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Note

Bulbimidazoles A−C, Antimicrobial and Cytotoxic Alkanoyl Imidazoles from a Marine Gammaproteobacterium *Microbulbifer* Species

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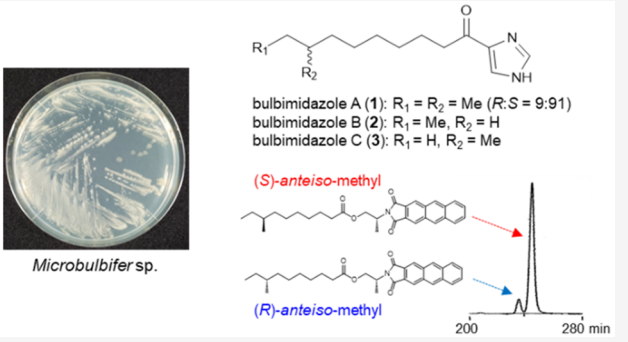
ABSTRACT: Three new alkanoyl imidazoles, designated bulbimidazoles A−C (**1**−**3**), were found from the culture extract of the gammaproteobacterium *Microbulbifer* sp. DC3-6 isolated from a stony coral of the genus *Tubastraea*. The absolute configuration of the *anteiso*-methyl substitution in **1** was established to be a mixture of (*R*)- and (*S*)-configurations in a ratio of 9:91 by applying the Ohrui−Akasaka method. Compounds **1**−**3** displayed unique broad-spectrum antimicrobial activity against Gram-positive and -negative bacteria and fungi with MICs ranging from 0.78 to 12.5 μg/mL. They also exhibited cytotoxicity toward P388 murine leukemia cells with IC_{50} in the micromolar range.



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Marine bacteria associating with marine invertebrates are regarded as a promising yet underexplored source of new chemical entities. Compared to the marine animals, the number of new compounds from microorganisms associated with or symbiotic to the host invertebrates is still small.[1] However, marine animal-associated bacteria are attracting attention because of the accumulating evidence that bacteria are the true producers of a number of marine natural products.[2] One of the striking examples of marine natural products isolated from both invertebrates and bacteria is the anticancer cyclic depsipeptide didemnin B. It was first isolated from a tunicate[3] and was reisolated from a cultured *Tistrella* bacterium collected from a sediment sample.[4]

Besides sponges and tunicates, corals are another rich source of natural products.[5] Natural products obtained from corals have unique structures and potent biological activities, while coral-associated microorganisms are underexplored in natural product discovery.[6] Bacteria of the genus *Microbulbifer* are marine obligate, Gram-negative, strictly aerobic, and rod-shaped, belong to the class *Gammaproteobacteria*,[7] and are commonly found in marine habitats such as salt marshes, intertidal sediments, solar salterns, marine sediments, and marine algae.[8] According to the latest genomic data, some *Microbulbifer* species possess biosynthetic genes for the production of nonribosomal peptides and siderophores,[9] but there are merely two reports to date regarding the compounds produced by this genus: benzoate derivatives from a sediment-derived strain[10] and an unsaturated fatty acid from a coral-derived strain.[11]

In the course of our continuing search for new bioactive compounds with therapeutic potential from marine microbes, HPLC peaks displaying UV absorption maximum around 254 nm were detected in a culture extract of a strain of the genus *Microbulbifer* isolated from a scleractinian (stony) coral, *Tubastraea* sp., collected off the south shore of central Japan. As the UV spectrum was not present in our in-house UV database, HPLC/UV-guided purification was conducted, and three new alkanoyl imidazoles designated bulbimidazoles A−C (**1**−**3**) were identified. These compounds are the deamino congeners of nocarimidazoles A (**4**) and B (**5**) previously isolated from a marine actinomycete of the genus *Nocardiopsis*.[12] Herein, we report the isolation, structure determination, and biological activities of **1**, **2**, and **3**.

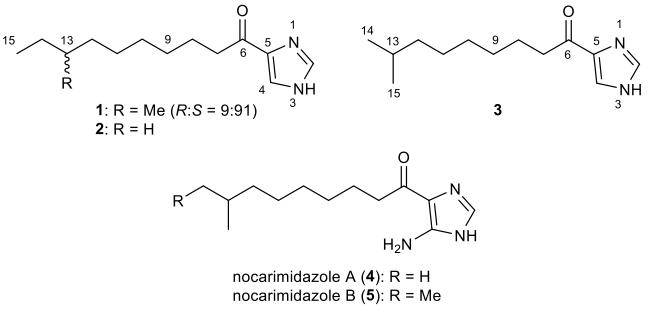

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Table 1. ^{1}H and ^{13}C Spectroscopic Data for Bulbimidazoles A−C (1−3) in DMSO-d_6 with TFA

