JOURNAL OF NATURAL PRODUCTS

Article

pubs.acs.org/jnp

11-Hydroxylation of Protoberberine by the Novel Berberine-Utilizing Aerobic Bacterium *Sphingobium* sp. Strain BD3100

Hisashi Takeda,[†] Kazuki Ishikawa,[†] Daigo Wakana,[†] Masao Fukuda,[‡] Fumihiko Sato,[§] and Tomoo Hosoe*[,†]

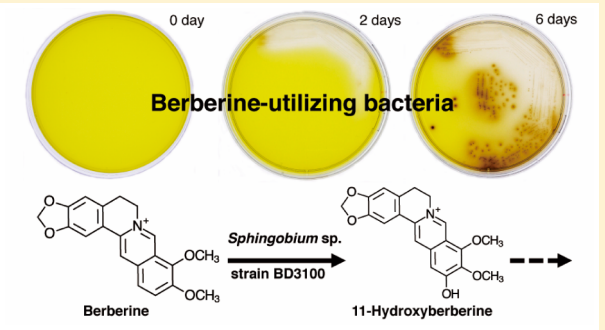
[†]Department of Organic Chemistry, Hoshi University, 2-4-41 Ebara, Shinagawa, Tokyo 142-8501, Japan

[‡]Department of Bioengineering, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka, Niigata 940-2188, Japan

[§]Division of Integrated Life Science, Graduate School of Biostudies, Kyoto University, Kitashirakawa, Sakyo, Kyoto 606-8317, Japan

Supporting Information

ABSTRACT: Protoberberine alkaloids, including berberine, palmatine, and berberrubine, are produced by medicinal plants and are known to have various pharmacological effects. We isolated two berberine-utilizing bacteria, *Sphingobium* sp. strain BD3100 and *Rhodococcus* sp. strain BD7100, from soil collected at a natural medicine factory. BD3100 had the unique ability to utilize berberine or palmatine as the sole carbon and energy source. BD3100 produced demethyleneberberine in berberine-supplemented medium. In a resting-cell incubation with berberine, BD3100 produced 11-hydroxyberberine; the structure of 11-hydroxyberberine was determined by detailed analysis of NMR and MS spectroscopic data. α-Naphthoflavone, miconazole, and ketoconazole, which are known inhibitors of cytochrome P450, interfered with BD3100 metabolism of berberine in resting cells. Inhibition by miconazole led to the production of a new compound, 11-hydroxydemethyleneberberine. In a resting-cell incubation with palmatine, BD3100 generated 11-hydroxypalmatine. This work represents the first report of the isolation and characterization of novel berberine-utilizing aerobic bacteria for the production of 11-hydroxylation derivatives of berberine and palmatine.



Many protoberberine alkaloids are produced in medicinal plants and are known to have various pharmacological effects.[1−4] Berberine (**1**) and its analogues, palmatine (**2**) and berberrubine (**3**), are protoberberine alkaloids isolated from herbal medicines such as *Rhizoma Coptidis*, *Cortex Phellodendri*, and *Mahonia aquifolium* (Figure 1). Berberine (**1**) has been used medicinally because of it is antibiotic activity against *Staphylococcus aureus*, *Streptococcus sanguis*, and *Escherichia coli*.[5] It is reported that the anti-*Staph. aureus* activity of berberine was potentiated in the presence of the multidrug pump inhibitor 5′-methoxyhydrocarpin.[6] Berberine (**1**) also has been reported to improve colitis,[7] to be cytotoxic against cancer cells, including ovarian tumors,[8] and to decrease plasma cholesterol and triglyceride levels in hyperlipidemic hamsters.[9] Palmatine (**2**) and berberrubine (**3**) also have been reported to have various biological activities.[10] Palmatine (**2**) exerts a wide range of biological effects arising from its ability to interact with proteins and nucleic acids.[11] Berberrubine (**3**) exhibits antitumor activity in animal models,[12] and its activity has been attributed to inhibition (as demonstrated in vitro) of topoisomerase II.[13] Thus, protoberberine alkaloids display many potential medical applications.

Until now protoberberine alkaloids have been produced mainly by isolation from plants and from synthesis[14,15] or by microbial production.[16−19] We report the characterization of two berberine-utilizing bacteria, *Sphingobium* sp. BD3100 and *Rhodococcus* sp. BD7100, and the production of new derivatives of berberine and palmatine by *Sphingobium* sp. BD3100.

RESULTS AND DISCUSSION

Berberine (**1**)-utilizing bacteria were isolated from soil at the dumping site for bark residues generated in the extraction of berberine. During the incubation of soil with berberine in liquid shaking culture, the yellow broth turned brown. Culturing this broth on agar plates containing berberine yielded several single colonies. One isolate, designated BD3100, grew as a yellow colony; a second isolate, designated as BD7100, formed a dry and rough colony.

