1   *Note*

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3   **Subterminal oxidation of *n*-alkanes in achlorophyllous alga *Prototheca* sp.**

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6   Eiji Sakuradani,[1]∗ Yusuke Natsume,[1] Yasushi Takimura,[2] Jun Ogawa,[1] and Sakayu

7   Shimizu[1]

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10   Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University,

11   Oiwake-cho, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan[1]; Tochigi Research

12   Laboratories of Kao Corporation, 2606 Akabane, Ichikai, Haga, Tochigi 321-3497,

13   Japan[2]

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15   **Corresponding author.** TEL: +81-75-753-6114; fax: +81-75-753-6128. E-mail:

16   esakura@kais.kyoto-u.ac.jp (E. Sakuradani).

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1 **Abstract**

2 Some *Prototheca* sp. are known to be involved in *n*-hexadecane degradation.   Two

3 derivatives derived from *n*-hexadecane in such *Prototheca* sp. were identified as

4 5-hexadecanone and 5-hexadecanol.   *n*-Hexadecane was assumed to be converted to

5 5-hexadecanol and then to 5-hexadecanone through a unique subterminal oxidation

6 pathway in such *Prototheca* sp.

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8 **Keywords**

9 Prototheca; *n*-alkane; subterminal oxidation; secondary alcohol; ketone

1   The genus *Prototheca* comprises achlorophyllous heterotrophic microorganisms closely

2   related to a photosynthetic green alga, *Auxenochlorella protothecoides*, on the

3   phylogenetic tree of 18S rDNA sequences (1).   In the 1970s, *Prototheca*

4   *hydrocarbonea* was firstly found to utilize hydrocarbons in crude oil for growth by

5   Kocková-Kratochívlová and Havelková (2).   Walker et al. (3-5) reported that a *P. zopfii*

6   strain isolated from Chesapeake Bay grew on crude oil and mixed hydrocarbons, being

7   able to utilize a large amount of crude oil as carbon sources in comparison with in the

8   case of the hydrocarbon heterotrophy between *P. zopfii* and some crude oil-degrading

9   bacteria.   Koenig and Ward (6) established optimum conditions as to pH, temperature,

10   and salinity for acetate-supported growth of a new isolate, *P. zopfii* Krüger strain

11   UMK-13.   Ueno et al. (7) isolated a thermotolerant strain, *P. zopfii* RND16, which

12   assimilated *n*-alkanes at 38°C and grew well on a mixture of *n*-alkanes and polycyclic

13   aromatic hydrocarbons at 35°C, and established a means of repeated batch cultivation of

14   *P. zopfii* RND16 immobilized in polyurethane foam for bioremediation.

15   *n*-Alkanes are assimilated by a wide variety of microorganisms such as bacteria,

16   yeasts, filamentous fungi, and algae.   Two major pathways for *n*-alkane degradation

17   are known (8): the terminal oxidation pathway, which is more general among

18   microorganisms, and the subterminal oxidation pathway, which, in contrast, has been

19   found in limited examples reported.   Subterminal oxidation involves the hydroxylation

20   at a middle carbon in an *n*-alkane, a secondary alcohol being generated that is then

21   oxidized by an alcohol dehydrogenase to the corresponding ketone.   The ketone is

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1 converted to an ester by a Baeyer-Villiger monooxygenase, that is then cleaved into a

2 primary alcohol and a fatty acid by an esterase.   The primary alcohol is oxidized to the

3 corresponding fatty acid and then enters the β-oxidation pathway.

4   Although *P. hydrocarbonea* and *P. zopfii* are well-known to assimilate

5 hydrocarbons, the metabolic pathway for hydrocarbons has not been elucidated.   In the

6 present study, we examined the *n*-hexadecane biodegradation ability of twenty-one

7 *Prototheca* sp., which were obtained from the Incorporated Administrative Agency

8 National Institute of Technology and Evaluation Biological Resource Center (NBRC)

9 and the Riken Bioresource Center Japan Collection of Microorganisms (JCM) in Japan,

10 and identified derivatives derived from *n*-hexadecane.   All *Prototheca* strains were

11 cultivated in 5 ml of Medium GY (0.5% glucose and 0.5% yeast extract, pH 6.2)

12 supplemented with 2% (v/v) *n*-alkane in a test tube at 28ºC for 5 d with shaking (300

13 strokes/min), unless otherwise stated.