	1			2		3	
no.	δ_C[a]	δ_H mult (J in Hz)[b]	HMBC[b,c]	δ_C[a]	δ_H mult (J in Hz)[b]	δ_C[a]	δ_H mult (J in Hz)[b]
2	137.6, CH	9.15, d (0.6)	4, 5, 6	137.5, CH	9.24, d (0.6)	137.5, CH	9.20, d (0.6)
4	125.2, CH	8.55, d (0.6)	2, 5, 6	125.1, CH	8.61, d (0.6)	125.1, CH	8.57, d (0.6)
5	131.8, C			131.5, C		131.5, C	
6	190.6, C			190.5, C		190.5, C	
7	38.6, CH_2	2.89, t (7.3)	5, 6, 8, 9	38.5, CH_2	2.89, t (7.3)	38.5, CH_2	2.89, t (7.3)
8	23.5, CH_2	1.59, quint (7.3)	6, 7, 9, 10	23.5, CH_2	1.60, quint (7.2)	23.5, CH_2	1.60, quint (7.3)
9	28.6, CH_2	1.29, m	8, 10	28.5, CH_2	1.22−1.30[e]	28.5, CH_2	1.28, m
10	29.2, CH_2	1.23, m		28.7,[d] CH_2	1.22−1.30[e]	29.0, CH_2	1.24, m
11	26.4, CH_2	1.22, m	13	28.8,[d] CH_2	1.22−1.30[e]	26.7, CH_2	1.24, m
12	36.0, CH_2	1.05, m; 1.24, m	11, 13	28.9,[d] CH_2	1.22−1.30[e]	38.4, CH_2	1.11, m
13	33.8, CH	1.26, m	12, 15, 16	31.3, CH_2	1.22, m	27.4, CH	1.47, sept (6.7)
14	29.0, CH_2	1.09, m; 1.27, m	12, 13, 15, 16	22.1, CH_2	1.23, m	22.5, CH_3	0.82, d (6.7)
15	11.3, CH_3	0.80, t (7.3)	13, 14	14.0, CH_3	0.83, t (6.9)	22.5, CH_3	0.82, d (6.7)
16	19.1, CH_3	0.79, d (6.7)	12, 13, 14				

[a]Recorded at 125 MHz (reference δ_C 39.5). [b]Recorded at 500 MHz (reference δ_H 2.49). [c]HMBC correlations are from proton(s) stated to the indicated carbon. [d]Assignment may be interchanged. [e]Overlapping signals.

The producing strain, coded DC3-6, was cultured in A11M seawater medium at 30 °C for 5 days. The extract, obtained by 1-butanol extraction of the whole culture broth, was subjected to consecutive fractionation using silica gel and ODS column chromatographies, followed by reversed-phase HPLC purification, yielding bulbimidazoles A (**1**, 11.3 mg), B (**2**, 3.2 mg), and C (**3**, 2.5 mg) from 3 L of culture.

The molecular formula of **1** was determined to be $C_{14}H_{24}N_2O$ based on a protonated molecule $[M + H]^+$ at m/z 237.1964 by an HR-ESITOFMS measurement. The calculated four degrees of unsaturation was in good agreement with an observed UV absorption maximum at 253 nm, implying the presence of a conjugated system with three or more double bonds. Moreover, the IR spectrum showed absorption bands at 3300 and 1665 cm^{-1}, corresponding to NH group(s) and a carbonyl functionality, respectively. The ^{1}H NMR spectrum showed two distinctive doublet resonances of sp^2 protons at δ_H 8.55 and 9.15, one doublet and one triplet methyl proton at δ_H 0.79 and 0.80, one aliphatic methine at δ_H 1.26, and several methylene multiplets, confirming the presence of an aromatic group and a methyl-branched alkyl chain in **1**. However, a ^{13}C NMR spectrum recorded in DMSO-d_6 exhibited only 10 aliphatic carbons and failed to detect the rest of the resonances (Figure S6), which were expected to be of the sp^2 carbons. These carbons were detected in HSQC and HMBC spectra as cross-peaks from the aforementioned sp^2 protons and some of the aliphatic protons (Figures S9 and S11). We suspected that the lack of resonances for sp^2 carbons in the ^{13}C NMR spectrum was due to the signal broadening and splitting caused by tautomeric exchange in the unidentified functional group. To convert the envisaged tautomers into a (few) resonating structure(s), a trace amount of trifluoroacetic acid (TFA) was supplemented to the NMR solvent. Gratifyingly, the anticipated four resonances appeared at δ_C 190.6, 137.5, 131.8, and 125.2 in the ^{13}C NMR spectrum (Figure S5). In combination with HSQC data, the assignment of all 14 carbon signals was made as one deshielded carbonyl carbon, one nonprotonated sp^2 carbon, two sp^2 methines, seven sp^3 methylenes, one sp^3 methine, and two methyl groups (Table 1). Analysis of the COSY spectrum elucidated three spin-systems, two of which were a trimethylene unit (H_2-7−H_2-8−H_2-9) and a six-carbon fragment with an *anteiso*-branching (H_3-15−H_2-14−H-13(H_3-16)−H_2-12−H_2-11) (Figure 1). These aliphatic fragments were joined via a methylene group at C-10 by a series of HMBC correlations from

