Microbial Degradation of Disinfectants. A New Chlorhexidine Degradation Intermediate (CHDI), CHDI-C, Produced by *Pseudomonas* sp. Strain No. A-3

Teruo Tanaka,*,[a] Sawa Murayama,[a] Natuki Tuda,[a] Masami Nishiyama,[a] Kazuko Nakagawa,[b] Yasuko Matsuo,[b] Youichiro Isohama,[b] and Yutaka Kido[a]

[a]*Division of Instrumental Analysis, Institute of Resource Development and Analysis, and* [b]*Laboratory of Medicinal Microbiology, Faculty of Pharmaceutical Sciences, Kumamoto University, 5–1, Oe-honmachi, Kumamoto 862–0973, Japan*
(Received November 18, 2004; Accepted January 24, 2005)

To clarify the degradation pathway of chlorhexidine by a microbe, *Pseudomonas* sp. Strain No. A-3, the isolation and identification of microbial chlorhexidine degradation products were attempted. A new chlorhexidine degradation intermediate (CHDI), named CHDI-C, was isolated by extraction with ethylacetate, *n*-butanol, column chromatography using Silica gel, and purified by preparative thin layer chromatography. The chemical structure of this product was examined by infrared, ^{1}H NMR, ^{13}C NMR and fast atom bombardment mass spectra studies. Based on the spectroscopic data, this product was assumed to be a modified compound of chlorhexidine (molecular weight, MW; 530). From the proposed structure, CHDI-C was assumed to be a new chlorhexidine degradation intermediate.

Key words —— chlorhexidine, disinfectant, degradation products, chlorhexidine degradation intermediate-C, chlorhexidine degradation intermediate, modified degradation pathway

*To whom correspondence should be addressed: Division of Instrumental Analysis, Institute of Resource Development and Analysis, Kumamoto University, 5–1, Oe-honmachi, Kumamoto 862–0973, Japan. Tel. & Fax: +81-96-371-4809; E-mail: tfujii@gpo.kumamoto-u.ac.jp

INTRODUCTION

Chlorhexidine (N, N″-Bis(4-chlorophenyl)3-12-diamino-2, 4, 11, 13-tetraazatetradecanediimidamide, CH) is active against Gram-positive, -negative bacteria and fungi. CH is released into the environment through its widespread use as a disinfectant in hospitals and medical schools (amount used 60.8 t, 2003. 4–2004. 3, Sumitomo Chemical Industries, Ltd.). The microbial degradation of CH, however, has not yet been clarified, though its treatment with activated sludge has been reported.[1,2] We attempted to isolate from activated sludge the microorganisms able to degrade CH, and to examine the chlorhexidine-degrading activity of isolates.[3] We also reported the treatment by activated sludge of CH[4] and determination of CH in waste waters by HPLC.[5] *Pseudomonas* sp. strain No. A-3 isolated from the sludge was able to utilize CH as the sole nitrogen source for growth.[6] This strain was found to form transient intermediates, detectable on HPLC, during microbial degradation.[6] To clarify the mechanisms of action on CH by this strain, we attempted to isolate the chlorhexidine degradation intermediates (CHDI) and chlorhexidine aromatic degradation products (CHADP). CHDI-B, CHDI-BR and CHDI-D were the pyruvate bond compounds to CH[7] and CHADP-5[8] was product of direct degradation of CH. From these results, we concluded that CH was degraded *via* two pathways (one is a modified degradation pathway and the other is a direct degradation pathway) by strain No. A-3. To clarify the two degradation pathways of CH by this strain, we attempted to isolate the chlorhexidine degradation products from the cultue filtrate. In this paper, the isolation and chemical structure of CHDI-C, are described, together with the relationship between CHDI-B and C.

MATERIALS AND METHODS

Materials —— Chlorhexidine gluconate (20% solution) was purchased from Sumitomo Chemical Industries, Ltd. (Osaka, Japan) and was purified before using. All other chemicals were guaranteed to be of the best grade commercially available.

