Transformation of shikonin by a cell-free extract of *Eubacterium* sp. A-44, a human intestinal bacterium

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Abstract

For the purpose of investigating the metabolic processes of shikonin (**1**) by human intestinal bacteria, we prepared a sonicated bacterial cell suspension and a crude enzyme preparation from *Eubacterium* sp. A-44, one of the intestinal bacteria capable of transforming **1** to various metabolites. After anaerobic incubation with the suspension for 1 hr, most of **1** was transformed to prometaboshikonin (**2**), and metaboshikonins I (**3**) and II (**4**). However, under aerobic conditions, the dimers, shikometabolins A (**5**) and B (**6**), were predominantly formed.

In the presence of the crude enzyme preparation, formation of **2**-**4** was inhibited by oxygen, but markedly enhanced by the addition of NADH. On the other hand, formation of **5** and **6** was appreciably accelerated by the addition of NAD^+. In the absence of the crude enzyme preparation, NADH and/or NAD^+ showed no ability to transform **1** to the any metabolites, as in the case of a thermally inactivated preparation. Accordingly, the two different metabolic processes leading to compounds **2**-**4** and compounds **5** and **6** by *Eubacterium* sp. A-44 are concluded to be enzyme-dependent in the presence of NADH and NAD^+.

Key words *Eubacterium*, human intestinal bacteria, metabolism, prometaboshikonin, shikonin.

Abbreviations NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; FAD, flavin adenine dinucleotide; HR-EIMS, high resolution electron impact mass spectra; MeCN, acetonitrile; HPLC, high performance liquid chromatography; DMSO, dimethyl sulfoxide; NMR, nuclear magnetic resonance.

Introduction

Shikonin (**1**), a major naphthoquinone pigment isolated from Radix Lithospermi was found to demonstrate a broad spectrum of biological activities.[1-5] Shikonin (**1**) showed a significant anti-amebic activity,[2] and induced cleavage of mammalian topoisomerase II-mediated DNA.[3,4] Moreover, the azoxymethane-induced intestinal tumor was significantly reduced after oral administration of **1** to rats.[5] Recently, the shikonin metabolites, deoxyshikonin, and shikometabolin D, obtained by anaerobic incubation with human intestinal bacteria demonstrated to be more potent in cytotoxicity than **1** against various human tumor cell lines.[6]

As regards the metabolism of shikonin (**1**), a few studies have been conducted; Li *et al.*[7] reported on the metabolism of **1** by rat liver microsomes, in which the main metabolites were mono- and di-hydroxylated derivatives of **1**. In addition, we reported that human intestinal bacteria had the ability of transforming **1** to a variety of metabolites (at least ten metabolites were identified).[8,9] However, the mecha-

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nism of the formation of these metabolites was not yet clear.

The present experiment aimed to investigate the metabolic processes of **1** by human intestinal bacteria using a cell-free extract of *Eubacterium* sp. A-44,[10] which showed potent ability to metabolize shikonin (**1**) *in vitro*.

Materials and Methods

Apparatus: Melting points were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. ^{1}H-and ^{13}C-NMR spectra were measured with a Varian Gemini 300 spectrophotometer (Varian Co., Palo Alto, U.S.A.) and chemical shifts are given in δ ppm relative to tetramethylsilane (TMS). HR-EIMS and EIMS were measured with a JMX-AX 505 HAD mass spectrometer (JEOL Co., Tokyo) at an ionization voltage of 70 eV. A sonicator, Sonifier 250 (Branson Co., Danbury, CT, USA) was used to disrupt bacterial cells. A centrifugal concentrator, Centriprep-10 (Amicon, Beverly, MA, USA) was used for ultrafiltration of the crude enzyme (10,000 molecular weight cut-off).