BD3100 showed better growth than BD7100 on berberine-supplemented W minimal (WM) medium. Liquid culture of BD3100 and BD7100 in berberine-supplemented WM medium (Figure 2) showed fair growth and gradual conversion of the yellow to brown, suggesting the ability of BD3100 and BD7100 to utilize berberine as a sole source of energy. The isolated organisms were identified as *Sphingobium* sp. strain BD3100 and *Rhodococcus* sp. strain BD7100. A dendrogram showing the

Received: May 7, 2015

DOI: 10.1021/acs.jnatprod.5b00408
J. Nat. Prod. XXXX, XXX, XXX−XXX

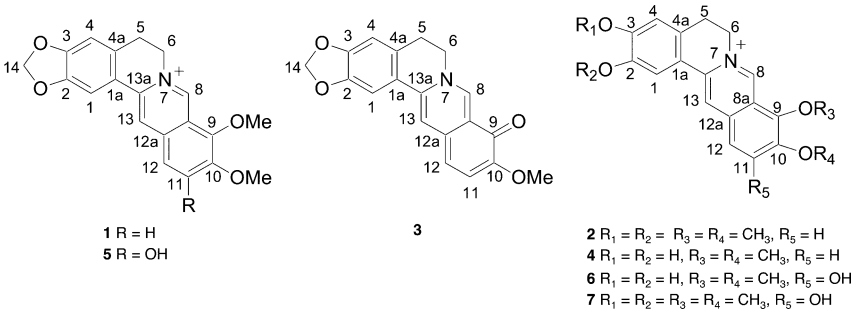


Figure 1. Structure of protoberberine alkaloids.

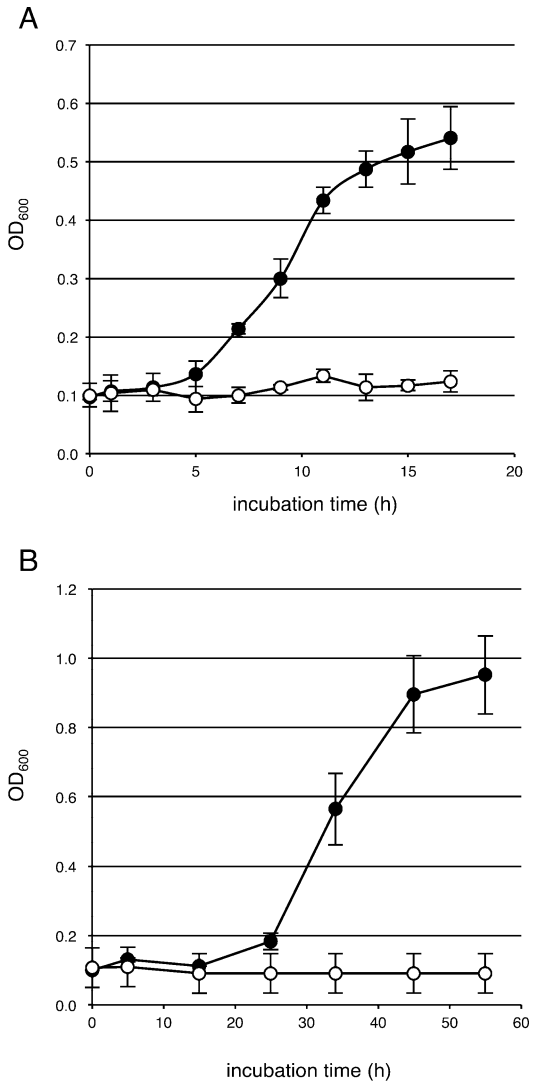


Figure 2. Growth of strain BD3100 (A) and BD7100 (B) on WM medium (open circles) and berberine-supplemented WM medium (solid circles). Results are means from at least three experiments. Vertical lines indicate standard deviations from the means.

close relation of BD3100 and BD7100 with 20 species of *Sphingobium* type-strains and *Rhodococcus* type-strains is provided as Figure S1 in the Supporting Information, respectively.

To confirm the unique ability of BD3100 and BD7100 to utilize berberine, these two strains were grown on berberine-supplemented LB agar and berberine-supplemented WM agar in parallel with four related species of BD3100 and three related species of BD7100 (Table 1). Whereas BD3100 and BD7100

Table 1. Growth of Related Strains on an Agar Plate Containing Berberine

strain	WM	LB
Sphingobium sp. BD3100	+[a]	+
S. yanoikuyae ATCC 51230	−[b]	+
S. yanoikuyae NBRC 15163	−	+
S. chlorophenolica ATCC 33790	−	−
S. japonicum UT26S	−	−
Rhodococcus sp. BD7100	+	+
R. erythropolis PR4	−	+
R. erythropolis ATCC 15963	−	+
R. jostii RHA1	−	+

[a]Growth was observed. [b]No growth was observed.

grew well on both types of agar plates, other related species, such as *Sphingobium yanoikuyae* ATCC 51230 and NBRC 15163, *Rhodococcus erythropolis* PR4 and ATCC 15963, and *Rhodococcus jostii* RHA1, showed growth only on berberine-supplemented LB, yielding no growth on berberine-supplemented WM. *Sphingobium chlorophenolicum* ATCC 33790 and *Sphingobium japonicum* UT26S did not grow on either berberine-supplemented medium. These results clearly indicated the unique ability of both BD3100 and BD7100 to utilize berberine as a carbon source.

BD3100 and BD7100 also showed growth on palmatine- or berberrubine-supplemented LB agar plates, although a growth difference between BD3100 and BD7100 was observed on berberrubine-supplemented LB. On WM agar plates, palmatine supported weak growth of BD3100 and no growth of BD7100 (Figure S2 in the Supporting Information). Neither BD3100 nor BD7100 grew on berberrubine-supplemented WM. These results suggested that both BD3100 and BD7100 preferentially utilize berberine, whereas BD3100 has limited ability to utilize palmatine.

