Treatment of **19** with H₂O₂ followed by concomitant *syn*-elimination of the resulting selenoxide **20** provided (*Z*)-**12** in a geometrically pure state. The other geometrical isomer (*E*)-**12** was obtained by following the same sequence of reactions as employed for the synthesis of (*Z*)-**12**, except that the aldol reaction in the first step was conducted *anti*-selectively to afford **14'** [18]. The geometrical configurations of (*Z*)-**12** and (*E*)-**12** were corroborated by NOE correlations depicted in Scheme 1. As shown in Fig. 5, the ³J_{CH} coupling constant between H7 and C9 was 3.1 Hz for (*Z*)-**12** and 7.3 Hz for (*E*)-**12**, which strongly supported the *Z*-configuration for the enol form of **7** (³J_{CH}, 4.1 Hz).

Compounds **6** and **7** were subjected to several biological testings. Preadipocyte differentiation into adipocytes is considered beneficial to insulin resistance and type 2 diabetes because matured adipocytes have an ability to secrete adiponectin, which promotes glucose uptake and improves insulin-sensitivity in type 2 diabetic patients [19]. Inducing potential of **6** and **7** toward murine ST-13 preadipocyte differentiation was thus evaluated at the concentrations of 10–40 μM by measuring the lipid droplet accumulation [20, 21]. **6** was weakly active (50% differentiation) at 40 μM, whereas **7** induced 69 and 85% differentiation at 10 and 20 μM, respectively. In addition, **6** and **7** were subjected to autophagy inducing activity assay. Autophagy is the

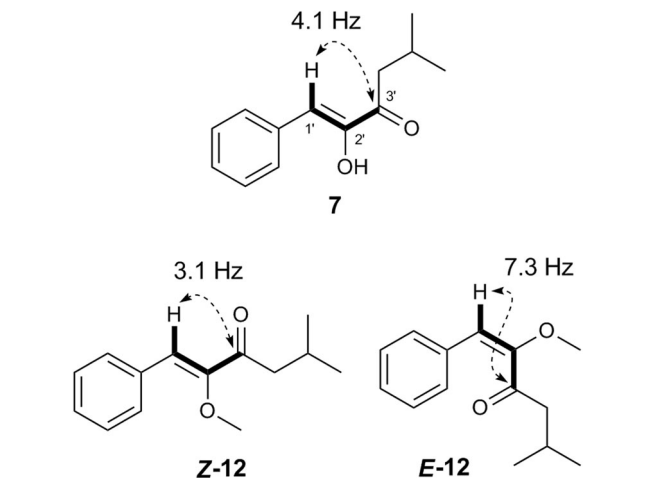


Fig. 5 Determination of double-bond geometry of **7** based on the ³J_{CH} coupling

cellular mechanism that eliminates unnecessary or malfunctioning proteins by peptide degradation. Since impairment of autophagy is considered as a cause of an onset or development of cancer, neurodegenerative and inflammatory diseases, autophagy inducers are expected to prevent or treat these kinds of illnesses [22]. Autophagy inducing activity of **6** and **7** was assessed by using PC12 cells that express mCherry-GFP-LC3 tandem fluorescent construct (tf-LC3) [23]. According to the fluorescence imaging

experiments, **7** significantly increased red puncta area at 1–10 μM after 24 h, indicating the activation of autophagy flux (Fig. 6). **6** was inactive in this assay. Both **6** and **7** showed no cytotoxicity against melanoma A375 cells at 10 μM and no appreciable activity in antimicrobial assay against *Escherichia coli*, *Kocuria rhizophila*, and *Candida albicans*.