Chromatography: Column chromatography was carried out over silica gel 60 (70-230 mesh, Merck). HPLC was performed on a CCPM-II (Tosoh, Tokyo) equipped with a Tosoh UV-8020 spectrometer and a Shimadzu C-R 6A chromatopac (Shimadzu, Kyoto). Column, Finepack SIL C1-5 (JASCO Co., Tokyo, 250 mm×4.5 mm i.d.); mobile phase, MeCN (solvent system A) and H_2O containing 1% H_3PO_4 (solvent system B) in a linear gradient mode (30%→70% in 25 min); flow rate, 1.0 ml/min; detection, UV 280 nm.

Chemicals: Shikonin (**1**), NADH, NAD^+ and NADPH were purchased from Tokyo Kasei Organic Chemicals (Tokyo), and general anaerobic medium (GAM) was from Nissui (Tokyo). FAD^+ and riboflavin were from Sigma-Aldrich Japan (Tokyo). Compounds **3**-**6** were previously isolated from **1** after incubation with *Bacteroides fragilis* subsp. *thetaotus* in our laboratories.[8,9]

A crude enzyme preparation and transformation of shikonin (1): *Eubacterium* sp. A-44 has been previously isolated from human feces,[10] and was maintained in GAM broth. Twenty ml of the culture were transferred to 10 volumes of the medium and incubated for 18 hr in an anaerobic incubator. The bacterial cells were harvested by centrifugation at 1500×g for 10 min, and the pellets were suspended in 50 mM K-phosphate buffer (pH 7.3, 90 ml). The bacterial cells were disrupted by sonication (60 sec×2), and part of the sonicated bacterial cell suspension (30 ml) was further centrifuged at 22500×g for 30 min to obtain a bacterial cell-free extract, which was kept on ice and used as a crude enzyme preparation.

Shikonin (**1**, 50 mg in 1 ml of DMSO) was added to the sonicated bacterial suspension (60 ml) and the reaction mixture was incubated in an anaerobic incubator for 2 hr. After acidification with 1 N HCl to *ca.* pH 5.0, the reaction mixture was extracted with EtOAc (200 ml×5). The EtOAc layer was washed with H_2O, dried over $MgSO_4$ and then evaporated *in vacuo* to give a residue. The residue was applied to a column of silica gel. Elution was started with hexane-Me_2CO (9:1→7:3) and then $CHCl_3$ with increasing % of MeOH to give 46 fractions. Compounds **3** (6 mg) and **4** (4 mg) were obtained from Fr. 2-8, **2** (18 mg) from Fr. 17-21, and **5** (4 mg) and **6** (4 mg) from Fr. 42-46.

Metabolite **2** Orange needles from hexane, mp. 120-123 °C. EI-MS *m/z* (rel. int.): 290 $[M]^+$ (20), 222 (100), 192 (75), 175 (25), 137 (30), 91 (15) and 69 (15). ^{1}H-NMR ($CDCl_3$) δ: 1.64 and 1.75 (3H each, 2×CH_3), 2.39 (1H, m, H_b-12), 2.41 (1H, br s, 11-OH), 2.62 (1H, m, H_a-12), 3.05 (4H, s, H_2-6 and H_2-7), 5.01 (1H, m, H-11), 5.17 (1H, dd, J=5.4 and 1.1 Hz, H-13), 7.43 (1H, s, H-3), 11.99 and 12.45 (1H each, s, 1-OH and 4-OH). ^{13}C-NMR ($CDCl_3$) δ: 18.3 (C-15), 26.2 (C-16), 36.4 (C-6 and C-7), 69.2 (C-11), 116.9 (C-10), 117.3 (C-9), 119.1 (C-13), 125.2 (C-3), 136.8 (C-14), 144.7 (C-2), 152.5 (C-4), 155.3 (C-1), 200.9 and 201.9 (C-5 and C-8).

Time course for the transformation of shikonin (1) by a crude enzyme preparation: A reaction mixture in a total volume of 200 μl contained 1 μmol of **1**, and a crude enzyme preparation (150 μl) in the presence or absence of 2 μmol of either NADH and/or 40 nmol of FAD. The mixture was incubated under anaerobic conditions.