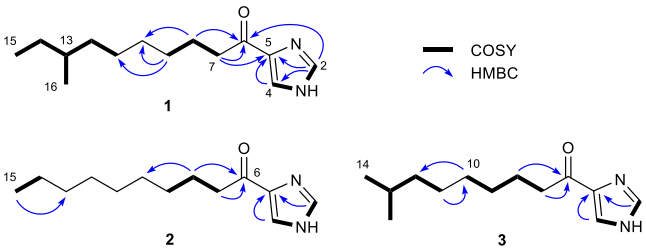

Figure 1. COSY and key HMBC correlations for **1**−**3**.

H_2-8 to C-10 and H_2-9 to C-10 and C-11. Furthermore, long-range correlations were observed from H_2-7 and H_2-8 to the carbonyl carbon (C-6), thereby establishing an 8-methyldecanoyl moiety. The last spin-system consisted of the two aromatic protons, H-2 and H-4, which were long-range coupled with J = 0.6 Hz, exchanged HMBC correlations with the corresponding carbons, and were further correlated with the nonprotonated carbon at δ_C 131.5 (C-5). Considering the remaining elemental composition HN_2 with two double-bond equivalents, an imidazole ring was the only option to satisfy these requirements. Finally, the alkanoyl moiety and imidazole were connected by HMBC correlations from the imidazole protons H-2 and H-4 to the carbonyl carbon C-6 and from the alkanoyl α-proton H_2-7 to the imidazole carbon C-5, thus completing the planar structure of **1**. The carbon chemical shifts of C-2, C-4, C-5, and C-6[13,14] as well as the UV maximal absorption[14] were in good accordance with those reported for synthetic alkanoyl imidazoles.

In order to establish the absolute configuration of the single stereogenic center C-13, the Ohrui−Akasaka method was employed (Figure 2).[15] This method is one of the few analytical means to elucidate the absolute configuration of an isolated stereogenic center with a methyl substituent in a long alkyl chain system. To generate a carboxyl group for derivatization, the imidazole ring was oxidized with $RuCl_3$−$NaIO_4$ in CCl_4−MeCN−H_2O,[16] which yielded 8-methyldecanoic acid (*nat*-**4**). This was derivatized with (*R*)-2-(anthracene-2,3-dicarboximido)propanol [(*R*)-2A1P] to give ester *nat*-**4**-(*R*)-2A1P (Figure 2c). The authentic (*S*)-**4**-(*R*)-2A1P and (*S*)-**4**-

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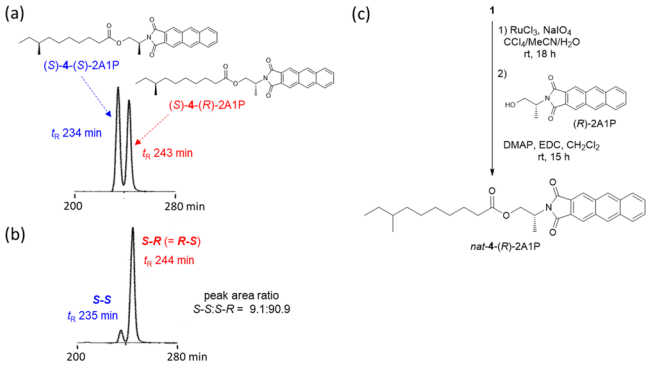

Figure 2. Results of Ohrui−Akasaka analysis. (a) Standard (*S*)-**4**-(*R*)-2A1P and (*S*)-**4**-(*S*)-2A1P. (b) *nat*-**4**-(*R*)-2A1P derived from **1**. (c) Oxidative degradation of **1** and derivatization with (*R*)-2A1P.

