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Microbial Degradation of Disinfectants: Two New Aromatic Degradation Products of Chlorhexidine, Chlorhexidine Aromatic Degradation Product (CHADP)-4 and CHADP-6, Produced by *Pseudomonas* sp. Strain No. A-3

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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. Two aromatic degradation products of chlorhexidine, named chlorhexidine aromatic degradation product (CHADP)-4 and CHADP-6, were isolated by column chromatography using Diaion HP-10, and purified by column chromatography using Diaion HP-20SS and Sephadex LH-20. The chemical structures of both compounds were examined by infrared, ^{1}H NMR, ^{13}C NMR and fast atom bombardment (FAB) mass spectra studies. Based on the spectroscopic data, CHADP-4 (molecular weight 335) and CHADP-6 (molecular weight 377) were found to be direct degradation products of chlorhexidine and were thought to be cleavage partners of *p*-chlorophenylurea (CHADP-5) and *p*-chloraniline (p-CA), respectively. Antimicrobial activity of CHADP-6 are similar to that of chlorhexidine, but antimicrobial activity of CHADP-4 decreased to 1/5–1/10 that of chlorhexidine.

Key words —— chlorhexidine, *Pseudomonas* sp., microbial degradation, direct degradation pathway

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INTRODUCTION

Chlorhexidine (N, N″-bis(4-chlorophenyl)3-12-diamino-2,4,11,13-tetraazatetradecanediimid-amide, CH) is released into the environment through its widespread use as a disinfectant in hospitals and medical schools. 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] *Pseudomonas* sp. Strain No. A-3 isolated from the sludge was able to utilize CH as the sole nitrogen source for growth.[4] We also reported the treatment by activated sludge of CH[5] and determination of CH in waste waters by HPLC.[6] *Pseudomonas* sp. Strain No. A-3 was found to form transient intermediates, detectable on HPLC, during microbial degradation.[4] To clarify the mechanisms of action on CH by this strain, we attempted to isolate the chlorhexidine products.[7–9] 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. Chlorhexidine degradation intermediates (CHDI), CHDI-B, CHDI-C, CHDI-D and CHDI-BR were modified compounds of CH. CHDI-B, CHDI-BR and CHDI-D were the pyruvate bond compounds of chlorhexidine.[8] CHDI-C was the degradation product of CHDI-B by *Pseudomonas* sp. Strain No. A-3.[9] On the other hand, chlorhexidine aromatic degradation product (CHADP), CHADP-5[7] was a direct degradation compound of CH. To clarify the two degradation pathways of CH by this strain, we attempted to isolate the CHADPs from the culture filtrate. In this paper, the isolation and chemical structure of CHADP-4 and CHADP-6, are described, together with the antimicrobial activities of both compounds.

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 CHADPs —— HPLC was done using jacketed stainless steel analytical column (2500 × 4 mm i.d.) packed with Lichrosorb RP-select B (7 μm). The

Table 1. Antimicrobial Activities of CH and Chlorhexidine Aromatic Degradation Products (CHADP-4 and CHADP-6)

Microorganism	MIC (μg/ml)		
	CH	CHADP-4	CHADP-6
Bacillus subtilis PCI-219	10	100	10
Staphylococcus aureus IFO-3060	10	50	10
Escherichia coli IFO-3301	20	200	20
Proteus vulgaris IFO-3167	20	200	20
Pseudomonas aeruginosa IFO-3923	100	1000	100
Serratia marcescens IFO-3046	100	$>$ 1000	100

mobile phase was methanol-water (70 : 30, v/v, pH 4.0) containing 0.005 M heptanesulfonic acid.[3] Detection and measurement of CHADPs were measured by HPLC using the method of Huston[10] 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 estimated by using a JEOL JMS-DX303HF MS spectrometer. ^{1}H, ^{13}C NMR, ^{1}H-^{1}H correlation spectroscopy (COSY), heteronuclear multiple quantum coherance (HMQC) and heteronuclear multiple bond correlation (HMBC) spectra with trimethylsilyl (TMS) as an internal standard were taken in CD_3OD at 400 MHz on a JEOL JNM GX-400 spectrometer.