In addition to **6** and **7**, seven more benzenoid metabolites were isolated from the culture extract of strain NBRC 105916: sattabacin (**2**) [24], hazakacin (**3**) [9], 2-(1'*H*-indole-3'-carbonyl)-thiazole-4-carboxylic acid (**4**) [11], 3-phenylpropanoic acid (**5**), *N*-acetylthranilic acid (**8**), *N*-propionylthranilic acid (**9**), benzenepropanoic acid (**10**), and cinnamic acid (**11**) (Figs. 1, 2). Compounds **8** and **9** were previously found from a thermophilic bacterium *Laceyella sacchari* as its major metabolites in our laboratory [12]. Isolation of **2** and **3** from *T. hazakensis* SK20-1^T was reported by Kuzuyama et al. [9]. They characterized an enzyme responsible for the synthesis of these acyloins. The enzyme catalyzed a carbon–carbon bond formation between two α -ketoacids, which coupled phenylpyruvic acid and 2-oxobutyric acid to yield an α -hydroxy- β -keto acid, a hypothetical intermediate of **3** (Fig. 7). α -Keto acids are known to be formed from the corresponding amino acids by oxidative deamination and the following hydrolysis: for example, L-phenylalanine is a precursor amino acid of phenylpyruvic acid, whereas ethylglycine, a plausible precursor amino acid of 2-ketobutyric acid, is quite uncommon in nature. To investigate the origin of 2-ketobutyric acid, incorporation of plausible ¹³C-labeled amino acid precursors was examined by feeding experiments. As expected, L-[4-¹³C]phenylalanine and [4-¹³C, 3-*d*₂]-2-ketobutyric acid were incorporated into **3**, labeling C1 and C5', respectively (Fig. 8, Table 3). Then, we tested the feeding of labeled threonine because 2-oxobutyric acid is generated from L-threonine as an intermediate for isoleucine biosynthesis (Fig. 7) [25]. Actually, the feeding of L-[1,2,3,4-¹³C]threonine gave the labeling of C3', C4', and C5' with high efficiency of incorporation (Fig. 7, Table 3). To the best of our knowledge, this is the first example of utilization of 2-oxobutyric acid as a building block of secondary metabolites.

In conclusion, our chemical investigation on *T. hazakensis* NBRC 105916 led to the discovery of two structurally unique benzenoid-type secondary metabolites. Cyclopentenone, the core structure of ktedonoketone (**6**), is widely distributed in nature but those that have a phenyl substitution at the α -position is limited: only two examples, alternarienoic acid [26] and sydowins [27] are known both from fungi. 2'-Oxasattabacin (**7**) is a dehydro-congener of sattabacin (**2**) and is likely produced by the oxidation of **2**. **7** is known as a product of chemical synthesis [28] or enzymatic reaction [29], but this is the first finding as a natural

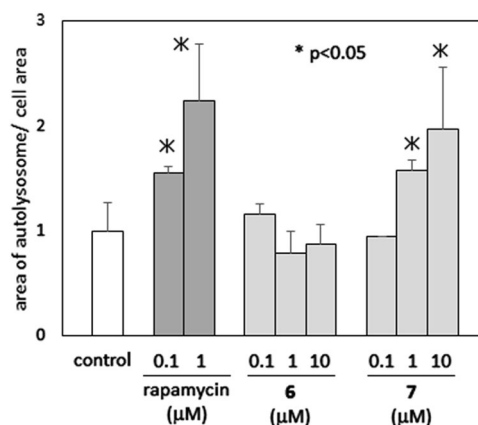


Fig. 6 Autophagy inducing activity of **6** and **7**

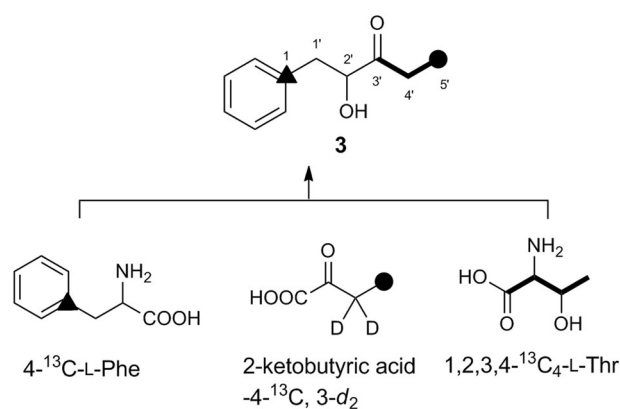


Fig. 7 Biosynthesis of hazakacin (**3**) in *Thermosporothrix hazakensis*

product. Many of the compounds characterized in this study are originated from amino acids, featuring the unique metabolism developed in the genus *Thermosporothrix*.