Apparatus, Analysis, and Measurement of Chlorhexidine Degradation Intermediates —— HPLC was done using jacketed stainless steel analytical column (2500 × 4 mm i.d.) packed with Lichrosorb RP-select B (7 μm). The mobile phase

Table 1. Antimicrobial Activities of Chlorhexidine and Chlorhexidine Degradation Intermediates (CHDI-B and -C)

Microorganism	MIC (μg/ml)		
	Chlorhexidine	CHDI-B	CHDI-C
Bacillus subtilis PCI-219	10	100	100
Staphylococcus aureus IFO-3060	10	50	50
Escherichia coli IFO-3301	20	200	200
Proteus vulgaris IFO-3167	20	200	200
Pseudomonas aeruginosa IFO-3923	100	1000	1000
Serratia marcescens IFO-3046	100	$>$ 1000	$>$ 1000

was methanol-water (75 : 25, v/v, pH 4.0) containing 0.005 M heptanesulfonic acid.[3] Detection and measurement of chlorhexidine degradation intermediates were measured by HPLC using the method of Huston[9] as described in the previous paper.[3] The IR spectrum was taken in KBr tablets on a JEOL JIR-6500W infrared spectrophotometer. Fast atom bombardment mass spectrum (FAB-MS) with glycerol as a matrix was recorded with a JEOL JMS-DX303HF ion source (3 kV accelerating potential) and the JMS 3500 data system. A xenon atom beam source (6 kV accelerating potential) was used. ^{1}H, ^{13}C NMR, ^{1}H-^{1}H correlation spectroscopy (COSY) and heteronuclear multiple quantum coherance (HMQC) spectra were taken on JEOL JNM GX-400 spectrometers in CD_3OD solution with trimethylsilyl (TMS) as an internal standard.

Microorganisms and Fermentation —— Strain No. A-3 isolated from activated sludge, as described in the previous paper,[3] was used for the microbial degradation of CH. Stock cultures of this strain were maintained in nutrient agar slants containing CH at 2000 μg/ml or growth medium agar slants[3] containing CH at 200 μg/ml. The growth medium used for nitrogen-limited growth was described in the previous paper.[3] Shaking culture fermentations were carried out with 30 ml of a growth medium in 200 ml Erlenmeyer flasks on a rotary shaker (180 rpm). Suspension from a slant culture were inoculated into a growth medium containing CH (100 μg/ml). The seed culture was incubated for 48 hr at 37°C on a rotary shaker (180 rpm). 500 ml of the resultant seed culture was inoculated in a 30-l jar fermentor containing 13 l of growth medium containing CH (100 μg/ml). Fermentation was carried out for 96 hr or 1 week at 37°C with agitation (350 rpm) and aeration (15 l/min).

Purification of CHDI-C —— All the purification steps were monitored by HPLC. The culture filtrate (12 l) was used as a starting material for the isolation of CHDI-C. The culture filtrate was adjusted to pH 9.0 with 1 N NaOH and extracted twice with ethylacetate (1/3 volume of culture filtrate) to remove CH. Aqueous layer was extracted twice with *n*-butanol (1/4 volume of aqueous layer). The *n*-butanol layer was concentrated in vacuo to obtain crude powder. The crude powder was then subjected to column chlomatography on silica gel. The column was developed with solvent mixture (*n*-propanol-ethylacetate-2.5% aqueous ammonia = 3 : 4 : 1) to obtain crude CHDI-C. Purification of CHDI-C was carried out on preparative TLC used with solvent mixture A (*n*-propanol-ethylacetate-2.5% aqueous ammonia = 3 : 2 : 1) and B (*n*-butanol-ethylacetate-water = 3 : 4 : 1). CHDI-C fraction was eluted with methanol from TLC and concentrated in vacuo to obtain purified CHDI-C (6.0 mg).

Minimum Inhibitory Concentration (MIC) of CHDI-C —— The serial agar dilution method[10] was applied in this study using a nutrient agar as an assay medium. The test organisms used for the study are given in Table 1.

