Discovery of a Marine Bacterium Producing 4-Hydroxybenzoate and Its Alkyl Esters, Parabens

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Chemically synthesized 4-hydroxybenzoate (4HBA) is widely used in the chemical and electrical industries as a material for producing polymers such as those of the liquid crystal type. Its alkyl esters, called parabens, have been the most widely used preservatives by the food and cosmetic industries. We report here for the first time a microorganism, a marine bacterium, which biosynthesizes these petrochemical products. The marine bacterial strain, A4B-17, which was found to belong to the genus *Microbulbifer* on the basis of its rRNA and *gyrB* sequences, was isolated from an ascidian in the coastal waters of Palau. Strain A4B-17 was, surprisingly, found to produce 10 mg/liter of 4HBA, together with its butyl (24 mg/liter), heptyl (0.4 mg/liter), and nonyl (6 mg/liter) esters. We therefore characterized 23 other marine bacteria belonging to the genus *Microbulbifer*, which our institute had previously isolated from various marine environments, and found that these bacteria also produced 4HBA, although with low production levels (less than one-fifth of that produced by A4B-17). We also show that the alkyl esters of 4HBA produced by strain A4B-17 were effective in preventing the growth of yeasts, molds, and gram-positive bacteria.

Many marine bacteria are free living, while others are symbiotic in character or live in close association with macroorganisms. Thousands of secondary metabolites have been isolated from marine organisms and have been shown to exhibit one or more of a wide range of activities that can be broadly categorized as either attractant, deterrent, or protectant (13, 30). The purpose of our current project is to isolate currently unknown microorganisms from marine environments and to search for novel and/or industrially useful secondary metabolites that they may produce. We have been collecting sponge, alga, ascidian, sea glass, soft coral, sediment, and seawater samples from the Pacific waters of Japan, Palau, Micronesia, and Indonesia. More than 15,000 microorganisms have been isolated from the collected samples and subjected to phylogenetic analysis and screening processes. We report here for the first time a microorganism, a marine bacterium, which produces petrochemicals, i.e., 4-hydroxybenzoate (4HBA) and its alkyl esters (parabens).

MATERIALS AND METHODS

Collection of marine bacteria. Marine organisms and other marine samples, which were found to include bacteria accumulating 4HBA, had been collected in the years 1997 to 2004, as described in Table 1. Seawater was directly spread onto a 1.5% agar plate containing each medium and incubated at 30°C. Colonies that appeared on the plate were collected, and each pure culture was obtained. Marine organisms were disrupted physically, dissolved in seawater, and then used for isolating marine bacteria as described above. All isolates listed in Table 1 were able to grow well in Difco Marine Broth 2216 (MB) at 30°C with shaking.

HPLC analysis. A portion of the culture (200 μ l) was collected each day and mixed with an equal amount of methanol. The mixture was subjected to high-

performance liquid chromatography (HPLC) analysis after being filtered through a 0.45- μ m-pore-size membrane.

An Alliance HPLC instrument (Waters, Milford, Mass.) with an octadecyl silica reverse-phase column (30 cm in length and 3.9 mm in diameter; Waters) was used at 40°C. A mobile phase consisting of solvent A (0.1% trifluoroacetate in water) and solvent B (acetonitrile) was used at a flow rate of 1 ml/min. After injection of a sample, 90% of solvent A was run through the column for 4 min, the solvent gradient was then programmed from 10% to 100% of solvent B over 6 min, and 100% of solvent B was finally run for 4 min. The absorbance at 255 nm of the eluate was monitored with a photodiode array detector (Waters 2996). The respective retention times of 4HBA, butyl 4HBA, heptyl 4HBA, and nonyl 4HBA were 4.8, 10.4, 12.8, and 13.7 min.

GC-MS analysis. A portion of the culture (200 μ l) was collected each day and acidified with hydrochloric acid to pH 2. The metabolites were extracted with 300 μ l of ethyl acetate, and the extract was dried in vacuo and then trimethylsilylated. The resulting sample was subjected to gas chromatography-mass spectrometry (GC-MS) analysis (QP5050A instrument; Shimadzu, Kyoto, Japan) with a DB-5 column (30 m in length and 0.25 mm in diameter; J & W Scientific). The column temperature was initially kept at 50°C for 5 min before being increased to 300°C at a rate of 2.5°C/min. The injection and detection temperatures were both 300°C.