Experimental section

General experimental procedures

Optical rotation was measured using a JASCO DIP-3000 polarimeter. UV spectra were recorded on a Hitachi U-3210 spectrophotometer. IR spectra were measured on a Perkin Elmer Spectrum 100. NMR spectra were obtained on a Bruker AVANCE 400 or a Bruker AVANCE 500 spectrometer. HRESITOFMS were recorded on a Bruker micro-TOF focus. Cosmosil 75C18-PREP (Nakalai Tesque, Inc., 75 μm) was used for ODS column chromatography.

Microorganism

T. hazakensis NBRC 105916 was purchased from Biological Resource Center, National Institute of Technology and

Fig. 8 Incorporation of ^{13}C -labeled precursors into hazakacin (**3**)

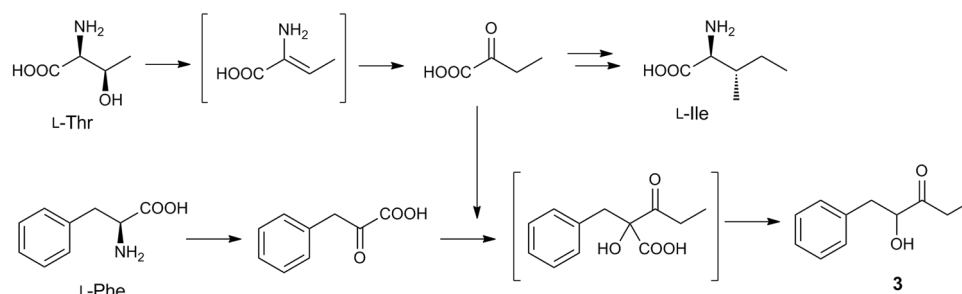


Table 3 Incorporation of ^{13}C -labeled precursors into hazakacin (**3**)

Relative enrichment				
position	$\delta_{\text{C}}^{\text{a}}$	4- ^{13}C -L-Phe	$^{13}\text{C}_4$ -L-Thr	4- ^{13}C -3,3- d_2 -2-ketobutyric acid
1	139.1	8.36	0.44	0.29
2, 6	130.7	1.37	1.68	1.46
3, 5	129.4	1.96	1.65	1.52
4	127.6	0.95	0.44	0.85
1'	41.3	1.00	1.00	1.00
2'	79.0	1.02	4.01	0.96
3'	215.4	0.36	29.2	0.24
4'	32.9	1.01	31.8	0.88
5'	7.7	0.93	32.4	27.7

^aRecorded in CD_3OD at 100 MHz (reference δ_{C} 49.2)

Evaluation, Chiba, Japan. The strain was maintained on a Bn-2 agar medium at 50 °C and transferred to a fresh medium every 2 weeks. Well-grown culture was also preserved at 4 °C.

Fermentation

T. hazakensis NBRC 105916 cultured on a Bn-2 medium consisting of soluble starch 0.5%, glucose 0.5%, meat extract (Kyokuto Pharmaceutical Industrial Co., Ltd) 0.1%, yeast extract (Difco Laboratories) 0.1%, NZ-case (Wako Chemicals USA, Inc.) 0.2%, NaCl 0.2%, CaCO_3 0.1%, and agar 1.5% (pH 7.0) at 50 °C was inoculated into 500-ml Erlenmeyer flasks with baffles each containing 100 ml of the seed medium consisting of soluble starch 1.0%, glucose 0.5%, NZ-case 0.3%, yeast extract 0.2%, Tryptone (Difco Laboratories) 0.5%, K_2HPO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%, and CaCO_3 0.3% (pH 7.0). The flasks were placed on a rotary shaker (130 rpm) at 50 °C for 4 days. The seed culture (20 ml) was transferred into 2-l Erlenmeyer flasks with baffles each containing 500 ml of the A-11M production medium consisting of glucose 0.5%, glycerol 2.0%, soluble starch 2.0%, Pharmamedia (Traders Protein) 1.5%, yeast extract 0.3%, and Diaion HP-20 (Mitsubishi Chemical Co.) 1.0%. The pH of the medium was adjusted to 7.0 before

sterilization. The inoculated flasks were placed on a rotary shaker (60 rpm) at 50 °C for 6 days.