RESULTS AND DISCUSSION

Detection of Chlorhexidine Degradation Intermediate, CHDI-B and C, Produced by *Pseudomonas* sp. Strain No. A-3

The HPLC chromatograms of culture filtrate of strain No. A-3 are shown in Fig. 1. The retention times of CHDI-C, CHDI-B and CH were 4.1, 5.2 and 6.4 min, respectively. At first, CHDI-B was formed with the decrease of CH. On the other hand, CHDI-C appeared after 36 hr of incubation. In the case of the CHDI-B instead of CH as the sole nitrogen source for growth, the HPLC chromatograms of culture filtrate of strain No. A-3 also revealed the

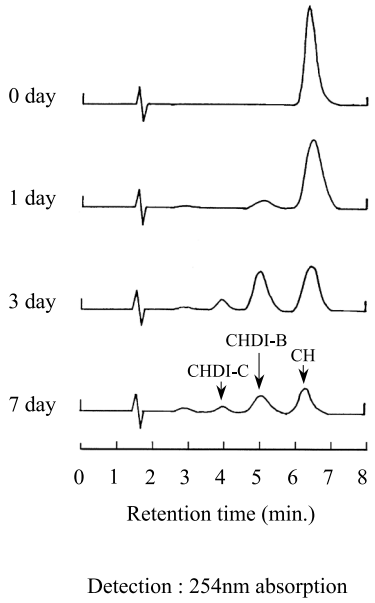

Fig. 1. HPLC Chromatograms of Culture Filtrate of Strain No. A-3

decrease of CHDI-B and emergence of CHDI-C. It was assumed that CHDI-B was converted to CHDI-C.

Structure Elucidation of CHDI-C

CHDI-C is a white powder with a mp of 140°C. It was positive to the sakaguchi reaction. The compound was soluble in methanol or ethanol, but insoluble water and chloroform. The IR absorptions were observed at 3480, 1500, 1640 and 1430 cm^{-1} in the IR spectrum due to the amino, imino and methylene groups, respectively. The ^{1}H and ^{13}C-NMR spectra of CHDI-C are shown in Fig. 2. From the results of ^{1}H, ^{13}C and HMQC experiment, single methylene (δ_H 4.2, δ_C 124) and methine (δ_H 4.6, δ_C 125) signals were recognized in addition to those of chlorhexidine. In the ^{1}H-^{1}H COSY spectrum, a correlation between the methylene and methine signals was recognized. The molecular ion peak of CHDI-C was obtained at *m/z* 531 $(M + H)^+$ by positive FAB-MS. The molecular weight of CH and CHDI-B is 504, and 576, respectively. From the result of the CHDI-B and C production (Fig. 1), the structure of CHDI-B[8] and FAB-MS of CHDI-C, the CHDI-C was thought to be produced by decarboxylation of CHDI-B and the loss of carbon dioxide (Fig. 3).

Biological Properties of CHDI-C

The antimicrobial spectra of CHDI-B and C are shown in Table 1. The antimicrobial activity of