To identify the metabolite(s) of berberine, BD3100 was grown in berberine-supplemented LB liquid medium. Growth was monitored by optical density (OD), and the broth was analyzed by HPLC. Decreased levels of berberine were observed with the increase of OD_{600}, especially after the middle growth stage, and berberine was completely depleted within 10 h (Figure 3A). HPLC analysis showed that the peak of berberine [retention time (t_R) at 17.7 min] was gradually decreased, while two new peaks [(**6**, t_R = 14.9 min) and (**4**, t_R = 15.3 min)] appeared on the HPLC chromatogram after 9 h of incubation (Figure 3B). Peak **4** was isolated and identified as demethyleneberberine (**4**)[20] by comparison of the retention

DOI: 10.1021/acs.jnatprod.5b00408
J. Nat. Prod. XXXX, XXX, XXX−XXX

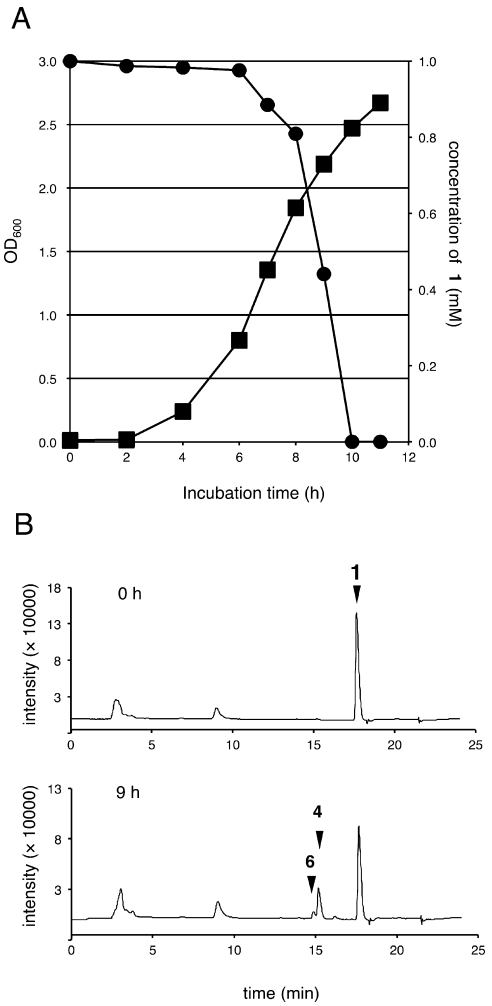


Figure 3. Detection of metabolites in the growing-cell assay of berberine. (A) Growth on berberine-supplemented LB medium and concentration of berberine in the broth. (B) HPLC chromatograms at 280 nm at the initial time (upper) and 9 h (bottom) of growing-cell assay.

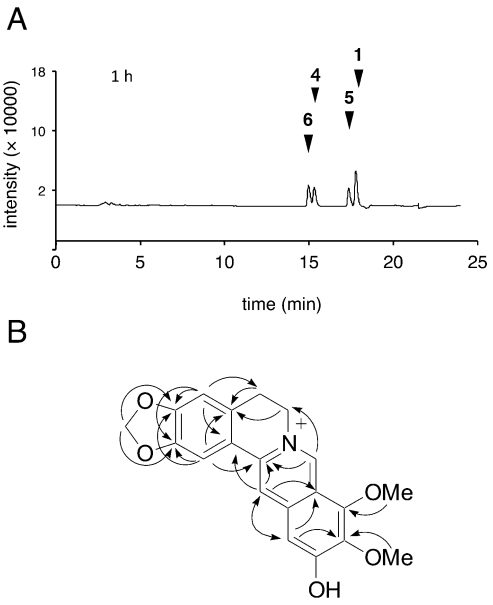


Figure 4. Detection of metabolites in the resting-cell assay of berberine. (A) HPLC chromatogram of the resting-cell assay at 280 nm after 60 min. (B) Structure of **5**. Black arrows show HMBC. Double-headed arrows indicate a cross-peak in the HMBC.

Table 2. Effect of Cytochrome P450 Inhibitors in Resting-Cell Assay[a]

	inhibition in metabolism of berberine (%)	
inhibitor	10 μM	100 μM
fluconazole	0	0
ketoconazole	23 (±7)	54 (±16)
miconazole	0	50 (±8)
α-naphthoflavone	0	99 (±2)
erythromycin	0	0
quinine	0	0

[a]The data are averages based on triplicate experiments. Values of ± indicate standard deviations.

time using an authentic standard on HPLC chromatograms (Figure S3 in the Supporting Information).

Since degradation of berberine occurred rapidly in the growing-cell assay in LB medium, berberine was incubated with the resting cells of BD3100 grown in berberine-supplemented LB medium. As shown in Figure 4A, the resting-cell suspension with berberine showed more peaks than that generated by the growing cells, and a new peak (**5**, t_R= 17.3 min) was observed. The metabolite **5** was purified and determined to be 11-hydroxyberberine (**5**) by MS and NMR spectroscopic data, including HSQC and HMBC (see Figure 4B and Figures S5 to S8 in the Supporting Information). To our knowledge, 11-hydroxyberberine (**5**) has not previously been reported in the literature.

As the metabolism of berberine in mammals has been attributed to the cytochrome P450 class of enzymes,[21] berberine metabolism in BD3100 cells in the presence of cytochrome P450 inhibitors was investigated. Specifically, we tested the effects of ketoconazole, miconazole, fluconazole, α-naphtoflavone, erythromycin, and quinine. Among cytochrome P450 inhibitors tested in the resting-cell assay (Table 2), α-naphthoflavone completely inhibited the metabolism of berberine, whereas ketoconazole and miconazole showed about 50% inhibition. In contrast, fluconazole, erythromycin, and quinine did not show any inhibition of the metabolism of berberine.