(*S*)-2A1P was prepared from (*S*)-8-methyldecanoic acid [(*S*)-**4**], which was synthesized from commercially available (*S*)-6-methyloctanol in four steps (Scheme S1). Retention times of these reference compounds were 243 min for (*S*)-**4**-(*R*)-2A1P and 234 min for (*S*)-**4**-(*S*)-2A1P, which is chromatographically equivalent to (*R*)-**4**-(*R*)-2A1P, whereas *nat*-**4**-(*R*)-2A1P showed two peaks for (*R*)-**4**-(*R*)-2A1P and (*S*)-**4**-(*R*)-2A1P in a ratio of 9.1:90.9 (Figure 2). Therefore, **1** was concluded to be an enantiomeric mixture consisting of 9% (*R*)- and 91% (*S*)-isomers.

Bulbimidazole B (**2**) was obtained as a pale yellow, amorphous solid. The molecular formula was determined to be $C_{14}H_{22}N_2O$ by HR-ESITOFMS, which was one methylene (14 amu) fewer than **1**. 1D and 2D NMR data were in overall similarity to those for **1**, except for the absence of the doublet methyl and a methine (H_3-16 and H-13 in **1**) resonance and the appearance of one additional methylene resonance (δ_C 31.3/δ_H 1.22, CH_2-13). COSY correlations between neighboring protons in an *n*-propyl spin-system H_3-15−H_2-14−H_2-13 and HMBC correlations from H_3-15 to C-13 and C-14 and H_2-14 to C-12 and C-13 established a nonbranching linear alkyl terminus in **2** (Table S1, Figure 1). The ^{1}H and ^{13}C NMR chemical shifts for the imidazole ring were mostly the same as those observed for **1** (Table 1).

Bulbimidazole C (**3**) was also obtained as a pale yellow, amorphous solid. It has the same molecular formula ($C_{14}H_{22}N_2O$) and UV maximum absorption (254 nm) as **2**, indicating that **3** is an alkyl chain isomer of **2**. The ^{1}H NMR spectrum showed a doublet methyl resonance (δ_H 0.82 d, J = 6.7 Hz) accounting for six protons, and this methyl group was correlated with a septet methine proton (δ_H 1.47, J = 6.7 Hz) in the COSY spectrum, confirming the presence of an isopropyl group. The intervening methylene chain part and the imidazole moiety were connected by COSY and HMBC correlations, thus establishing the structure of **3** (Table S1, Figure 1).

Compounds **1**−**3** were tested against two Gram-positive bacteria (*Kocuria rhizophila* ATCC9341 and *Staphylococcus aureus* FDA209P JC-1), three Gram-negative bacteria (*Escherichia coli* NIHJ JC-2, *Rhizobium radiobacter* NBRC14554, and *Tenacibaculum maritimum* NBRC16015), and three fungi (*Candida albicans* NBRC0197, *Glomerella cingulata* NBRC5907, and *Trichophyton rubrum* NBRC5467). Compounds **1**−**3** inhibited the growth of *K. rhizophila* and *S. aureus* with MICs of 0.78−3.12 μg/mL. They were inactive against *E. coli* and *R. radiobacter*, but were active against *T. maritimum*, a causative agent of fish skin ulcer, with MICs ranging from 3.12 to 12.5 μg/mL. Compounds **1**−**3** also showed relatively potent activity against three fungi tested in this study with MIC values ranging from 0.78 to 12.5 μg/mL. Overall, compounds **1** and **2** showed better activity than **3**, implying a key role of the alkyl chain length in exerting antimicrobial activity. In addition, **1**−**3** exhibited moderate cytotoxicity against P388 murine leukemia cells with IC_{50} values of 5.0, 5.8, and 7.0 μM, respectively.

Bulbimidazoles A−C (**1**−**3**) are new members of imidazole-containing natural products in which an alkyl chain and an imidazole ring are coupled through a keto group. These compounds, together with the 4-amino-substituted congeners, nocarimidazoles A (**4**) and B (**5**) from a marine-derived actinomycete *Nocardiopsis* sp.,[12] are the only natural products of this class. As synthetic compounds, alkanoyl imidazoles were reported as synthetic intermediates or structural motifs of a variety of drugs/drug candidates such as adrenoceptor agonists or HIV-1 protease inhibitors.[13,17]

Bacterial *anteiso*-fatty acids have been regarded to have an (*S*)-configuration based on the biosynthesis of the starter unit, 2-methylbutanoyl-CoA, from L-isoleucine.[18,19] However, this idea seems not applicable to secondary metabolites: nocapyrone L, a metabolite of *Nocardiopsis*, is a mixture of (*R*)- and (*S*)-enantiomers in a ratio of 2:3;[20] compound **1** is the second example of this kind. Both of these compositions were unambiguously elucidated by a sound analytical technique. The occurrence of (*R*)-*anteiso* enantiomers implies that the enantiospecificity of chain elongation enzymes toward the substrate, 2-methylbutanoyl CoA, is lower than expected, or even is lacking.[19] In the upstream pathway, conversion of L-isoleucine into both of the enantiomers of 2-methylbutanoyl CoA takes place in *Streptomyces*,[21,22] rat skin,[23] and apple (Scheme S2).[24] It is no wonder that the downstream pathway is also shaped to accept and process both enantiomers.