16S rRNA gene sequence determination and analysis. Genomic DNA was extracted by using the InstaGene matrix (Bio-Rad Laboratories, Hercules, Calif.). PCR-mediated amplification of the 16S rRNA gene was carried out by using the universal primers 27F and 1492R (11). The PCR products were purified with a Suprec-02 cartridge (Takara Biochem, Kyoto, Japan) and sequenced with a BigDye terminator cycle sequencing kit, version 3.1 (Applied Biosystems, Foster City, Calif.). An Applied Biosystems 3730 genetic analyzer was used for sequencing. The 16S rRNA sequence was classified by using the Classifier online tool, while the aligned 16S rRNA sequence was created by using the Hierarchy Browser online tool. Both online tools were provided by the Ribosomal Database Project II database release 9 (3). The aligned sequence was edited with the BioEdit program (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and used to create a phylogenetic tree by the neighbor-joining method (19), based on genetic distances calculated from the Kimura two-parameter model (8) by MEGA version 3 (10). The robustness of the topology was evaluated by the neighbor-joining method through 1,000 bootstrap replications (4).

***gyrB* gene sequence determination and analysis.** The *gyrB* gene fragment covering positions 274 to 1525 in the *Escherichia coli gyrB* gene was amplified by using the universal primer set UP1Ei (5'-GAA GTC ATC ACC GTT CTG CAY GSI GGI GGI AAR TTY RA-3') and UP2ri (5'-AGC AGG GTA CGG ATG TGC GAG CCR TCI ACR TCI GCR TCI GTC AT-3'). A total of 35 cycles of amplification

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TABLE 1. 4HBA production in the isolates and type strains

Isolate or type strain	4HBA production (%) ^a	Isolation medium	Geographic origin	Source	Accession no. for <i>gyrB</i> gene
A4B-17	100.0	Seawater + 0.1% alanine	Palau	Ascidian	AB243185
A4A-72	0.6	Seawater	Palau	Sponge	AB243174
V296	8.2	MA	Yap	Sponge	AB243175
A4B-20	1.2	Seawater + 0.1% alanine	Palau	Alga	AB243176
A2C-113	15.5	1/10 MA	Palau	Sponge	AB243177
A4A-79	5.7	Seawater	Palau	Ascidian	AB243178
AM222	9.2	1/10 MA	Palau	Sediment	AB243179
AM292	0.8	1/10 MA	Palau	Sediment	AB243180
A4A-68	4.7	Seawater	Palau	Sponge	AB243181
A2D-17	4.5	Bennett-maltose	Palau	Sponge	AB243182
I-1876	4.4	1/10 MA	Ishigaki, Japan	Alga	AB243183
C-91	3.4	1/10 MA	Yap	Alga	AB243184
HG111	9.5	1/10 MA	Miura, Japan	Sponge	AB243186
F-104	6.9	1/10 MA	Palau	Alga	AB243187
V32	5.0	1/10 MA	Yap	Sponge	AB243188
HG125	1.7	MA	Miura, Japan	Sponge	AB243189
A3G-2	8.1	1/10 MA	Palau	Ascidian	AB243190
A3P2-23-3-2	9.1	Seawater	Palau	Ascidian	AB243191
AM220	0.6	Seawater	Kamaishi, Japan	Sediment	AB243192
YM3-0188	6.4	MA	Palau	Sponge	AB243193
A2D-15	8.4	Bennett-maltose	Palau	Sediment	AB243194
HG869	0.3	1/10 MA	Palau	Ascidian	AB243195
HG279	6.6	MA	Miura, Japan	Sponge	AB243196
ssthio04PA2-36a	7.2	1/10 MA	Palau	Sponge	AB243197
<i>M. maritimus</i> JCM 12187 ^T	18.2		Yellow Sea, Korea	Sediment	AB243198
<i>M. elongatus</i> DSM 6810 ^T	0.2		Braunschweig, Germany		AB243199
<i>M. salipaludis</i> JCM 11542 ^T	8.2		Yellow Sea, Korea	Sediment	AB243200
<i>M. hydrolyticus</i> ATCC 700072 ^T	1.5		Augusta, Ga.	Lignin-rich pulp mill waste	AB243201