Miconazole incubation resulted in the production of **5** and **6** (Figure 5A). Following isolation by preparative HPLC, **6** (t_R = 14.9 min) was determined to be 11-hydroxydemethyleneberberine (**6**) by detailed analysis of ESIMS and NMR spectroscopic data (Figure 5B and Figures S10 to S14 in the Supporting Information). To our knowledge, 11-hydroxydemethyleneberberine (**6**) is also a new compound and has not previously been reported in the literature.

Incubation of palmatine (**2**) with cells grown in berberine-supplemented LB medium, palmatine-supplemented LB medium, and LB medium without berberine and palmatine resulted in the production of 11-hydroxypalmatine (**7**), although the production rate of 11-hydroxypalmatine is different in each case (Figure 6).

Antibacterial activity of berberine, 11-hydroxyberberine, 11-hydroxydemethyleneberberine, and 11-hydroxypalmatine against *Staph. aureus* was measured by the paper disk method. 11-Hydroxyberberine, 11-hydroxydemethyleneberberine, and 11-hydroxypalmatine showed no activity at 250 nmmol per disk although the diameter of inhibition circle for berberine was

DOI: 10.1021/acs.jnatprod.5b00408
J. Nat. Prod. XXXX, XXX, XXX−XXX

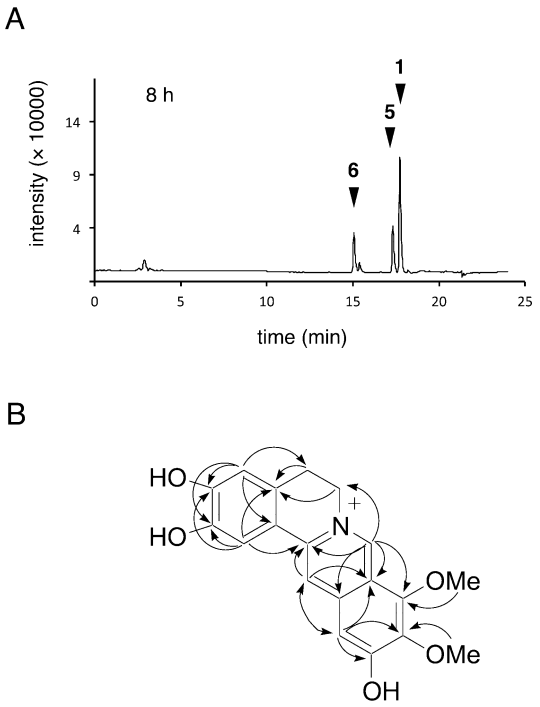


Figure 5. Detection of metabolites in the resting-cell assay of berberine treated with miconazole. (A) HPLC chromatogram at 280 nm of the resting-cell assay containing cytochrome P450 inhibitor miconazole in the reaction time at 8 h. (B) Structure of **6**. Black arrows show HMBC. Double-headed arrows indicate a cross-peak in the HMBC.

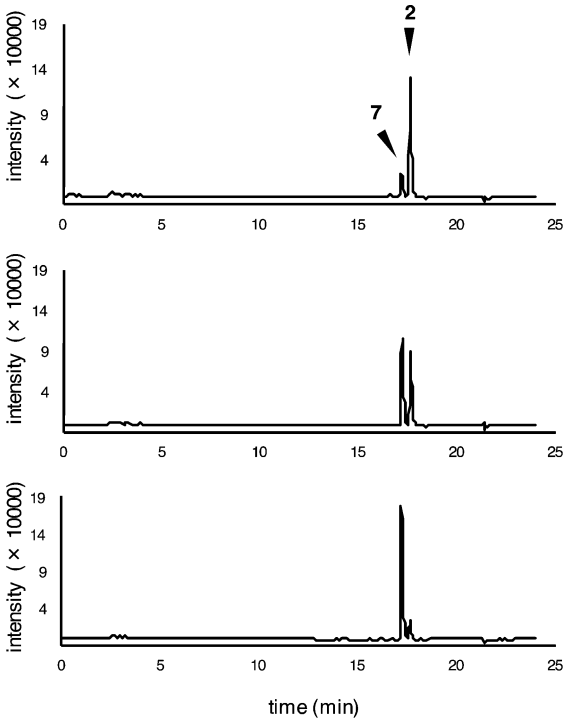


Figure 6. HPLC chromatograms at 280 nm after 30 min in the resting cell assay of palmatine using resting cells of BD3100 grown in LB (upper), palmatine-supplemented LB (middle), and berberine-supplemented LB (bottom).

12 mm at 125 nmol per disk (Figure S15 in the Supporting Information).

To our knowledge, this report is the first isolation and identification of berberine-utilizing bacteria, although fungi are known to produce 7-*N*-oxide berberine.[22] 11-Hydroxylated protoberberine alkaloids are unusual, and 11-hydroxypalmatine isolated from tubers of *Stephania glabra* is the only example.[23] The antimicrobial activity of berberine against *Staph. aureus* has been reported, and this is enhanced by 13-alkylation or 9-ethoxylation of berberine.[15] However, 11-hydroxylation of berberine and its analogues results in compounds that are inactive against *Staph. aureus*, as shown here.

EXPERIMENTAL SECTION

General Experimental Procedures. ESIMS data were measured on a Micromass ZQ mass spectrometer (Nihon Waters K. K., Tokyo, Japan). ^{1}H and ^{13}C NMR spectra were recorded using a Bruker AVANCE-400 spectrometer (400.13 MHz for ^{1}H, 100.61 MHz for ^{13}C, Bruker Biospin K. K., Kanagawa, Japan). Chemical shifts (δ) were measured in ppm using tetramethylsilane as an internal standard.