14   Total lipids were extracted from the reaction mixture with chloroform-methanol

15 (1:2, v/v) according to the Bligh-Dyer method (9). The resultant lipids were analyzed

16 with a GC-17A gas-liquid chromatograph (GLC; Shimadzu, Kyoto, Japan) equipped

17 with a TC-WAX capillary column (0.25 mm I.D. × 30 m; GL Sciences, Tokyo, Japan)

18 and a flame ionization detector.   The initial column temperature of 180°C was raised at

19 2°C/min to 240°C and then held for 10 min at 240°C.   1-Hexadecanol was used as an

20 internal standard for quantitative analysis.

21   Total lipids were applied to a Sep-Pak Plus Silica Cartridge (Waters Co., Milford,

1   MA) to remove residual *n*-hexadecane.   *n*-Hexadecane was eluted with 10 ml of

2   *n*-hexane, and then other lipids including the derivatives derived from *n*-hexadecane

3   were eluted with 10 ml of methanol.   The collected fraction containing the derivatives

4   was concentrated with a centrifugal evaporator.   The lipids were separated by high

5   performance liquid chromatography (HPLC, LC-10A system, Shimadzu) on a

6   CLC-ODS (M) column (250 x 4.6 mm I.D.; Shimadzu). The mobile phase was

7   acetonitrile-H_2O (8:2, v/v), the flow rate was 1.0 ml/min, and the effluent was

8   monitored with a refractive index detector.   The purified compounds were subjected to

9   structural determination by means of gas chromatography-mass spectrometry (GC-MS)

10   using a GCMS-OP5050 (Shimadzu) operating at an ionization voltage of 70 eV and

11   proton nuclear magnetic resonance (^{1}H-NMR).   ^{1}H-NMR and [1]H- two dimensional

12   nuclear Overhauser effect spectroscopy ([1]H-NOESY) analytical experiments were

13   performed on a Bruker Biospin DMX-750 (750 MHz for ^{1}H; Bruker Co., Billerica, MA),

14   and chemical shifts were assigned relative to the solvent signal. Compounds were

15   dissolved in dichloromethane-d_2 and the diameter of the tube was 5 mm.

16   Total lipids of *Prototheca* sp. cultivated in Medium GY supplemented with 2%

17   *n*-hexadecane were analyzed by GLC.   Two unknown materials, designated as UK1

18   and UK2, were detected as major compounds on GLC chromatography for *P. zopfii*

19   JCM9400 and *P. zopfii* NBRC6998, respectively (Fig. 1).   These compounds were   Fig. 1

20   assumed to be derivatives of *n*-hexadecane due to their detection with just addition of

21   *n*-hexadecane to the medium.   UK1 and UK2 from *P. zopfii* JCM9400 and *P. zopfii*

1   NBRC6998, respectively, were purified by means of HPLC for their structural

2   identification.   They were analyzed by means of GC-MS, as shown in Fig. 1.   The

3   molecular ion peak of UK1 was detected at m/z 240 (Fig. 1C).   The fragment ion peak

4   at m/z 183 was characteristic of 5-hexadecanone.   The fragment ion peak pattern of

5   UK1 corresponded to that of commercially available 5-hexadecanone used as an

6   authentic standard.   The fragment ion peak of UK2 at m/z 224 indicated its molecular

7   ion peak, from which the molecular weight of H_2O is deducted, as shown in Fig. 1D.

8   The fragment ion peak at m/z 185 was characteristic of 5-hexadecanol.   The fragment

9   ion peak pattern of UK2 corresponded to that of commercially available 5-hexadecanol

10 as an authentic standard.   UK1 and UK2 were purified and then analyzed by ^{1}H-NMR

11 (see supplementary data).   The signals (2.38 ppm, *m*, 4H; 1.56 ppm, *m*, 4H) of UK1

12 indicated the existence of a ketone group.   The signal (3.59 ppm, *m*, 1H) of UK2

13 indicated the existence of a hydroxyl group characteristic of a secondary alcohol.