^a One hundred percent is defined as 10 mg/liter, the concentration produced by strain A4B-17.

was performed, with template DNA denaturation at 95°C for 1 min, primer annealing at 58°C for 1 min, and primer extension at 72°C for 2 min. The PCR products were purified with Montage PCR_μ96 (Millipore, Bedford, Mass.). Sequencing was performed by using UP1s (5'-GAA GTC ATC ACC GTT CTG CA-3') and UP2rs (5'-AGC AGG GTA CGG ATG TGC GAG CC-3') as described by Yamamoto and Harayama (27). The nucleotide sequence was determined after purifying the products of the DNA sequencing reaction by using Montage SEQ96 (Millipore). ClustalX version 1.83 (21) was used for multiple alignment of the *gyrB* nucleotide sequences. A phylogenetic tree was constructed by the same method as was used for the 16S rRNA sequences.

Antimicrobial activity assay for strain A4B-17. *Bacillus subtilis* subsp. *subtilis* IFO1379^T was incubated with Luria-Bertani medium (10 g/liter of Bacto tryptone, 5 g/liter of yeast, and 5 g/liter of NaCl) overnight at 30°C with shaking. Two hundred microliters of the culture was inoculated into 20 ml of Difco Marine Agar 2216 (MA), which had been kept at 50°C after autoclaving, and the mixture was poured onto a plate. Strain A4B-17 (100 μl), which had been cultured with

MB overnight with shaking, was dropped onto the center of the plate and incubated overnight at 30°C.

Investigation of the MIC. Parabens were purchased from Wako Pure Chemical Industries (Osaka, Japan). They were dissolved in methanol as 10% solutions and used for preparing plates. The microbial strains tested in this study are listed in Table 2 and were purchased from each culture collection center. The culture medium and conditions for each strain were as recommended by each culture collection center. Each strain was cultured overnight and plated on a 1.5% agar plate containing the an alkyl ester of 4HBA before being incubated again overnight. The concentration in the plate on which fewer than five colonies had appeared is defined as the MIC. More than 500 colonies appeared on the control plate containing no alkyl ester.

Nucleotide sequence accession numbers. The nucleotide sequence of 16S rRNA has been deposited in the DDBJ, EMBL, and GenBank sequence databases under accession no. AB243106. The accession numbers for the *gyrB* genes are listed in Table 1.

TABLE 2. Antimicrobial activities of the alkyl esters of 4HBA produced by strain A4B-17

Microorganism	MIC (mg/L)			Combined inhibition by the three esters (%) ^a
	Butyl 4HBA	Heptyl 4HBA	Nonyl 4HBA	
<i>Bacillus subtilis</i> subsp. <i>subtilis</i> IFO 1379 ^T	75	0.4	1.5	100
<i>Proteus vulgaris</i> ATCC 13315	60	2.5	50	30
<i>Staphylococcus aureus</i> subsp. <i>aureus</i> ATCC 6538P	75	0.5	4	100
<i>Aspergillus niger</i> ATCC 9642	55	0.8	50	100
<i>Penicillium chrysogenum</i> ATCC 10002	45	0.8	>400	100
<i>Candida molischiana</i> ATCC 2516	60	0.8	>400	60
<i>Saccharomyces cerevisiae</i> IFO 10217 ^T	52	0.5	20	100
<i>Escherichia coli</i> JM109	>1,200	>100	>400	
<i>Pseudomonas putida</i> ATCC 12633 ^T	>1,200	>100	>400	

^a The respective concentrations of butyl, heptyl, and nonyl 4HBAs produced by strain A4B-17 were 24, 0.4, and 6 mg/liter. These three parabens were mixed at the final concentrations shown to examine the inhibitory effect. One hundred percent means that the three alkyl esters completely inhibited the growth of the microorganism.

TABLE 3. GC-MS analysis of the secondary metabolites accumulated in the culture medium of strain A4B-17

Secondary metabolite ^a	Retention time (min)	<i>m/z</i> of major ion peaks (% ^b)
4HBA	19.1	282 (M ⁺) (22), 267 (100), 223 (81), 193 (59), 126 (19), 73 (54)
Butyl 4HBA	20.8	266 (M ⁺) (32), 251 (34), 210 (82), 195 (100), 193 (77), 151 (34), 135 (20), 89 (31), 73 (58)
Heptyl 4HBA	24.0	211 (30), 210 (100), 195 (87), 193 (51), 73 (35)
Nonyl 4HBA	26.0	211 (38), 210 (100), 195 (81), 193 (48), 73 (33)

^a All secondary metabolites are trimethylsilyl derivatives and were identified by comparing their retention times and mass spectra with those of standards.