Extraction and isolation

At the end of the fermentation period, 500 ml of 1-butanol were added to each flask, and the flasks were allowed to shake for 1 h. The mixture was centrifuged at 4000 rpm for 8 min, and the organic layer was separated from the aqueous layer containing the mycelium. Evaporation of the solvent gave ~5.67 g of extract from 6 l of culture. The crude extract was subjected to silica gel column chromatography with a step gradient of $\text{CHCl}_3/\text{MeOH}$ (1:0, 20:1, 10:1, 4:1, 2:1, 1:1, and 0:1 v/v). Fraction 3 (10:1) was concentrated to provide 1.45 g of brown oil, which was further purified by ODS column chromatography with a gradient of $\text{MeCN}/0.1\% \text{HCO}_2\text{H}$ solution (2:8, 3:7, 4:6, 5:5, 6:4, 7:3, and 8:2 v/v), and MeOH . Fractions 3 (4:6), 4 (5:5), and 5 (6:4) were evaporated separately, and the resultant aqueous solution was extracted with EtOAc . After drying with anhydrous Na_2SO_4 , the EtOAc layer was concentrated to give brown oil (Fr. 3: 134 mg; Fr. 4: 148 mg; Fr. 5: 254 mg). Fr. 4 was subjected to HPLC purification (Nakalai Tesque, Cosmosil C18-AR-II, 20 × 250 mm, 15 ml min^{-1} , UV detection at 210 nm) with $\text{MeCN}/0.1\% \text{HCO}_2\text{H}$ (20:80), followed by evaporation and extraction with EtOAc , to give benzenepropanoic acid (**10**, 6.5 mg, t_{R} 18.7 min), hazakacin (**3**, 37.0 mg, t_{R} 21.5 min), and ktedonoketone (**6**, 2.9 mg, t_{R} 27.3 min). HPLC purification from Fr. 5 using the same column condition with $\text{MeCN}/0.1\% \text{HCO}_2\text{H}$ (42:58) followed by evaporation and extraction with EtOAc gave satabacin (**2**, 105.9 mg, t_{R} 24.7 min). *N*-Acetylanthranilic acid (**8**, 1.3 mg, t_{R} 13.1 min) and *N*-propionylanthranilic acid (**9**, 4.3 mg, t_{R} 24.4 min) were obtained from Fr. 3 by HPLC purification (Nakalai Tesque, Cosmosil C18-AR-II, 10 × 250 mm, 4 ml min^{-1} , UV detection at 210 nm) with $\text{MeCN}/0.1\% \text{HCO}_2\text{H}$ (20:80), followed by evaporation and extraction with EtOAc . The silica gel fraction 2 (20:1) was evaporated to give 31.4 mg of yellow oil, which was then fractionated by LH-20 gel filtration (CH_2Cl_2 - MeOH = 1:1) to yield 2'-oxosatabacin (**7**, 3.2 mg). Silica gel column fractions Fr. 6 (1:1) and Fr. 7 (0:1) were combined and

concentrated *in vacuo*. The residue (1.90 g) was fractionated by ODS column chromatography in a similar manner as described above. Fraction 4 (5:5) was concentrated and extracted with EtOAc to give a brown solid (25 mg) which was purified by HPLC (Nakalai Tesque, Cosmosil C18-AR-II, 10 × 250 mm, 4 ml min⁻¹, UV detection at 254 nm) with MeCN/0.1% HCO₂H solution (33:67), followed by evaporation and extraction with EtOAc to give 2-(1'*H*-indole-3'-carbonyl)-thiazole-4-carboxylic acid (**4**, 4.3 mg, *t*_R 17.1 min).