Berberine (**1**) sulfate (>98%) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Berberrubine (**3**) and demethyleneberberine (**4**) were chemically synthesized according to the references.[14,22] The experimental details are shown in the Supporting Information (Figures S16 and S17). Ketoconazole and α-naphthoflavone were purchased from Sigma-Aldrich Japan (Tokyo, Japan). All other chemicals used in this study were purchased from Wako Pure Chemicals Industry, Ltd. (Tokyo, Japan).

Semiquantitative analysis of berberine and the derivatives in the liquid samples was performed by photodiode array (PDA)-HPLC using a PU-980 and PU-1580 pump (flow rate 1 mL/min, JASCO Co., Ltd., Tokyo, Japan) and Insertsil ODS-3 column (5 μm, 4.6 × 250 mm, GL Science Inc., Tokyo, Japan) in a CO-2065 Plus column oven (at 30 °C, JASCO Co., Ltd., Tokyo, Japan), equipped with an MD-2010 Plus photodiode array (JASCO Co., Ltd., Tokyo, Japan). The column used a gradient system of the mobile phase; initial conditions were 15% B with a linear gradient to 50% B from 5 to 15 min, followed by a linear gradient to 100% B at 15.1 min, with the last condition maintained until 18 min. The mobile phase was returned to initial conditions at 18.1 min and maintained until the end of the run at 25 min (A: 0.1% TFA/H_2O; B: acetonitrile). Isolation of metabolites was performed by preparative HPLC using a Senshu SSC-3160 pump (Senshu Scientific Co., Ltd., Tokyo, Japan) and an ULTRA PACK ODS-SM-50B column (50 μm, 26 × 300 mm, Yamazen Corp., Osaka, Japan); preparative HPLC then was performed using an LC-20AT prominence pump (flow rate 2 mL/min, Shimadzu Corp., Kyoto, Japan) and an Inertsil ODS-P column (5 μm, 10 × 250 mm, GL Science Inc., Tokyo, Japan) in the CO-965 column oven (at 30 °C, JASCO Co., Ltd., Tokyo, Japan), equipped with an SPD-20A UV detector (Shimadzu Corp., Kyoto, Japan).

Determinations of molecular weight and molecular formula in the liquid samples were performed by LC-ESI-TOF-MS using a 1200 series system (flow rate 0.2 mL/min, column temperature 30 °C, Agilent Technologies, Inc., Tokyo, Japan) and Inertsil ODS-4 column (3 μm, 2.1 × 250 mm, GL Science Inc., Tokyo, Japan). The column used a gradient system of the mobile phase; initial conditions were 15% B with a linear gradient to 90% B from initial to 10 min, which then was maintained until 12 min. The mobile phase was returned to initial conditions at 12.1 min and maintained until the end of the run at 18 min (mobile solvent A: 0.1% TFA in H_2O; B: acetonitrile).

The OD_{600} of the broth culture was monitored continuously using an OD Box-A (TAITEC Co., Ltd., Saitama, Japan) and an Ultrospec 2100 Pro ultraviolet (UV)−visible spectrophotometer (Amersham Biosciences Ltd., Japan) at 600 nm. BioShaker BR43-FH (TAITEC Co., Ltd., Saitama, Japan) and FTW-502 M (Hirayama Manufacturing Corp., Saitama, Japan) were used for incubation of BD3100 and BD7100.

Bacteria. *S. yanoikuyae* strain ATCC 51230 was obtained from the Japan Collection of Microorganisms at RIKEN Bioresource Center (Wako, Japan). *S. yanoikuyae* NBRC 15163, *S. chlorophenolicum* ATCC

DOI: 10.1021/acs.jnatprod.5b00408
J. Nat. Prod. XXXX, XXX, XXX−XXX

Table 3. NMR Spectroscopic Data for Compounds 5 (Methanol-d_4) and 6 (DMSO-d_6)

	5				6			
position	δ_C[a]	δ_H (J in Hz)[b]			δ_C[a]	δ_H (J in Hz)[b]		
1	106.1	7.60	s		112.9	7.54	s	
1a	121.8				117.8			
2	149.8				145.5			
3	152.1				149.3[c]			
4	109.4	6.94	s		114.9	6.8	s	
4a	132.1				127.4			
5	28.4	3.21	t	(6.1)	26.0	3.06	dd	(5.8, 5.4)
6	56.1	4.78	m		54.5	4.75	dd	(5.8, 5.4)
7								
8	144.3	9.41	s		143.3	9.48	s	
8a	118.4				116.1			
9	151.1				149.4[c]			
10	143.5				141.8			
11	163.7				161.7			
12	106.6	7.22	s		104.7	7.26	s	
12a	139.0				136.9			
13	118.5	8.36	s		116.1	8.42	s	
13a	140.8				139.4			
14	103.6	6.09	s					
9-OMe	62.7	4.24	s		62.1	4.14	s	
10-OMe	61.8	4.02	s		61.0	3.94	s	

[a]100 MHz for ^{13}C NMR. [b]400 MHz for ^{1}H NMR. [c]Exchangeable peak.

33790, *S. japonicum* UT26S, *R. jostii* RHA1, *R. erythropolis* PR4, and *R. erythropolis* ATCC 15963 were obtained from the National Institute of Technology and Evaluation Biological Resource Center (NBRC) in Japan.