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 was 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 1 week at 37°C with agitation (350 rpm) and aeration (15 l/min).

Purification of CHADP-4 and CHADP-6 —— All the purification steps were monitored by HPLC. The culture filtrate (12 l) was used as a starting material for the isolation of CHADP-4 and CHADP-6. The culture filtrate was adsorbed on a Diaion HP-10 column (500 $\times$ 34 mm i.d.). The column was washed with 500 ml water and developed with 500 ml each of 60, 80, and 100% methanol. Crude CHADP-4 and CHADP-6 were eluted with 80% methanol and 100% methanol. The fractions were evaporated *in vacuo*. The crude fractions were dissolved with a small amount of methanol and evaporated *in vacuo* with a small amount of Diaion HP-20SS (1 g). This sample was added on a Diaion HP-20SS column (700 $\times$ 20 mm i.d.). The column was washed with 200 ml of water and eluted with 80% methanol containing 0.05 N HCl. The respective fractions of CHADP-4 or CHADP-6 concentrated *in vacuo* were dissolved in a small amount of methanol and applied to Sephadex LH-20 column (700 $\times$ 15 mm i.d.). The fractions containing CHADP-4 or CHADP-6 were pooled and concentrated *in vacuo* to obtain purified CHADP-4 (9.0 mg) or CHADP-6 (7.2 mg).

Minimum Inhibitory Concentration (MIC) of CHADP-4 and CHADP-6 —— The serial agar dilution method[11] 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 Patterns of CH by *Pseudomonas* sp. Strain No. A-3

The HPLC chromatogram of CH in the incubation mixture of Strain No. A-3 is shown in Fig. 1. The retention times of CHADP-4, CHADP-6 and CH were 4.2, 5.1 and 12.0 min, respectively. Products of the modified degradation pathway (CHDI-B, CHDI-C, CHDI-BR, CHDI-D) varied in retention times from 8 to 11 min.[8,9]

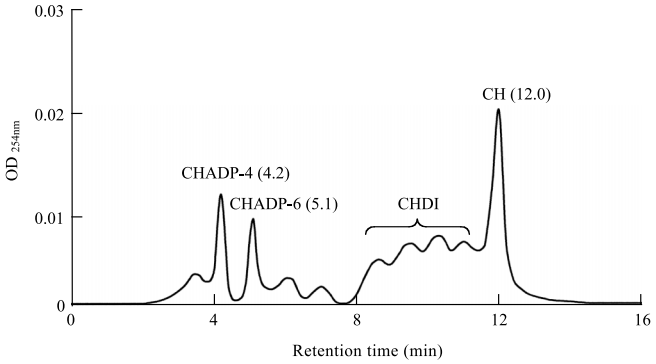

Fig. 1. HPLC Chromatogram of Chlorhexidine Degradation by *Pseudomonas* sp. Strain No. A-3