Ktedonoketone (6): pale yellow oil; [α]_D²³ + 12 (*c* 0.04, CHCl₃); IR ν_{\max} 3378, 1703 cm⁻¹; UV (MeOH) λ_{\max} (log ϵ) 202 (4.06), 223 (3.73), 300 (2.99); For ¹H and ¹³C NMR data, see Table 1; HRESITOFMS [M+Na]⁺ 239.1043 (calcd for C₁₄H₁₆O₂Na, 239.1044).

2'-Oxosattabacin (7): pale yellow amorphous; IR ν_{\max} 3375, 1666, 1634 cm⁻¹; UV (MeOH) λ_{\max} (log ϵ) 233 (3.29), 309 (3.87); For ¹H and ¹³C NMR data, see Table 2; HRESITOFMS [M-H]⁻ 203.1081 (calcd for C₁₃H₁₅O₂, 203.1078).

Feeding experiment

L-[1,2,3,4-¹³C]Threonine (Sigma-Aldrich), L-[4-¹³C]phenylalanine (Cambridge Isotope Laboratories, Inc.), and [4-¹³C, 3,3-*d*₂]-2-ketobutyric acid sodium salt (Cambridge Isotope Laboratories, Inc.) were used for feeding experiments. In all cases, ¹³C-enriched carbon sources were dissolved in distilled water (4 mg ml⁻¹). Each solution was sterilized by filtration through 0.2- μ m filter (Minisart), and 5 ml was fed to 500 ml of growing culture in a 2-l Erlenmeyer flask of *T. hazakensis* NBRC 105916. Feeding of each labeled precursor (20 mg per flask) was commenced 2 days after inoculation and continued four times at 24-h intervals. After a total of 144-h (6 days) incubation, the culture broth was extracted and ¹³C-labeled hazakacin (**3**) was isolated as described in the Extraction and isolation section.

Adipocyte differentiation assay

The assay was carried out according to the reported procedure [20, 21]. In brief, mouse-derived ST-13 preadipocytes were plated at 1 × 10⁴ cells/ml in 24-well dishes containing 1 ml of a basal medium containing DME/F-12, 10% FBS, 100 units/ml penicillin, and 100 μ g ml⁻¹ streptomycin on day 1, and cultured at 37 °C in a humidified atmosphere containing 5% CO₂. Moreover, the fresh basal medium containing the indicated concentrations of test compounds (or 20 nM rosiglitazone (positive control)) was replaced on day 2, 5, and 8. On day 12, the ST-13 cells were washed three times with phosphate-buffered saline, fixed with 10% formalin neutral buffer solution at room temperature for 10 min, and then washed with distilled water to

remove formalin solution. Furthermore, the cells were rinsed with 60% isopropanol for 5 min, stained with 0.24 % oil red O at room temperature for 20 min, and then were photographed under a phase contrast microscope (×100 magnification) equipped a CCD camera (Leica Microsystems Japan, Tokyo, Japan).

Autophagy inducing assay

The assay was carried out according to the reported procedure [30]. In brief, autophagy induction was evaluated by fluorescence imaging with tandem fluorescent-tagged LC3 (mCherry-EGFP-LC3B, tf-LC3). Inducible tf-LC3 expressing clones was generated by transfection of pTRE2hyg/tf-LC3 plasmid vector to PC12/tet-on cells (631137, TaKaRa, Shiga, Japan). After 72 h differentiation with 100 ng ml⁻¹ of NGF, PC12/tet-on/tf-LC3 cells were co-treated with 1 μ g ml⁻¹ of doxycycline (D9891, Sigma-Aldrich, St. Louis, MO, USA) and the indicated concentrations of test compounds. After 24 h, the cells were fixed with 3% paraformaldehyde in PBS for 15 min. Fluorescence imaging was performed with a confocal laser scanning microscope system (Fluoview FV1000, Olympus, Tokyo, Japan) equipped with a ×60 oil objective lens and an inverted microscope (IX81, Olympus). To quantify autophagy induction, mCherry-positive-GFP-negative puncta area was calculated using ImageJ software (<http://rsb.info.nih.gov/ij/>).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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