^b The percent ion abundance is shown in parentheses.

RESULTS

Secondary metabolites of strain A4B-17. Of our collected microorganisms, marine bacterial strain A4B-17 was isolated from an ascidian in the coastal waters of Palau. Ascidian is a class in the Tunicata subphylum of sac-like marine filter feeders. Strain A4B-17 was cultured with 50 ml of MB for 7 days at 30°C to investigate which secondary metabolites would be produced. The culture medium was subjected to GC-MS and HPLC analyses. The GC-MS analysis indicated that strain A4B-17 produced four compounds, which were confirmed to be 4HBA and butyl, heptyl, and nonyl 4HBAs by comparing their retention times and mass spectra with those of standards (Table 3). As shown in Fig. 1, strain A4B-17 accumulated 10 mg/liter of 4HBA and 24, 0.4, and 6 mg/liter of butyl, heptyl, and nonyl 4HBAs, respectively.

Antimicrobial activities of strain A4B-17. It was considered very surprising that a microorganism produced the alkyl esters of 4HBA, which are more commonly known as the parabens, since the petrochemical products have activity toward a wide range of microorganisms and there had been no previous report describing their microbial synthesis. We can reasonably speculate that strain A4B-17 produces the alkyl esters of 4HBA in order to prevent other microorganisms from growing. The type strain, *Bacillus subtilis* subsp. *subtilis* IFO1379, was used to confirm this hypothesis. Strain A4B-17 was cultured overnight in MB, and 100 μ l of this culture medium was dropped into the center of an MA plate containing strain IFO1379^T. An inhibitory zone was formed around strain A4B-17 after the plate had been incubated overnight at 30°C (Fig. 2).

Antimicrobial activities of parabens produced by strain A4B-17. The MICs of the alkyl esters of 4HBA against several bacteria, molds, and yeasts which had been purchased from culture collection centers were examined (Table 2). Activities against gram-positive bacteria (*B. subtilis* and *Staphylococcus aureus*), yeasts (*Candida molischiana* and *Saccharomyces cerevisiae*), and fungi (*Aspergillus niger* and *Penicillium chrysogenum*) were apparent at a low concentration of each alkyl ester. Nonyl 4HBA alone had no inhibitory effect on the growth of *P. chrysogenum* and *C. molischiana*, while more than 1,200, 100, and 400 mg/liter of butyl, heptyl, and nonyl 4HBAs, respectively, had no inhibitory effect on the growth of the gram-negative bacteria *Escherichia coli* and *Pseudomonas putida*, although they did inhibit *Proteus vulgaris*. Haag and Loncrini (7) have also reported that parabens were more effective against yeasts and molds than against bacteria and were more

FIG. 1. Kinetics of the synthesis of 4HBA and its esters by strain A4B-17.

effective against gram-positive bacteria than against gram-negative bacteria.

When MICs of the three alkyl esters were compared with the concentrations of the alkyl esters accumulated by strain A4B-17 (Table 2), it was found that the inhibitory effects (heptyl 4HBA > nonyl 4HBA > butyl 4HBA) were significantly inversely correlated with the concentrations accumulated by strain A4B-17. A synergistic inhibitory effect was observed when the three alkyl esters were mixed at the concentrations accumulated by strain A4B-17. The mixture completely inhibited the growth of five microbial strains, as shown in Table 2. Aalto et al. (1) have also reported that two or more alkyl esters of 4HBA could effectively inhibit a microorganism's growth. The contents and proportions of the alkyl esters produced by strain A4B-17 seem to be rationally designed for preventing the growth of a wide range of yeasts, molds, and gram-positive bacteria.

Phylogenetic analysis of strain A4B-17. The results of 16S rRNA sequence analysis revealed strain A4B-17 to have the closest phylogenetic affiliation to the genus *Microbulbifer*, which was confirmed by a high bootstrap resampling value

FIG. 2. Inhibitory zone of strain A4B-17 with *Bacillus subtilis* subsp. *subtilis* IFO1379^T.