Media and Culture Conditions. Bacteria were grown in LB medium (10 g of Bacto-tryptone, 5 g of Bacto-yeast extract, and 5 g of NaCl in 1 L of distilled water) and/or WM medium [1.7 g of KH_2PO_4, 12.4 g of $Na_2HPO_4{\cdot}12H_2O$, 1.0 g of $(NH_4)_2SO_4$, 2.0 mg of $CaCO_3$, 1.4 mg of $ZnSO_4{\cdot}7H_2O$, 0.25 mg of $CuSO_4{\cdot}5H_2O$, 0.28 mg of $CoSO_4{\cdot}7H_2O$, 0.06 mg of H_3BO_4, 100 mg of $MgSO_4{\cdot}7H_2O$, 0.95 mg of $FeSO_4{\cdot}7H_2O$, and 10.7 mg of MgO in 1 L of distilled water]. All cultures were incubated at 30 °C, and liquid cultures were aerobically incubated with shaking at 180 rpm. Growth was monitored by an OD monitor (TAITECH, Tokyo, Japan) as absorption of 600 nm.

Isolation of Berberine-Utilizing Bacteria. Soil samples were obtained from the site where bark residues of *Cortex phellodendri* used for the extraction of medicinal properties, at an herbal-medicine factory, Naganoken Pharmaceutical Co., Ltd., in the village of Otaki in Japan, were discarded. Sample soil (0.5 g) was added directly to 100 mL of WM medium containing 0.25% (w/v) berberine and incubated at 30 °C for 6 days on a rotary shaker. An aliquot of the resulting culture was diluted 1:100 into 100 mL of fresh WM medium containing 0.25% (w/v) berberine and incubated at 30 °C for 6 days on a rotary shaker. This 1:100 dilution and outgrowth was repeated twice more (for a total of three times). An aliquot (100 μL) of the resulting culture was spread directly on a plate of WM agar containing 0.05% (w/v) berberine. Individual colonies were streaked on WM agar plates containing 0.05% berberine.

Identification of Berberine-Utilizing Bacteria. Each isolated bacterium was grown in LB medium at 30 °C for 16 h on a rotary shaker. Total DNA from strains BD3100 and BD7100 was extracted from each bacterial pellet with a Genomic DNA purification kit (Promega KK, Tokyo, Japan) according to the manufacturer's protocol. The 16S rDNA was amplified using the 27F (5′-GAG TTT GAT CCT GGC TCA G-3′) and 1525R (5′-GAG GTG ATC CAG CCG CAG G-3′) primer pair. PCR was performed with each extracted total DNA as the template for 16S rRNA gene amplification using KOD plus neo DNA polymerase (TOYOBO, Osaka, Japan) with the following program: initial denaturation at 96 °C for 120 s; 32 cycles of amplification (94 °C for 20 s, 55 °C for 20 s, and 68 °C for 45 s); and extension at 68 °C for 5 min. Amplified products were purified by the Wizard SV gel and PCR clean-up system (Promega KK) according to the manufacturer's protocol. The purified DNA fragments were cloned into the *Sma*I site of pUC19. Sequencing reactions were performed using the Big Dye Terminator Cycle Sequencing system (Applied Biosystems, CA, USA) on an automated ABI 3100 sequencer (Applied Biosystems). Sequencing primers were as follows: M13−20, 520F (5′-CAG CAG CCG CGT AAT AC-3′), 520R (5′-GTA TTA CCG CGG CTG CTG-3′), 920F (5′-AAA CTC AAA TGA ATT GAC GG-3′), 920R (5′-CCG TCA ATT CAT TTG AGT TT-3′), and M13-Rv. The 16S rRNA gene sequences of the isolated bacteria were submitted to the classifier program in the Ribosomal Database Project release 11.3 maintained by the Center for Microbial Ecology at Michigan State University (http://rdp.cme.msu.edu/).[24]

The 16S rRNA gene sequence of BD3100 showed high similarities to the 16S rRNA of some species of the genus *Sphingobium*. Thus, we classified the strain BD3100 as a member of the genus *Sphingobium* and named the strain *Sphingobium* sp. strain BD3100. On the other hand, the 16S rRNA gene sequence of BD7100 showed 100% similarity to the 16S rRNA of several strains of *Rhodococcus erythropolis*. Thus, we classified the BD7100 as a member of the genus *Rhodococcus* and named it *Rhodococcus* sp. strain BD7100. These strains are stored at −80 °C in a deep freezer in our laboratory.

Growing-Cell Assay. BD3100 was grown overnight at 30 °C in LB medium. An aliquot (1.0 mL) of preculture was added into 100 mL of LB medium containing 1 mM berberine (**1**), and the medium was incubated at 30 °C on a rotary shaker. Growth was monitored using an OD monitor (TAITECH, Tokyo, Japan). Aliquots of the culture (400 μL/time point) were periodically removed, and growth was stopped (cells lysed, reactions quenched) by the addition of an equal volume of MeOH. After centrifugation at 18800*g* at 4 °C for 10 min, the supernatants were analyzed by high-performance liquid chromatography (HPLC).

Resting-Cell Assay. BD3100 was grown at 30 °C with shaking for 16 h in 100 mL of LB medium or LB medium containing 0.5 mM berberine (**1**) or palmatine (**2**). The cells were washed twice with 50 mM sodium phosphate buffer (pH 7.0) and then suspended in 20 mL of 50 mM sodium phosphate buffer to give a turbidity of 10 at 600 nm. Substrate was added to a final concentration of 0.5 mM, and the

DOI: 10.1021/acs.jnatprod.5b00408
J. Nat. Prod. XXXX, XXX, XXX−XXX

mixture of resting-cell suspension and substrate in the sodium phosphate buffer was incubated at 30 °C with shaking. Aliquots of the reaction (0.4 mL/time point) were periodically removed, and growth was stopped (cells lysed, reactions quenched) by the addition of an equal volume of a stop solution (MeOH containing 0.05 N HCl). After centrifugation at 18800*g* at 4 °C for 10 min, the supernatants were analyzed by HPLC.