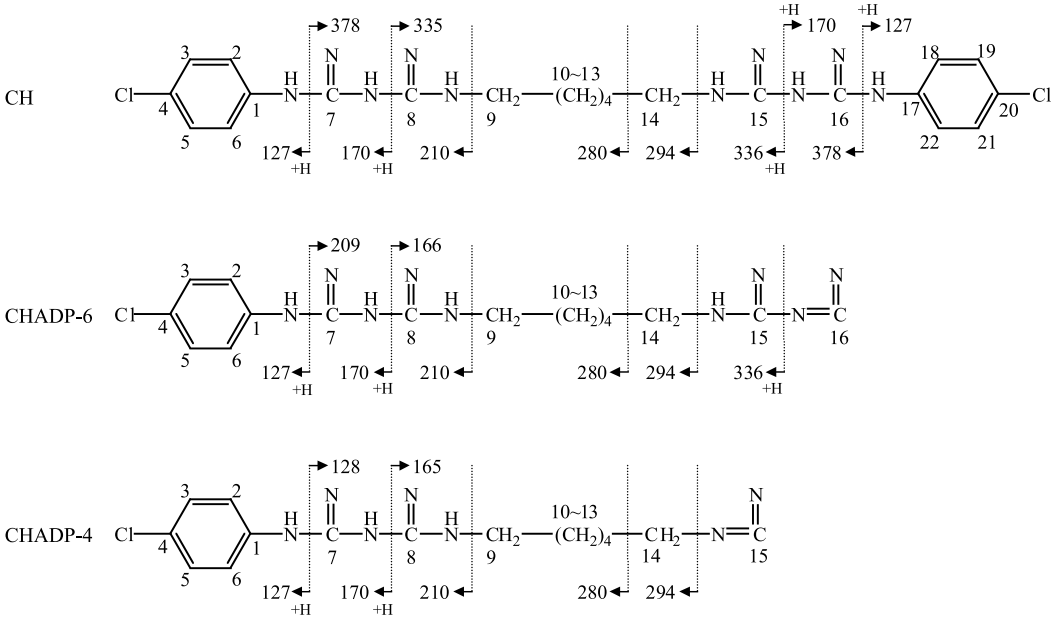

Fig. 2. Fragmentation Patterns of CH, CHADP-6 and CHADP-4 by Positive FAB-MS Spetra

Structure Elucidation of CHADP-6

CHADP-6 is a white powder with a mp of 159–162°C. It was positive to the Sakaguchi reaction. The compound was soluble in methanol or ethanol, but insoluble water, acetone and chloroform. The IR absorptions were observed at 3500, 3100, 1500 and 1620 cm^{-1} in the IR spectrum due to the amino and imino groups, respectively. The molecular ion peak of CHADP-6 was obtained at *m*/*z* 378 $(M + H)^+$ by positive FAB-MS. The pattern of fragmentation was similar to that of CH and the molecular ion of CHADP-6 was 127 mass units lower than that of CH (Fig. 2). The elementary analysis of CHADP-6 generated $C_{16}H_{24}N_9Cl{\cdot}2HCl$ as the molecular formula, which agreed with *m*/*z* 378 $(M + H)^+$ as ion peak on the positive FAB-MS. ^1H NMR spectrum of CHADP-6 revealed a loss of four benzene ring protons (δ_H 7.29 and δ_H 7.37) compared with those of CH, indicating that CHADP-6 had a loss of one benzene ring. Other proton signals were almost similar to those of CH. The ^{13}C NMR spectrum and proposed structure of CHADP-6 from the results of ^1H, ^{13}C, HMQC and HMBC experiments are shown in Table 2 and Fig. 2. The two carbon (C_{15} and C_{16}) signals of CHADP-6 revealed high field shift. CHADP-6 is thought to be a cleavage partner of *p*-chloraniline (Fig. 3).

Structure Elucidation of CHADP-4

CHADP-4 is a white powder with a mp of 176–178°C. It was positive to the Sakaguchi reaction. The compound was soluble in methanol or ethanol,

but insoluble water, acetone and chloroform. The IR absorption was similar to that of CHADP-6. The molecular ion peak of CHADP-4 was obtained at *m*/*z* 336 $(M + H)^+$ by positive FAB-MS. The pattern of fragmentation was similar to that of CH. The elementary analysis of CHADP-4 generated $C_{15}H_{22}N_7Cl{\cdot}HCl$ as the molecular formula, which agreed with *m*/*z* 336 $(M + H)^+$ as ion peak on the positive FAB-MS. ^1H NMR spectrum of CHADP-4 also revealed a loss of four benzene ring protons (δ_H 7.29 and δ_H 7.37) compared with those of CH, indicating that CHADP-4 had a loss of one benzene ring. Other proton signals were almost similar to those of CH. The ^{13}C NMR spectrum and proposed structure of CHADP-4 from the results of ^1H, ^{13}C, HMQC and HMBC experiments are shown in Table 2 and Fig. 2. One carbon (C_{15}) signal of CHADP-4 also revealed high field shift. CHADP-4 is thought to be a cleavage partner of 4-chlorophenylurea[7] (Fig. 3).