FIG. 3. Phylogenetic trees based on the neighbor-joining method using the 16S rRNA sequences, demonstrating the phylogenetic position of strain A4B-17 in the *Microbulbifer* genus. Distances were estimated by the Kimura two-parameter model. Bootstrap percentages after 1,000 simulations are shown. The bar represents a 2% estimated sequence divergence.

(Fig. 3). Strain A4B-17 showed 95% 16S rRNA sequence identity with *M. hydrolyticus* IRE-31^T and *M. salipaludis* SM-1^T and 94% 16S rRNA sequence identity with *M. elongatus* ATCC 10144^T.

Physiological and biological characteristics of strain A4B-17.

An electron micrograph of strain A4B-17 is shown in Fig. 4; the strain is aerobic, gram negative, nonmotile, non-spore forming, rod shaped, and approximately 0.3 to 0.5 μm wide and 10 to 100 μm in length. It grew at 15 to 37°C, grew optimally at 30°C, grew at pH 5.5 to 9.5, grew optimally at pH 7.5 to 8.5, and grew optimally in the presence of 1 to 3% NaCl. The guanine-plus-cytosine content was 52.4 mol%, which was determined by HPLC. Starch was hydrolyzed, and gelatin was slowly liquefied. No hydrogen sulfide, colored pigment, or indole was produced, and citrate and agar were not utilized. Catalase, oxidase, lipase, and nitrate reduction were positive. Weak DNase activity was detected, and the strain was negative for the urease β -galactosidase. The respiratory lipoquinone was composed of two unsaturated ubiquinones, 83.9% of a ubiquinone of eight isoprene units (Q8) and 9.1% of a ubiquinone of seven isoprene units (Q7). The fatty acid profile was as follows: 9:0,

0.8%; 10:0, 2.9%; 11:0 iso, 10.7%; 11:0, 1.0%; 10:0 2OH, 1.0%; 10:0 3OH, 2.8%; 12:0, 14.2%; 11:0 iso 3OH, 27.3%; 11:0 3OH, 1.4%; 13:0 iso, 5.8%; 12:0 3OH, 4.8%; 14:0, 0.6%; 13:0 iso 3OH, 0.6%; 15:0 iso, 5.0%; 16:0, 6.6%; 17:1 iso ω 9c, 3.7%; 17:0 iso, 2.3%; 17:0, 0.6%; 18:1 ω 7c, 3.3%; and 17:0 iso 3OH, 1.1%. These characteristics are similar to those of the type strain of *M. salipaludis*, SM-1 (28). Based on these results and phylogenetic analysis, we have named strain A4B-17 *Microbulbifer* sp. strain A4B-17.

Strain A4B-17 had no ability to degrade parabens and 4HBA and no ability to grow with them as sole carbon and energy source.

Characterization of another 23 isolates belonging to *Microbulbifer*. There are 23 other strains belonging to genus *Microbulbifer* that have been isolated from different samples. We collected four type strains of the genus *Microbulbifer*, i.e., *M. maritimus* JCM 12187 (29), *M. elongatus* DSM 6810 (28), *M. salipaludis* JCM 11542 (29), and *M. hydrolyticus* ATCC 700072 (6), from each collection center. The *gyrB* gene fragments of these strains were amplified and sequenced to construct a phylogenetic tree (Fig. 5). This shows that most of our isolates could be grouped separately from the four type strains. The production of 4HBA was observed with all strains, although the amounts were less than 20% of that produced by strain A4B-17 and no parabens could be detected (Table 1).

DISCUSSION

Parabens are stable, effective over a wide pH range, and relatively active against a broad spectrum of microorganisms (7). Several parabens are generally recognized as safe compounds, with a maximum permissible use quantity of 0.1% (7). Parabens have been widely used as preservatives in a wide variety of food, pharmaceutical, and cosmetic products due to their low toxicity to humans and their outstanding antimicrobial activities. Although the mechanism of action of the para-

FIG. 4. Electron micrograph of strain A4B-17, taken with a Hitachi H7000 instrument operated at an accelerating voltage of 75 kV.