Effect of Cytochrome P450 Inhibitors. Stock solutions of cytochrome P450 inhibitors (fluconazole, ketoconazole, miconazole, α-naphthoflavone, erythromycine, and quinine) were formulated by dissolving the respective compound at 10 mM in dimethyl sulfoxide. Aliquots (100 μL/reaction) of P450 inhibitor stock solution or 10-fold-diluted P450 inhibitor stock solution were added to 9.4 mL of resting cells (i.e., to a final inhibitor concentration of 100 or 10 μM) that had been suspended in 50 mM sodium phosphate buffer (pH 7.0) to give a turbidity of 5 at 600 nm. Inhibitor-free controls were generated by adding 100 μL of vehicle (dimethyl sulfoxide) to the suspension of resting cells. Substrate was added to each reaction to a final concentration of 0.5 mM, and the mixture was incubated at 30 °C with shaking. HPLC samples were prepared as described in the resting-cell assay.

Preparation and Isolation of 5. Resting-cell assay of berberine was performed in 50 mM sodium phosphate buffer (pH 7.0) at 30 °C for 5 h with shaking (180 rpm). The cells were removed by centrifugation (2610*g*, 15 min, room temperature). The supernatant was filtered and then evaporated in vacuo. Dried samples were resuspended in MeOH (5 mL) and assayed by HPLC. The HPLC conditions were as follows: column, Inertsil ODS-P; flow rate, 2.0 mL/min; oven temperature, 40 °C; detector, UV at 254 nm; mobile phase, 30% acetonitrile in H_2O containing 0.1% TFA. Peak **5** (t_R = 23.1 min) was fractionated using preparative HPLC.

*11-Hydroxyberberine (**5**):* colorless, amorphous solids; UV (MeOH) λ_{max} (log ε) 289 (4.17), 321 (4.11), 387 (3.73) nm; IR (KBr) ν_{max} 3421, 2922, 1634, 1617, 1433, 1396 cm^{-1} (Figure S4 in the Supporting Information); ^1H NMR (methanol-d_4, 400 MHz) and ^{13}C NMR (methanol-d_4, 100 MHz), see Table 3; HRESIMS *m*/*z* 352.1209 (calcd for $C_{20}H_{18}NO_5^+$, 352.1185)

Preparation and Isolation of 6. The resting-cell assay of berberine was performed in 50 mM sodium phosphate buffer (pH 7.5) containing 1 mM miconazole at 30 °C for 11 h with shaking (180 rpm). The cells were removed by centrifugation (2610*g*, 15 min, room temperature). The supernatant was filtered and then evaporated in vacuo. Dried samples were resuspended in MeOH (5 mL/sample) and assayed by HPLC. The HPLC conditions were as follows: column, Inertsil ODS-P; flow rate, 2.0 mL/min; oven temperature, 30 °C; detector, UV at 254 nm; mobile phase, 30% acetonitrile in H_2O containing 0.1% TFA. Peak **6** (t_R = 12.1 min) was fractionated using preparative HPLC.

*11-Hydroxydemethyleneberberine (**6**):* colorless, amorphous solids; UV (MeOH) λ_{max} (log ε) 292 (3.93), 322 (3.84), 383 (3.55) nm; IR (KBr) ν_{max} 3419, 2960, 1634, 1615, 1432, 1394 cm^{-1} (Figure S9 in the Supporting Information); ^1H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 100 MHz), see Table 3; HRESIMS *m*/*z* 340.1224 (calcd for $C_{19}H_{18}NO_5^+$, 340.1185).

Preparation and Isolation of 7. The resting-cell assay of palmatine was performed in 50 mM phosphate buffer at 30 °C for 11 h with shaking (180 rpm). The cells were removed by centrifugation (2610*g*, 15 min, rt). The supernatant was filtrated, and the filtrate was evaporated. MeOH was added to the residue; it was prepared as a sample for HPLC. The HPLC conditions were as follows; column, Inertsil ODS-P; flow rate, 2.0 mL/min; oven temperature, 30 °C; detector, UV detector at 254 nm and the gradient system of mobile phase; initial conditions, 35% B with a linear gradient to 60% B from 0 to 15 min, followed by a linear gradient to 100% B at 15.1 min, this proportion being maintained until 18 min. The mobile phase was returned to initial conditions at 18.1 min and maintained until the end of the run at 25 min (A: 0.1% TFA/H_2O; B: acetonitrile). The peak (t_R = 5.8 min) of 11-hydroxypalmatine (**7**) was fractionated using preparative HPLC. Compound **7** was identified by comparison of ESIMS and NMR spectroscopic data in a previous paper.[23]

Antibacterial Activity. A semiquantitative antibacterial assay was performed using the paper disk method against *Staph. aureus* strain H209P maintained in our laboratory. Kanamycin was used as a positive control. Test substances (berberine, 11-hydroxyberberine, and 11-hydroxydemethyleneberberine) and kanamycin were prepared to 1, 2, and 5 mM solutions, and each 50 μL solution was applied to the paper disk of 8 mm diameter. The disks were placed on LB agar plates including bacteria, and the plates were incubated at 30 °C. After 12 h of incubation, the zones of inhibition (the diameter measured in millimeters) were recorded.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b00408.