The wide antimicrobial spectrum of **1**−**3** against pathogens of humans (*S. aureus*, *C. albicans*, and *T. rubrum*), fish (*T. maritimum*), and plants (*G. cingulata*) poses a question regarding their molecular target(s). Finally, discovery of **1**−**3** warrants further investigation on the metabolites of the genus *Microbulbifer*, from which only two classes of compounds are known,[10,11] and other taxonomic groups of marine bacteria, which are not yet exploited as a source of drug discovery.

EXPERIMENTAL SECTION

General Experimental Procedures. The specific rotation was measured on a JASCO P-1030 polarimeter. UV spectra were obtained on a Shimadzu UV-1800 spectrophotometer. IR spectra were recorded on a PerkinElmer Spectrum 100 spectrophotometer. NMR spectra were obtained on a Bruker AVANCE 500 spectrometer in DMSO-d_6 supplemented with or without a trace amount of trifluoroacetic acid, using the signals of the residual solvent protons (δ_H 2.49) and carbons (δ_C 39.5) as internal standards for compounds **1**−**3** or in $CDCl_3$ using the signals of the residual solvent protons (δ_H 7.27) and carbons (δ_C 77.0) as internal standards for other compounds. HR-ESITOFMS spectra were recorded on a Bruker micrOTOF focus. An Agilent HP1200 system equipped with a diode array detector was used for analysis and purification.

Microorganism. The coral sample *Tubastraea* sp., collected as fishery waste at −10 to −15 m near the coast of Minami-Ise, Mie, Japan, was obtained through a local aquarium vendor. Strain DC3-6 was isolated according to the method described previously[25] and was identified as a member of genus *Microbulbifer* on the basis of 99.3% similarity in the 16S rRNA gene sequence (1455 nucleotides; DDBJ accession number LC498626) to *Microbulbifer echini* AM134^T (accession number KJ789957).

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Fermentation. Strain DC3-6 was maintained on Marine Agar 2216 (Difco). A loopful of strain DC3-6 was inoculated into a 500 mL K-1 flask containing 100 mL of Marine Broth 2216 as a seed culture. The seed culture was incubated at 30 °C on a rotary shaker at 200 rpm for 2 days. Then 3 mL each of seed culture was inoculated into 30 500 mL K-1 flasks containing 100 mL of A11M production medium, which consists of glucose 0.2%, soluble starch 2.5%, yeast extract 0.5%, polypeptone (Wako Pure Chemical Industries, Ltd.) 0.5%, NZ-amine (Wako Pure Chemical Industries, Ltd.) 0.5%, $CaCO_3$ 0.3%, and Diaion HP-20 (Mitsubishi Chemical Co.) 1% in natural seawater (collected from Toyama Bay, Japan). The pH of the medium was adjusted to 7.0 before sterilization. The inoculated flasks were incubated at 30 °C for 5 days with rotational shaking at 200 rpm.

Extraction and Isolation. After fermentation, 100 mL of 1-butanol was added to each flask, and the flasks were shaken for 1 h. The emulsified mixture was centrifuged at 6000 rpm for 10 min, and the organic layer was separated from the aqueous layer. Then, the organic layer was concentrated *in vacuo* to afford 4.3 g of extract from 3 L of production culture. The extract was chromatographed on a silica gel column with $CHCl_3$−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 0.35 g of brown oil, which was then fractionated by ODS column chromatography with a gradient of MeCN−0.1% HCO_2H aqueous solution (2:8, 3:7, 4:6, 5:5, 6:4, 7:3, and 8:2 v/v). Fraction 4 (5:5) was concentrated *in vacuo*, and the remaining aqueous layer was extracted with EtOAc. The organic layer was dried over anhydrous Na_2SO_4, filtered, and concentrated to give 83 mg of semipure material. Final purification was achieved by preparative HPLC (Cosmosil AR-II, Nacalai Tesque Inc., 10 × 250 mm, 4 mL/min, UV detection at 254 nm) with an isocratic elution of MeCN/0.1% HCO_2H aqueous solution (46:54) to afford **1** (11.3 mg, t_R 15.9 min), **2** (3.2 mg, t_R 11.2 min), and **3** (2.5 mg, t_R 10.5 min).