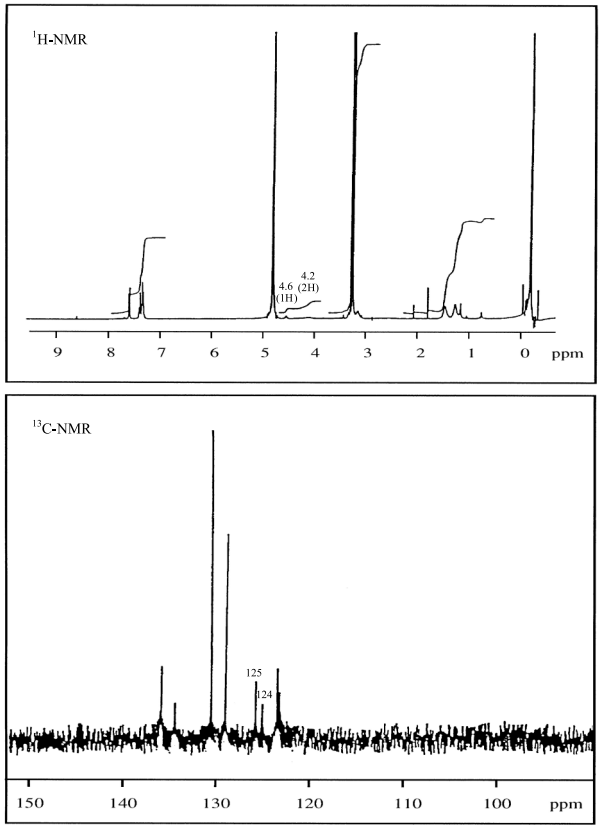

Fig. 2. ^{1}H- and ^{13}C-NMR Spectra of CHDI-C in CD_3OD (400 MHz)