The phylogenetic tree of BD3100 and BD7100, growth of BD3100 and BD7100 on the plate containing protoberberine, comparison of HPLC chromatograms using standard reagent of demethyleneberberine, and ^1H NMR, ^{13}C NMR, and 2D NMR spectra of compounds of 11-hydroxyberberine and 11-hydroxydemethyleneberberine (PDF)

Accession Codes

Sequences obtained in the present study have been deposited in the International Nucleotide Sequence Database Collaboration under Accession No. LC030231 for BD3100 and LC030232 for BD7100.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +81-3-5498-5788. Fax: +81-3-5498-5788. E-mail: hosoe@hoshi.ac.jp.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Mr. T. Yataka, former President and CEO of Naganoken Pharmaceutical Co., Ltd., for providing soil samples.

■ REFERENCES

(1) Singh, A.; Duggal, S.; Kaur, N.; Singh, J. *J. Nat. Prod.* **2010**, *3*, 64−75.

(2) Yu, X.; Gao, X.; Zhu, Z.; Cao, Y.; Zhang, Q.; Tu, P.; Chai, X. *Molecules* **2014**, *19* (9), 13042−13060.

(3) Chan, C. O.; Chu, C. C.; Mok, D. K.; Chau, F. T. *Anal. Chim. Acta* **2007**, *592* (2), 121−131.

(4) Vennerstrom, J. L.; Klayman, D. L. *J. Med. Chem.* **1988**, *31* (6), 1084−1087.

(5) Scazzocchio, F.; Cometa, M. F.; Tomassini, L.; Palmery, M. *Planta Med.* **2001**, *67* (6), 561−564.

(6) Stermitz, F. R.; Lorenz, P.; Tawara, J. N.; Zenewicz, L. A.; Lewis, K. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97* (4), 1433−1437.

(7) Yan, F.; Wang, L.; Shi, Y.; Cao, H.; Liu, L.; Washington, M. K.; Chaturvedi, R.; Israel, D. A.; Wang, B.; Peek, R. M.; Wilson, K. T.; Polk, D. B. *Am. J. Physiol Gastrointest Liver Physiol* **2012**, *302* (5), G504−G514.

(8) Marverti, G.; Ligabue, A.; Lombardi, P.; Ferrari, S.; Monti, M. G.; Frassineti, C.; Costi, M. P. *Int. J. Oncol.* **2013**, *43* (4), 1269−1280.

(9) Kong, W.; Wei, J.; Abidi, P.; Lin, M.; Inaba, S.; Li, C.; Wang, Y.; Wang, Z.; Si, S.; Pan, H.; Wang, S.; Wu, J.; Wang, Y.; Li, Z.; Liu, J.; Jiang, J. D. *Nat. Med.* **2004**, *10* (12), 1344−1351.

(10) Singh, I. P.; Mahajan, S. *Expert Opin. Ther. Pat.* **2013**, *23* (2), 215−231.

DOI: 10.1021/acs.jnatprod.5b00408
J. Nat. Prod. XXXX, XXX, XXX−XXX

(11) Maiti, M.; Kumar, G. S. *J. Nucleic Acids* **2010**, *2010*, 1−23.
(12) Ikekawa, T.; Ikeda, Y. *J. Pharmacobio-Dyn.* **1982**, *5* (7), 469−474.
(13) Kim, S. A.; Kwon, Y.; Kim, J. H.; Muller, M. T.; Chung, I. K. *Biochemistry* **1998**, *37* (46), 16316−16324.
(14) Grycová, L.; Dostál, J.; Marek, R. *Phytochemistry* **2007**, *68* (2), 150−175.
(15) Iwasa, K.; Kamigauchi, M.; Ueki, M.; Taniguchi, M. *Eur. J. Med. Chem.* **1996**, *31* (6), 469−478.
(16) Hawkins, K. M.; Smolke, C. D. *Nat. Chem. Biol.* **2008**, *4* (9), 564−573.
(17) Sato, F.; Kumagai, H. *Proc. Jpn. Acad., Ser. B* **2013**, *89* (5), 165−182.
(18) Minami, H.; Kim, J. S.; Ikezawa, N.; Takemura, T.; Katayama, T.; Kumagai, H.; Sato, F. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105* (21), 7393−7398.
(19) Chappell, J. *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105* (23), 7897−7898.
(20) Li, Y. H.; Li, Y. H.; Yang, P.; Kong, W. J.; You, X. F.; Ren, G.; Deng, H. B.; Wang, Y. M. X.; Wang, Y. M. X.; Jiang, J. D.; Song, D. Q. *Bioorg. Med. Chem.* **2010**, *18* (17), 6422−6428.
(21) Ma, J. Y.; Feng, R.; Tan, X. S.; Ma, C.; Shou, J. W.; Fu, J.; Huang, M.; He, C. Y.; Chen, S. N.; Zhao, Z. X.; He, W. Y.; Wang, Y.; Jiang, J. D. *J. Pharm. Sci.* **2013**, *102* (11), 4181−4192.
(22) Agusta, A.; Wulansari, D.; Praptiwi; Nurkanto, A.; Fathoni, A. *Procedia Chem.* **2014**, *13*, 38−43.
(23) Semwal, D. K.; Rawat, U.; Semwal, R.; Singh, R.; Singh, G. J. *J. Asian Nat. Prod. Res.* **2010**, *12* (2), 99−105.
(24) Wang, Q.; Garrity, G. M.; Tiedje, J. M.; Cole, J. R. *Appl. Environ. Microbiol.* **2007**, *73* (16), 5261−5267.

DOI: 10.1021/acs.jnatprod.5b00408
J. Nat. Prod. XXXX, XXX, XXX−XXX