FIG. 5. Phylogenetic tree based on the neighbor-joining method and using the *gyrB* sequence to demonstrate the phylogenetic position of strain A4B-17 in *Microbulbifer* and with respect to related bacteria. Estimated distances were obtained by the Kimura two-parameter model. Bootstrap percentages after 1,000 simulations are shown. The bar indicates an estimated 2% sequence divergence.

bens is not well understood, they have been proposed to act by disrupting the membrane transport system (5) or by inhibiting the synthesis of DNA and RNA (14) or of some key enzymes such as ATPases and phosphotransferases (12) in some bacterial species. Parabens are usually prepared industrially by reacting the corresponding alcohols with 4HBA in the presence of an acid catalyst. Bacterial strain A4B-17 seems to synthesize the parabens by a similar reaction but by using an enzyme such as esterase.

The genus *Microbulbifer* was proposed by Gonzalez et al. (6) for a novel rod-shaped and strictly aerobic marine bacterium. *Microbulbifer* belongs to the γ -3 subclass of the *Proteobacteria*, which includes the fluorescent pseudomonads and related genera, the genera *Oceanospirillum* and *Marinobacter*, an Antarctic gas-vacuolated bacterium, and the family *Halomonadaceae* (6). As shown in Table 1, production of 4HBA as a secondary metabolite seems to be a common characteristic of the genus *Microbulbifer*. 4HBA is widely used in the chemical and electrical industries as a material for producing polymers, specifically as a monomer for the formation of liquid crystalline polymers (9). 4HBA is currently synthesized from the petro-

leum product benzene via intermediates of cumene and phenol under high pressure and high temperature with a catalyst.

No native (nonrecombinant) microorganism has previously been reported to accumulate 4HBA as a secondary metabolite. On the other hand, the metabolic pathways for synthesizing 4HBA have been elucidated. Two routes were designed for synthesizing 4HBA by using the genes derived from microbes and plant cells. One involves chorismate lyase, encoded by the *ubiC* gene involved in the ubiquinone biosynthesis pathway in bacteria, which has the ability to directly convert chorismate, an intermediate of the shikimate pathway, into 4HBA (15). The other route involves plant cells and many bacteria having the gene for phenylalanine ammonia lyase, which is responsible for converting L-phenylalanine to cinnamate (25, 26). Cinnamate is then converted to benzoate through a coenzyme A-dependent non-beta-oxidative pathway (16). *A. niger* with a cytochrome P450 system is able to introduce a hydroxy group at the *para* position of benzoate to generate 4HBA (23).

The synthesis of 4HBA was observed when strain A4B-17 was grown in M9 minimal medium (20) containing 0.2% glucose as the sole carbon source. When L-tyrosine, L-phenylala-

nine, L-tryptophan, cinnamate, 4-coumarate, or benzoate was added to a final concentration of 0.1% to the culture medium of strain A4B-17 which had been cultured for 1 day and incubated with shaking for another 7 days, the amount of 4HBA produced was not apparently elevated by addition of any of these compounds (data not shown). These results could support the notion that strain A4B-17 utilized a synthetic pathway to 4HBA coming directly from an intermediate on the shikimate pathway, rather than from aromatic amino acids and other aromatic compounds.

On the other hand, a set of metabolic genes has recently been introduced into a *Pseudomonas* strain for biotransformation of the petroleum product toluene to synthesize 4HBA (17). The *ubiC* gene has also been employed for the biosynthesis of 4HBA from chorismic acid (2, 24). It is, however, important to note that strain A4B-17 without any genetic modification has the ability to synthesize 4HBA from renewable sources such as glucose. It is therefore expected that the genetic engineering of strain A4B-17 could improve the production of 4HBA and open up the possibility of replacing the current chemical processes.

It is a common characteristic of such soil bacteria such as *Comamonas*, *Pseudomonas*, *Rhodococcus*, and *Bacillus* species living on land to have the ability to degrade 4HBA, which is believed to be an intermediate in lignin degradation (16). A new esterase from *Enterobacter cloacae* hydrolyzing the esters of 4HBA has recently been reported (22). The degradation of 4HBA and its esters had been thought to have been acquired from the lignin degradation system. We have discovered for the first time in this study a marine bacterium that did not decompose but rather synthesized 4HBA and its alkyl esters. Although the alkyl esters of 4HBA were first found to have antimicrobial activity by Sabalitschka in 1924 (18) and have been widely used as antimicrobial agents since then, microorganisms having the ability to produce such chemicals have not previously been discovered.

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