*Bulbimidazole A (**1**):* pale yellow, amorphous solid; $[\alpha]^{23}_D$ +1.2 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 253 (4.32) nm; IR (ATR) ν_{max} 3125, 2957, 2925, 1665 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; HR-ESITOFMS *m*/*z* 237.1964 $[M + H]^+$ (calcd for $C_{14}H_{25}N_2O$, 237.1961).

*Bulbimidazole B (**2**):* pale yellow, amorphous solid; UV (MeOH) λ_{max} (log ε) 254 (4.32) nm; IR (ATR) ν_{max} 3127, 2957, 2925, 1667 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; HR-ESITOFMS *m*/*z* 223.1809 $[M + H]^+$ (calcd for $C_{13}H_{23}N_2O$, 223.1804).

*Bulbimidazole C (**3**):* pale yellow, amorphous solid; UV (MeOH) λ_{max} (log ε) 254 (4.28) nm; IR (ATR) ν_{max} 3260, 2958, 2929, 1668 cm^{-1}; ^1H and ^{13}C NMR data, Table 1; HR-ESITOFMS *m*/*z* 223.1809 $[M + H]^+$ (calcd for $C_{13}H_{23}N_2O$, 223.1804).

*Synthesis of (S)-8-Methyldecanoic Acid (**4**).* To a solution of (*S*)-6-methyl-1-octanol (50 mg, 0.35 mmol, Wako Pure Chemical Industries, Ltd.) in dry CH_2Cl_2 (5 mL) was added Dess-Martin periodinane (200 mg, 0.47 mmol) at room temperature (rt), and the resultant mixture was stirred for 2 h. The reaction mixture was quenched by adding saturated a $NaHCO_3$ solution and Na_2SO_3 solution and extracted with EtOAc. The organic layer was washed with H_2O and brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to afford (*S*)-6-methyloctanal (30 mg), which was used for the next reaction without further purification.

(*S*)-6-Methyloctanal (30 mg, 0.21 mmol) was then reacted with methyl (triphenylphosphoranylidene)acetate (140 mg, 0.42 mmol) in dry CH_2Cl_2 (1 mL) at rt. After stirring for 2 h, ice−water was added to the reaction mixture, which was then extracted with EtOAc. The organic layer was washed with H_2O and brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was chromatographed over a silica gel column (*n*-hexane−EtOAc = 1:0−1:1) to give methyl (*S*,*E*)-8-methyl-2-decenoate (12 mg, 17% yield): ^1H NMR ($CDCl_3$, 500 MHz) δ 0.83 (3H, d, *J* = 6.3 Hz), 0.85 (3H, t, *J* = 7.2 Hz), 1.08−1.15 (2H, m), 1.24−1.37 (5H, m), 1.40−1.45 (2H, m), 2.20 (2H, ddt, *J* = 1.7, 7.1, 7.1 Hz), 3.72 (3H, s), 5.81 (1H, dt, *J* = 15.6, 1.7 Hz), 6.97 (1H, dt, *J* = 15.6, 7.0 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 167.2, 149.8, 120.8, 51.4, 36.3, 34.3, 32.3, 29.4, 28.3, 26.6, 19.2, 11.4; HR-ESITOFMS *m*/*z* 221.1648 $[M + Na]^+$ (calcd for $C_{12}H_{22}O_2Na$, 221.1644).

A solution of methyl (*S*,*E*)-8-methyl-2-decenoate (12 mg, 60 μmol) in MeOH (3 mL) was vigorously stirred with Pd/C (20 mg) under a H_2 atmosphere at rt. After stirring for 8 h, the reaction mixture was passed through Celite and the eluent was concentrated under reduced pressure to give methyl (*S*)-8-methyldecanoate (10 mg, 50 μmol), which was further subjected to hydrolysis in MeOH−THF (2 mL each) containing 1 M NaOH (2 mL). After stirring at rt for 12 h, the solution was acidified with 2 M HCl and extracted with EtOAc. The EtOAc layer was washed with H_2O and brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. The residue was purified on a silica gel column (*n*-hexane−EtOAc = 1:0−1:1) to afford (*S*)-8-methyldecanoic acid (10 mg, 88% yield): ^1H NMR ($CDCl_3$, 500 MHz) δ 0.83 (3H, d, *J* = 6.3 Hz), 0.85 (3H, t, *J* = 7.2 Hz), 1.06−1.16 (2H, m), 1.24−1.37 (9H, m), 1.63 (2H, quint, *J* = 7.6 Hz), 2.34 (2H, t, *J* = 7.6 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 179.2, 36.5, 34.3, 33.9, 29.5, 29.4, 29.0, 26.8, 24.6, 19.1, 11.3.