14 These results corresponded to those for each authentic standard on ^{1}H-NMR analysis.

15 UK1 and UK2 were identified as 5-hexadecanone and 5-hexadecanol, respectively.

16   We measured time courses of decrease of *n*-hexadecane and accumulation of

17 5-hexadecanol and 5-hexadecanone during the cultivation of *P. zopfii* JCM9400 in the

18 medium containing 2% (v/v) *n*-hexadecane (Fig. 2).   *n*-Hexadecane gradually   Fig. 2

19 decreased and its concentration led to 0.2% at 6th day.   The concentrations of

20 5-hexadecanol and 5-hexadecanone reached 1.1 mg/L of broth at 3rd day and 82.6 mg/L

21 of broth at 4th day, respectively, followed by decrease of their amounts with a parallel to

1   the decrease of *n*-hexadecane.   These oxidized products were suggested to be derived

2   from *n*-hexadecane.   On the other hand, we recognized the generation of pentadecanoic

3   or hexadecanoic acid in addition with 1-pentadecanol or 1-hexadecanol to the culture

4   medium, respectively (data not shown).   This indicates that *P. zopfii* JCM9400 has a

5   conversion activity of a primary fatty alcohol to the corresponding fatty acid.   Even if

6   *n*-pentadecane was added to the culture medium for *P. zopfii* JCM9400, we detected

7   neither 1-pentadecanol nor pentadecanoic acid in the total lipids prepared according to

8   the Bligh-Dyer method (data not shown).   This indicates that *P. zopfii* JCM9400 has no

9   conversion activity of pentadecane to 1-pentadecanol as the first step of terminal

10   oxidization for *n*-alkanes.

11   Twenty-one strains of *Prototheca* sp. obtained from microbial type culture

12   collections in Japan were examined as to their *n*-hexadecane degradation abilities.

13   Some of them, capable of using *n*-hexadecane as a carbon source, accumulated

14   5-hexadecanone and 5-hexadecanol (Table 1).   *Prototheca wickerhamii*, *Prototheca*   Table 1

15   *moriformis*, and *Prototheca stagnora* JCM9641 did not accumulate any derivatives

16   derived from *n*-hexadecane.   *Prototheca zopfii* JCM9400 exhibited the highest

17   production (238.3 mg/L of cultural broth) of 5-hexadecanone.   *P. zopfii* NBRC6998, *P.*

18   *zopfii* NBRC7532, *P. zopfii* NBRC7533, and *Prototheca eriobotryae* NBRC32449

19   exhibited high 5-hexadecanol, rather than 5-hexadecanone, production.

20   Thus far, microbial degradation of hydrocarbons through the subterminal

21   oxidation pathway has been reported.   Bacteria and many methanotrophs co-oxidize

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short-chain alkanes via terminal as well as subterminal oxidation (10, 11). Subterminal oxidation has also been found for longer alkanes in *Penicillium*, *Bacillus*, *Pseudomonas* (12), and *Rhodococcus* sp. (13). However, microorganisms that accumulate 5th-positional-oxidation derivatives of *n*-hexadecane have not yet been reported. In the present study, we first found that *Prototheca* sp., especially *P. zopfii* JCM9400, exhibit high *n*-hexadecane degradation abilities, and accumulate unique oxidative products such as 5-hexadecanone and 5-hexadecanol. In *P. zopfii* JCM9400, *n*-hexadecane is presumed to be oxidized to 5-hexadecanol and then to 5-hexadecanone through general subterminal oxidation steps. Fatty alcohols like 5-hexadecanol can be a component of waxes by formation of an ester with some acids. Ketones like 5-hexadecanone can be converted to epoxy compounds by oxidation to be used as resin materials. As shown in Fig. 1, minor derivatives other than those identified in this study are present in some *Prototheca* sp. We need to elucidate the subterminal oxidation pathway for *n*-hexadecane in *Prototheca* sp. through identification of the minor derivatives.

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16

1   **Figure legends**

2

3   FIG. 1.   GLC chromatograms of lipids from *Prototheca zopfii* JCM9400 (A) and P.

4   zopfii NBRC6998 (B).   GC-MS spectra and deduced structural formulae of UK1 (C)

5   and UK2 (D). These strains were cultivated in medium supplemented with

6   *n*-hexadecane.