Table 2. ^{13}C NMR Spectral Data for CH, CHADP-6 and CHADP-4 in CD_3OD

position	CH	CHADP-6	CHADP-4
1	140.4	142.0	141.0
2	124.9	125.0	124.6
3	129.4	129.8	129.8
4	126.7	125.0	125.0
5	129.4	129.8	129.8
6	124.9	125.0	124.6
7	161.1	161.1	161.1
8	157.8	158.0	158.1
9	43.1	43.0	43.1
10	30.1	31.0	31.0
11	27.3	27.5	27.5
12	27.3	27.5	27.3
13	30.1	30.5	30.4
14	43.1	43.0	43.1
15	157.8	153.0	136.8
16	161.1	142.0	
17	140.4		
18	124.9		
19	129.4		
20	126.7		
21	129.4		
22	124.9		

Antimicrobial Activity of CHADP-4 and CHADP-6

The antimicrobial spectra of CHADP-4, CHADP-6 and CH as determined by the agar dilution method are shown in Table 1. The antimicrobial activity of CHADP-6 is similar to that of CH, but the antimicrobial activity of CHADP-4 decrease to 1/5–1/10 that of CH. The antimicrobial activity of CHDI-B, CHDI-C and CHDI-BR also decreased to 1/5–1/10 that of CH. CH and CHADP-6 have two guanido groups, but CHADP-4, CHDI-B, CHDI-C and CHDI-BR have a loss or modification of one guanido group of CH. From the results of the antimicrobial activities of those compounds, it is considered that two guanido groups of CH are necessary for its antimicrobial activity.

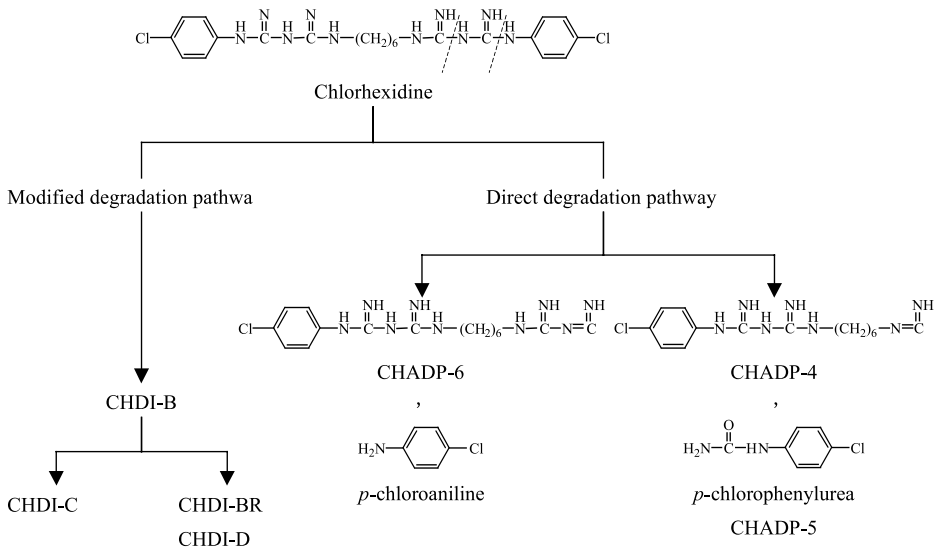

Fig. 3. Direct Degradation Pathway of Chlorhexidine by *Pseudomonas* sp. Strain No. A-3

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