Preparation of (*R*)- and (*S*)-2-(Anthracene-2,3-dicarboximido)propyl ester of (*S*)-8-Methyldecanoic acid [(*S*)-4-(*R*)-2A1P and (*S*)-4-(*S*)-2A1P]. (*S*)-8-Methyldecanoic acid (**4**, 5.0 mg, 26 μmol) was treated with (*R*)-2-(anthracene-2,3-dicarboximido)-propanol [(*R*)-2A1P] (8.0 mg, 26 μmol), EDAC (6.0 mg, 34 μmol), and DMAP (trace amount) in dry CH_2Cl_2 (4 mL) at rt for 17 h. The reaction was quenched with ice−water, and the mixture was extracted with EtOAc. The organic layer was concentrated *in vacuo*, and the residue was chromatographed over a silica gel column (*n*-hexane−EtOAc = 1:0−1:1) to give (*R*)-2-(anthracene-2,3-dicarboximido)-propyl ester of (*S*)-8-methyldecanoic acid [(*S*)-**4**-(*R*)-2A1P, 3.5 mg]: ^1H NMR ($CDCl_3$, 500 MHz) δ 0.74 (3H, d, *J* = 6.3 Hz), 0.78 (3H, t, *J* = 7.3 Hz), 0.98−1.22 (9H, m), 1.49 (2H, m), 1.57 (3H, d, *J* = 7.1 Hz), 2.22 (2H, t, *J* = 7.5 Hz), 4.43 (1H, dd, *J* = 11.2, 5.0 Hz), 4.63 (2H, dd, *J* = 11.2, 9.8 Hz), 4.74 (1H, m), 7.63 (2H, m), 8.09 (2H, m), 8.51 (2H, s), 8.64 (2H, s); HR-ESITOFMS *m*/*z* 496.2451 $[M + Na]^+$ (calcd for $C_{30}H_{35}NO_4Na$, 496.2458).

(*S*)-**4**-(*S*)-2A1P was prepared from (*S*)-**4** and (*S*)-2A1P in a similar manner to that described above: ^1H NMR ($CDCl_3$, 500 MHz) δ 0.73 (3H, d, *J* = 6.3 Hz), 0.78 (3H, t, *J* = 7.3 Hz), 0.98−1.25 (9H, m), 1.49 (2H, m), 1.57 (3H, d, *J* = 7.2 Hz), 2.22 (2H, t, *J* = 7.6 Hz), 4.43 (1H, dd, *J* = 11.2, 4.9 Hz), 4.63 (1H, dd, *J* = 11.2, 9.7 Hz), 4.74 (1H, m), 7.63 (2H, m), 8.09 (2H, m), 8.50 (2H, s), 8.64 (2H, s); HR-ESITOFMS *m*/*z* 496.2454 $[M + Na]^+$ (calcd for $C_{30}H_{35}NO_4Na$, 496.2458).

Oxidative Degradation of 1 and Derivatization with (*R*)-2A1P. A solution of bulbimidazole A (**1**, 0.5 mg, 2 μmol) in a mixture of MeCN (80 μL) and deionized H_2O (60 μL) was stirred with $NaIO_4$ (4.8 mg, 22 μmol) until the salt was dissolved. To this solution were added CCl_4 (80 μL) and a solution of $RuCl_3$ hydrate in 0.1 M sodium phosphate buffer (1 mg/mL, 60 μL, pH 7.6), and the biphasic mixture was vigorously stirred at rt for 18 h. The reaction mixture was passed through Celite, and the filter cake was washed with MeCN. After evaporation of the organic solvent from the filtrate, the aqueous solution was acidified with 2 M HCl and extracted with EtOAc. The EtOAc layer was washed with H_2O and brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo* to give (*S*)-8-methyldecanoic acid (0.8 mg), which was reacted with (*R*)-2A1P in a similar manner to that described for (*S*)-**4**-(*R*)-2A1P to give the ester derivative of 8-methyldecanoic acid derived from **1** [*nat*-**1**-(*R*)-2A1P, 0.4 mg]: HR-ESITOFMS *m*/*z* 496.2457 $[M + Na]^+$ (calcd for $C_{30}H_{35}NO_4Na$, 496.2458).