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8   FIG. 2.   Time courses of concentrations of *n*-hexadecane (solid circle), 5-hexadecanol

9   (open triangle), and 5-hexadecanone (open square) on the cultivation of *P. zopfii*

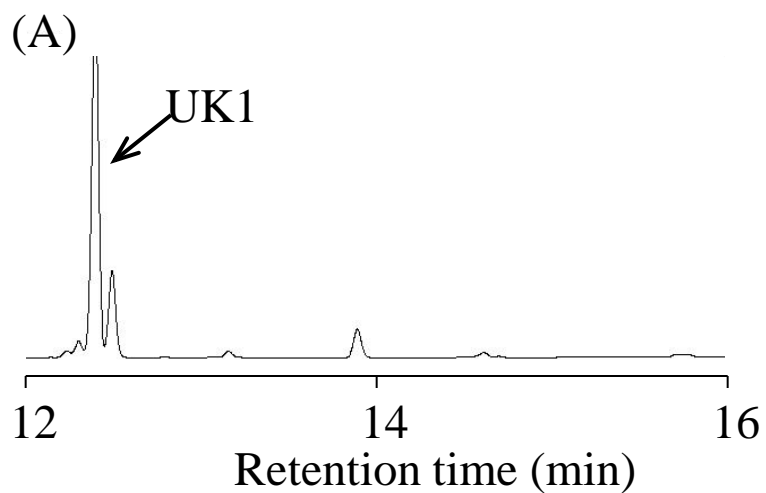10   JCM9400 in the medium containing 2% (v/v) *n*-hexadecane.
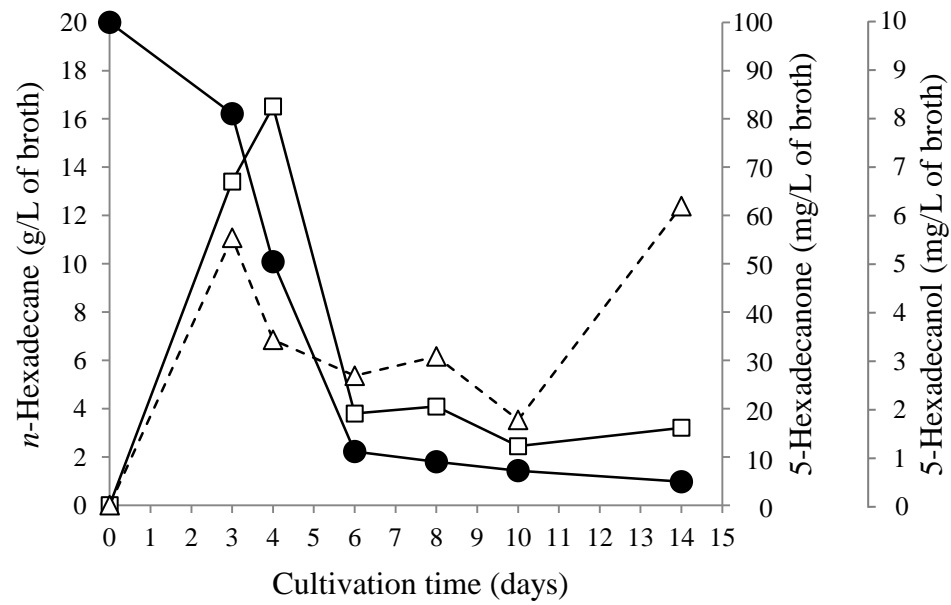
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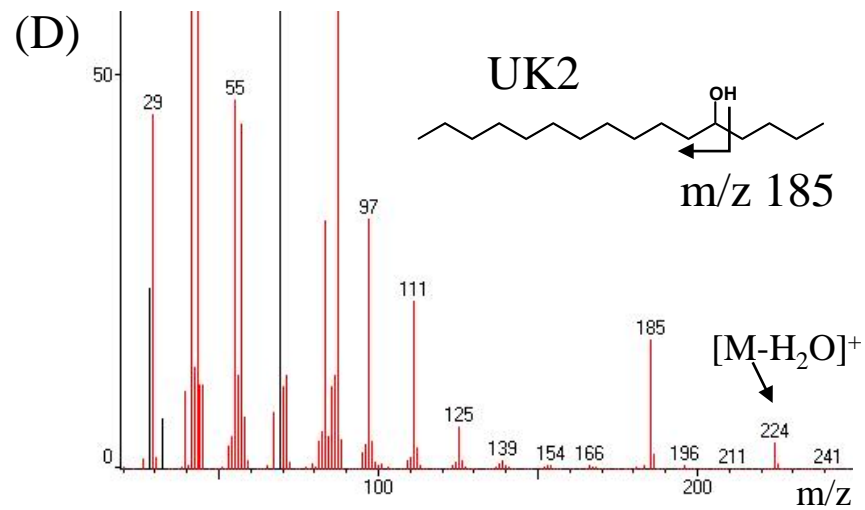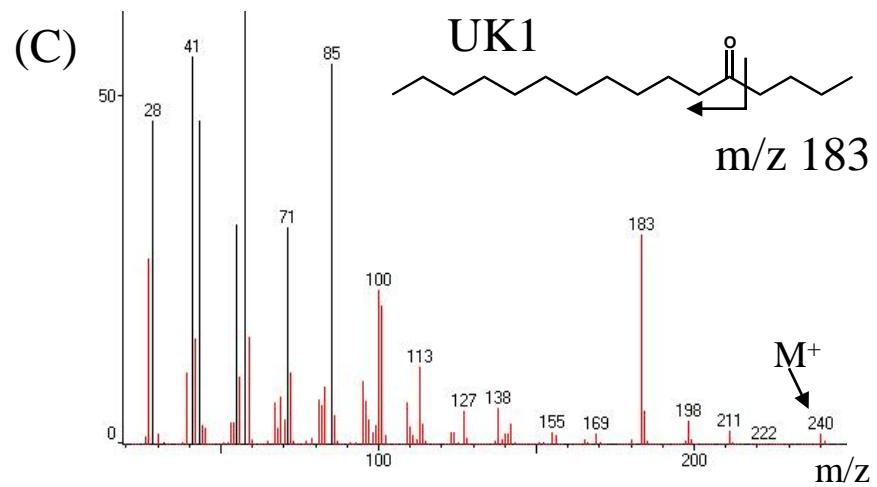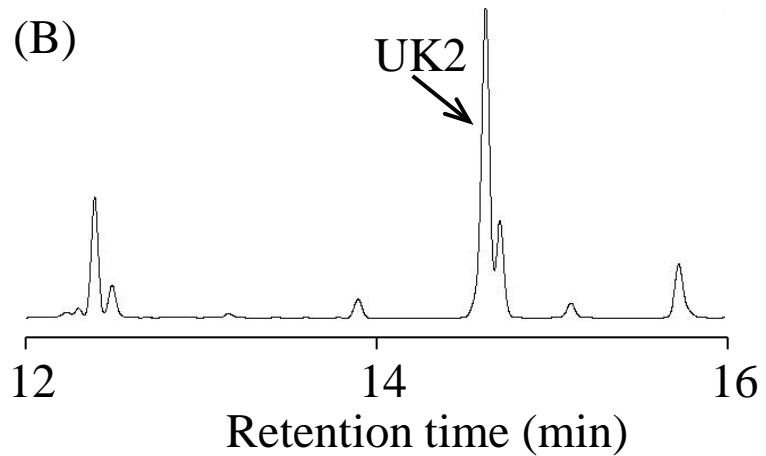
Fig. 1. Sakuradani et al.