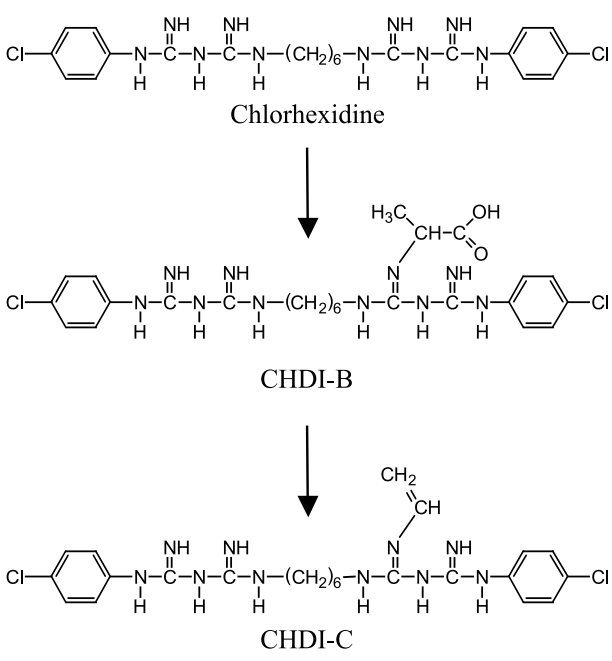

Fig. 3. Structure of Chlorhexidine and Proposed Structures of CHDI-B and CHDI-C

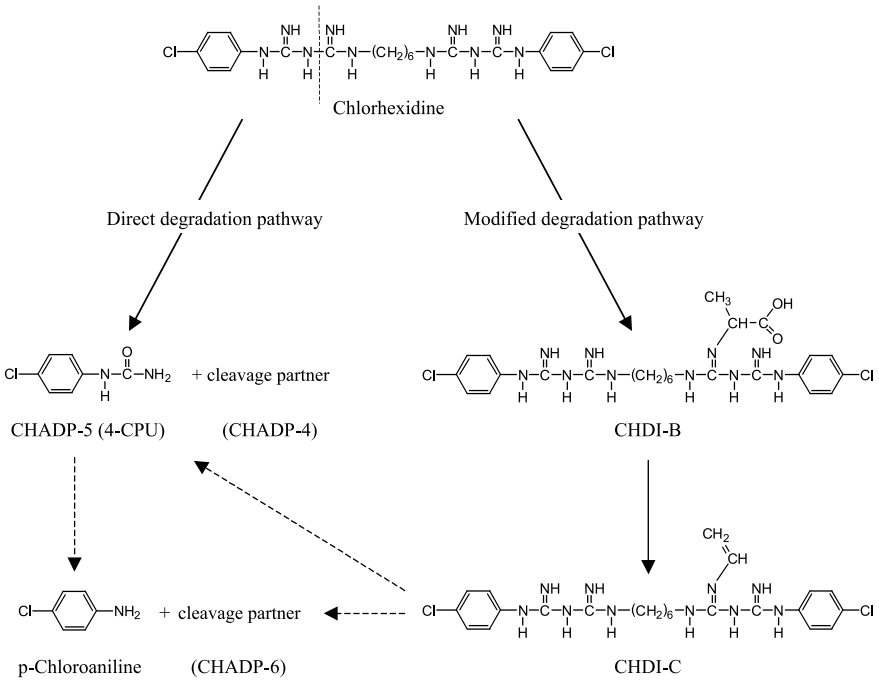

Fig. 4. Degradation Pathway of Chlorhexidine by Strain No. A-3

CHDI-B, -C, -BR[7] and D[7] decreased to 1/5–1/10 that of CH. It was suggested that A-3 attaches pyruvate to the guanidyl group of CH, and decreases the antimicrobial activities. *Serratia marcescens* IFO-3046 became resistant to CH and also modified CH to CHDI-B.[11] It was suggested that one mechanism of resistance to CH by some bacteria may be related to CHDI-B formation. On the other hand, CHADP-5 (*p*-chlorphenylurea) and *p*-chloraniline do not have antimicrobial activity. Strain No. A-3 directly utilizes CH as a sole nitrogen source for growth. It is interesting to note that this strain has two degradation pathways (Fig. 4), one is a modified degradation pathway and the other is a direct degradation pathway.

REFERENCES

1) Sayama, N. (1981) Microbiological studies on waste water treatment by medical school and hospitals. (III) Treatment of benzalkonium chloride and chlorhexidine. *Jpn. J. Hyg*., **35**, 869–873.
2) Sakagami, Y., Yokoyama, H. and Ose, Y. (1983) Degradation of some disinfectants by activated sludge. *Jpn. J. Toxicol. Environ. Health*, **29**, 342–351.
3) Kido, Y., Yokoyama, H., Uraki, F., Uyeda, M., Tsuruoka, M. and Shibata, M. (1988) Microbial degradation of disinfectants. I. chlorhexidine-degrading bacteria isolated from activated sludge. *Jpn. J. Toxicol. Environ. Health*, **34**, 10–14.
4) Kodama, H., Hashimoto, T., Tsuruoka, M., Kido, Y., Uyeda, M. and Shibata, M. (1988) Microbial degradation of disinfectant IV. Treatment by activated sludge of chlorhexidine. *Jpn. J. Toxicol. Environ. Health*, **34**, 408–413.
5) Kido, Y., Kodama, H., Uraki, F., Uyeda, M., Tsuruoka, M. and Shibata, M. (1988) Microbial degradation of disinfectants. III. Determination of chlorhexidine in waste waters by high-performance liquid chromatography after pretreatment. *Jpn. J. Toxicol. Environ. Health*, **34**, 319–324.
6) Kido, Y., Kodama, H., Uraki, F., Uyeda, M., Tsuruoka, M. and Shibata, M. (1988) Microbial degradation of disinfectants. II. Complete degradation of chlorhexidine. *Jpn. J. Toxicol. Environ. Health*, **34**, 97–101.
7) Kido, Y., Kodama, H., Isohama, Y., Uyeda, M. and Shibata, M. (1990) Microbial degradation of disinfectants. V. A new lipophilic degradation product of chlorhexidine. *Jpn. J. Toxicol. Environ. Health*, **36**, 139–142.
8) Uyeda, M., Shiozaki, T., Yokomizo, K., Suzuki, K., Uraki, F., Tanaka, T. and Kido, Y. (1996) Microbial degradation of disinfectants. Structures of chlorhexidine degradation intermediates, CHDI-B, CHDI-BR and CHDI-D, produced by *Pseudomonas* sp. Strain No. A-3. *Jpn. J. Toxicol. Environ. Health*,

42, 121–126.
9) Huston, C. E., Wainwright, P. and Cooke, M. (1982) High-performance liquid chromatographic method for the determination of chlorhexidine. *J. Chromatogr*., **237**, 457–464.
10) Waksman, S. A. and Reilly, H. C. (1945) Agar-streak method for assaying antibiotic substances. *Ind. Eng. Chem. Anal. ED*., **17**, 556–557.
11) Uyeda, M., Shiozaki, T., Enokida, T., Watanabe, K., Komiya, K., Yokomizo, K. and Suzuki, K. (1996) Microbial degradation of Chlorhexidine by Clinical isolates. *Jpn. J. Toxicol. Environ. Health*, **42**, 312–318.