Determination of the Absolute Configuration at the *anteiso*-Methyl Branching in 1. *nat*-**4**-(*R*)-2A1P and synthetic (*S*)-**4**-(*R*)-2A1P and (*S*)-**4**-(*S*)-2A1P were analyzed by HPLC under the following conditions. Column: tandemly connected Develosil ODS-HG-3 (3.0 mm i.d. × (250 + 150) mm, Nomura Chemical); mobile phase: MeCN−MeOH−THF = 3:1:1; column temperature: −42.5 °C; flow rate: 0.10 mL/min. The column was cooled by using Cryocool CC-100 (Neslab Instruments Inc.). HPLC peaks were detected by monitoring fluorescence intensity at 460 nm with the excitation at 298 nm by using an FP-4025 fluorescence detector (JASCO Corporation). Retention times were 234 min for (*S*)-**4**-(*S*)-2A1P and 243 min for (*S*)-**4**-(*R*)-2A1P, while *nat*-**4**-(*R*)-2A1P gave peaks at 235 and 244 min in a ratio of 9.1:90.9.

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Antimicrobial Assay. Antimicrobial activity was evaluated by the liquid microculture method using round-bottomed 96-well microtiter plates against five bacteria, *Kocuria rhizophila* ATCC9341, *Staphylococcus aureus* FDA209P JC-1, *Escherichia coli* NIHJ JC-2, *Rhizobium radiobacter* NBRC14554, and *Tenacibaculum maritimum* NBRC16015, and three fungi, *Candida albicans* NBRC0197, *Glomerella cingulata* NBRC5907, and *Trichophyton rubrum* NBRC5467, as indication strains. Mueller-Hinton broth (Difco), Sabouraud dextrose broth (Difco), and Marine Broth (Difco) were used for bacteria, fungi, and *T. maritimum* NBRC16015, respectively. Compounds **1**−**3**, reference drugs kanamycin sulfate for bacteria, sulfamethoxazole for *R. radiobacter* NBRC14554 and *T. maritimum* NBRC16015, and amphotericin B for fungi were made in 2-fold dilution series along the longer side of the plates by sequential transfer of 100 μL aliquots between the adjacent wells, to which the same amount of medium was predispensed. To each well was added a 100 μL suspension of the indication strains prepared at 1×10^4−10^5 cfu/mL from a culture at the logarithmic growth phase. The solvent vehicle added to the top rows was set at 0.5% of the final culture volume to avoid the effect on the growth of microbes. The plates were incubated at 37 °C for 20 h for bacteria, at 24 °C for *T. maritimum* NBRC16015, and at 25 °C for fungi. The experiments were done in triplicate, and the absorbance at 650 nm was measured using a microplate reader.

Cytotoxicity Assay. P388 murine leukemia cells were maintained in RPMI-1640 medium containing L-glutamine (product no. 186-02155) supplemented with 10% fetal bovine serum and 0.1 mg/mL gentamicin sulfate. Compounds **1**−**3** and doxorubicin as a reference were used to validate cytotoxicity in a 96-well round-bottom microtiter plate. To each well were seeded the cells at a final density of 1×10^4 cells/well, and 200 μL cultures thus made were incubated for 72 h at 37 °C in an atmosphere of 5% CO_2 in air with 100% humidity. Viability of the cells was visualized by addition of 50 μL of medium containing XTT (1 mg/mL) and PMS (40 μg/mL) to each well. After incubating for 4 h at 37 °C, medium was carefully removed by a suction aspirator, and formazan dye formed by respiratory reduction by living cells was quantified by measurement of absorption at 450 nm and read by a microplate reader to calculate the rate of cell growth inhibition at each concentration. The results of triplicate experiments were plotted on a graph to deduce IC_{50} values.

Table 2. Antimicrobial Activity of Bulbimidazoles A−C (1−3)

	MIC (μg/mL)		
microorganism	1	2	3
Kocuria rhizophila ATCC9341	0.78	0.78	1.56
Staphylococcus aureus FDA209P JC-1	1.56	1.56	3.12
Escherichia coli NIHJ JC-2	>100	>100	>100
Rhizobium radiobacter NBRC14554	>100	>100	>100
Tenacibaculum maritinum NBRC16015	3.12	6.25	12.5
Candida albicans NBRC0197	6.25	12.5	6.25
Glomerella cingulata NBRC5907	1.56	1.56	3.12
Trichophyton rubrum NBRC5467	0.78	1.56	3.12

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jnatprod.0c00082.

UV, IR, and 1D and 2D NMR spectra of **1**, **2**, and **3**; COSY and key HMBC correlations for **2** and **3**; assignment of ^1H and ^{13}C NMR chemical shifts with HMBC correlations for **2** and **3**; synthesis of (*S*)-8-methyldecanoic acid; possible biosynthetic routes to (*R*)- and (*S*)-2-methylbutanoyl CoA from L-isoleucine (PDF)

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Notes

The authors declare no competing financial interest.

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