Fig. 2. Sakuradani et al.

1   TABLE 1.   Comparison of the amounts of 5-hexadecanone and 5-hexadecanol
2   produced by *Prototheca* sp.[a]

Strain	5-Hexadecanone (mg/L)	5-Hexadecanol (mg/L)
Prototheca zopfii JCM9400	238.3	6.8
P. zopfii JCM9646	10.3	16.6
P. zopfii NBRC6998	14.2	22.6
P. zopfii NBRC7532	2.1	26.3
P. zopfii NBRC7533	2.5	22.2
P. zopfii NBRC7534	3.0	13.1
P. zopfii NBRC7535	43.3	7.8
P. zopfii NBRC7536	46.8	9.0
P. trispora NBRC6996	108.0	11.4
P. eriobotryae NBRC32449	13.8	26.1
P. thermodurica JCM8557	13.5	2.2
P. stagnora JCM9641	-[b]	-
P. stagnora JCM9642	11.0	2.6
P. portoricensis NIES-2182	2.3	5.9
P. moriformis JCM9640	-	-
P. moriformis JCM9729	-	-
P. moriformis NBRC6995	-	-
P. wickerhamii JCM9643	-	-
P. wickerhamii JCM9644	-	-
P. wickerhamii JCM9645	-	-
P. wickerhamii NBRC6997	-	-

29   [a]*Prototheca* sp. were cultivated in Medium GY supplemented with *n*-hexadecane (final
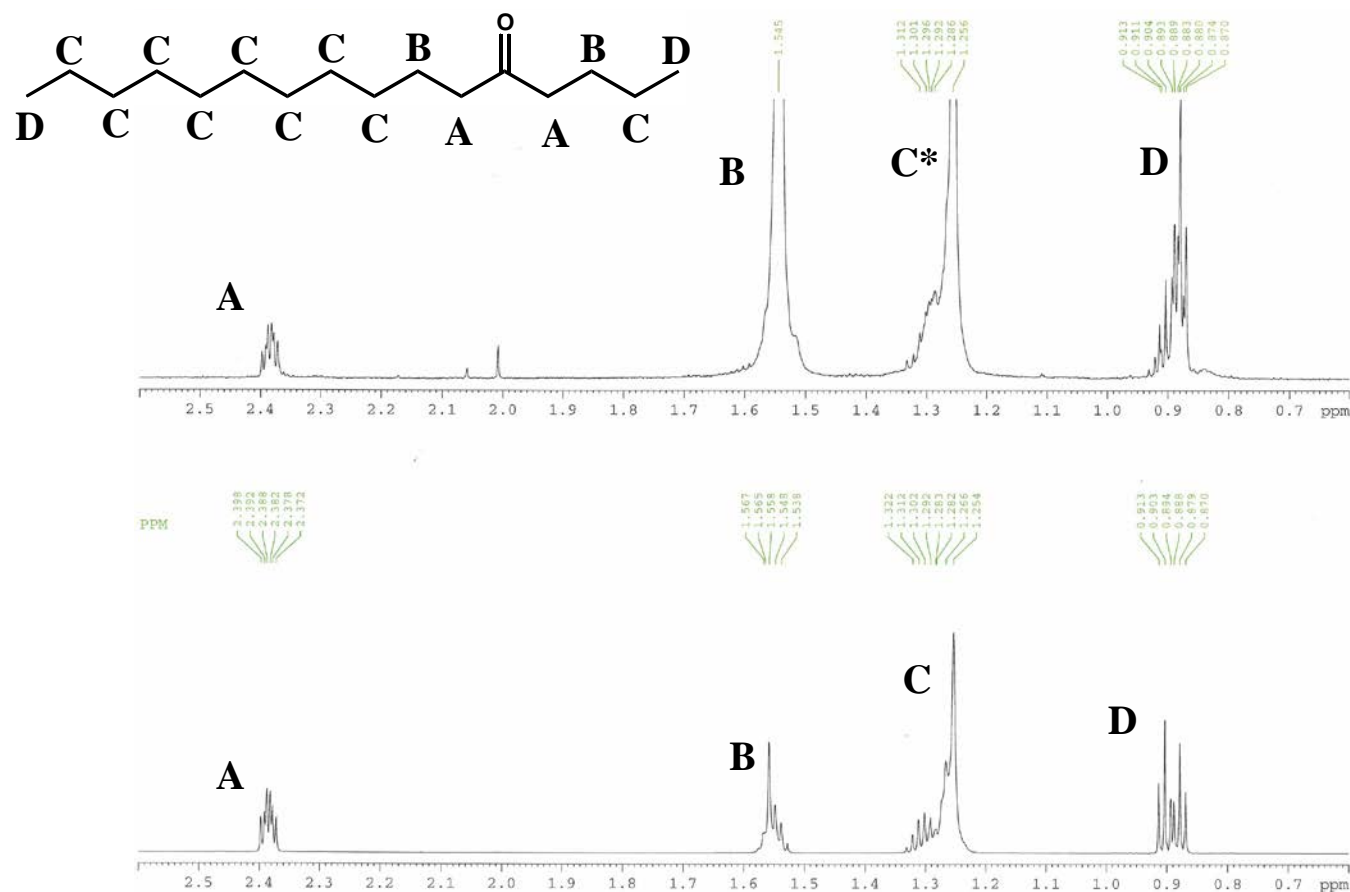30   concentration, 2%).
31   [b]-, not detected.

FIG. S1. Comparison of ^{1}H-NMR spectra of UK1 (top) and authentic 5-hexadecanone (bottom). The letters (A-D) indicate the protons corresponding to individual peaks. The C spectrum of UK1 (top) contains a signal derived from H_2O as an impurity.
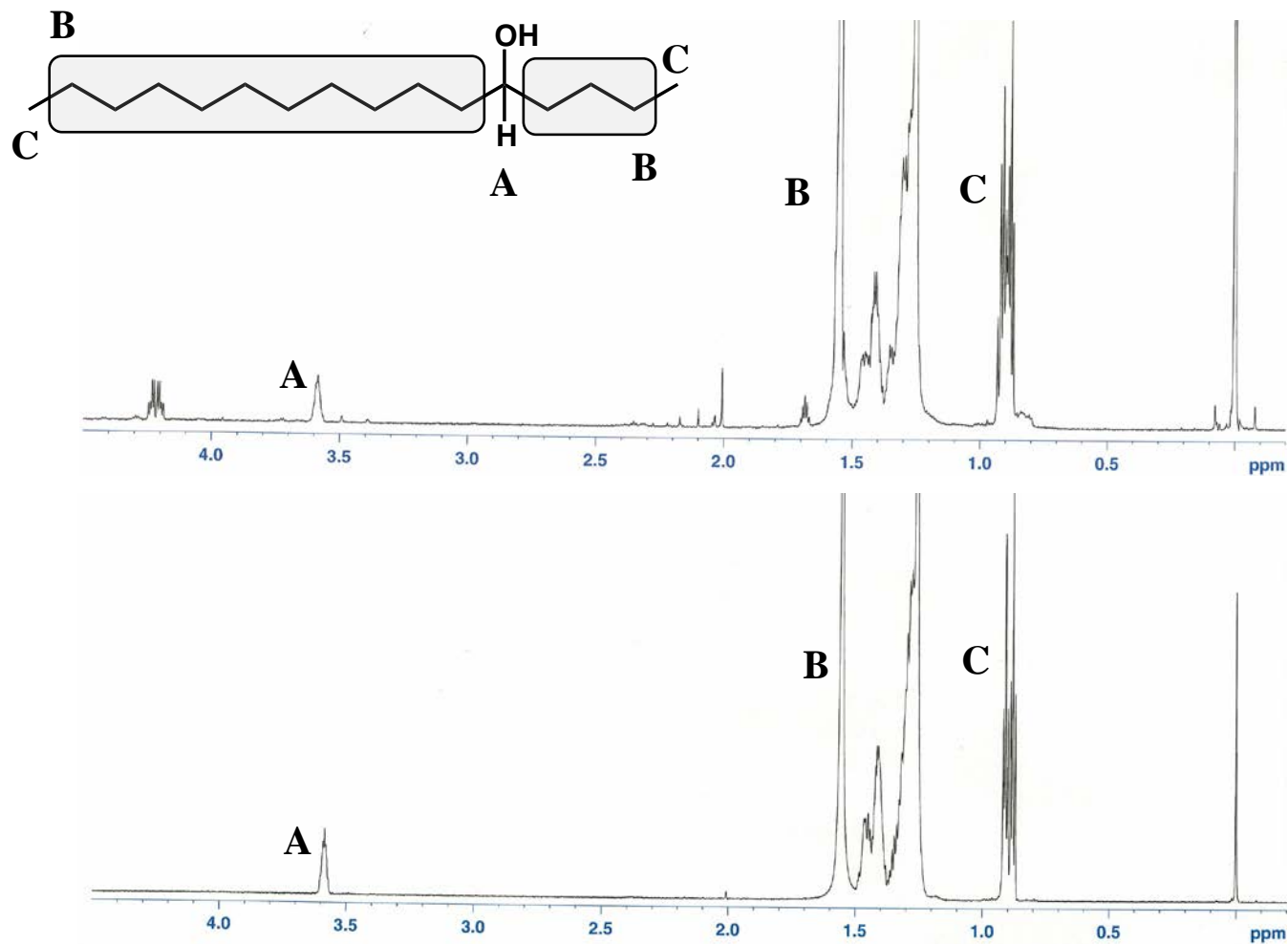
FIG. S2. Comparison of ^{1}H-NMR spectra of UK2 (top) and authentic 5-hexadecanone (bottom). The letters (A-C) indicate the protons corresponding to individual peaks.