In order to examine the effect of other co-factors, NADH was replaced by NADPH or NAD^+. Non-

enzymatic activity of the cofactors was also investigated in the absence of the crude enzyme preparation (replaced by 150 μl buffer) and in the presence of the crude enzyme preparation previously boiled for 10 min. The reaction mixture was incubated under aerobic or anaerobic conditions and portions were picked up at intervals after the start of incubation. The reaction was stopped by adding 1 N HCl (10 μl), and the mixture was extracted with EtOAc (200 μl) and centrifuged at 3000×g for 5 min. A 100 μl portion of the EtOAc layer was evaporated *in vacuo* to give a residue. The residue was dissolved in 5 μl of DMSO, and 95 μl of MeOH were added. The products obtained were analyzed by HPLC as mentioned above.

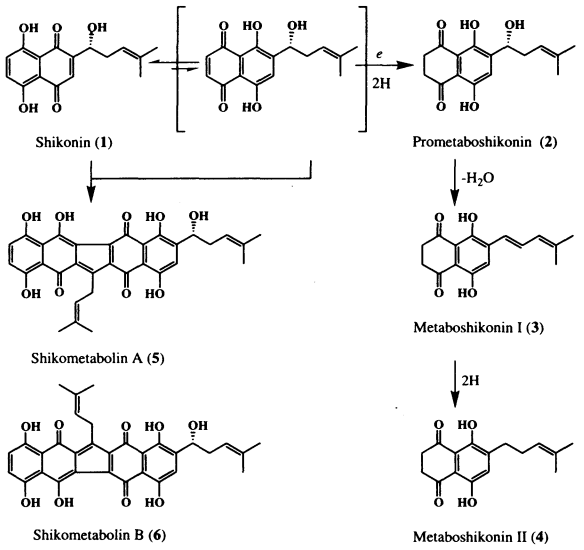

Chart 1. Possible metabolic pathways of shikonin (**1**) by a cell-free extract of *Eubacterium* sp. A-44

Results and Discussion

Eubacterium sp. A-44, an intestinal bacterium isolated from human feces, was found to demonstrate a potent activity to metabolize shikonin (**1**) after incubation for 15 hr as confirmed by HPLC (data not shown), when compared with that shown by 30 defined bacterial strains isolated from human feces in the previous experiment.[9]

After anaerobic incubation of **1** with a sonicated bacterial suspension of *E.* sp. A-44 for 2 hr, five metabolites (**2-6**) were isolated from the EtOAc-soluble fraction (Chart 1). The structures of **2-6** were determined by spectroscopic methods. Metabolites **3-6** were identified by direct comparison of their spectral data with those of authentic metaboshikonins I (**3**) and II (**4**), and shikometabolins A (**5**) and B (**6**), previously isolated from **1** after incubation with *B. fragilis* subsp. *thetaotus*,[9] while **2** is a new compound and its structure was determined as follows.

Metabolite **2** showed a molecular ion peak at *m/z* 290 $[M]^+$, consistent with the molecular formula $C_{15}H_{18}O_5$ with two mass units more than that of **1**. The ^{1}H-NMR spectrum of **2** showed signals characteristic for a side chain similar to that in **1**, namely, δ 1.64, 1.75, 2.39, 2.62, 5.00 and 5.20. Moreover, singlet signals were observed at δ 3.08 (for two methylenes), 7.43 (for an aromatic proton), 11.99 and 12.45 (for two *peri*-OH protons). The ^{13}C-NMR spectrum of **2** showed signals characteristic for two carbonyl carbons at δ 202.0 and 201.0, similar to those in **3** and **4**. From these findings, the structure of **2** was confirmed as (*R*)-1,4-dihydroxy-2-(1-hydroxy-4-methyl-3-pentenyl)-5,8-naphthoquinone, a reduced product of a tautomeric form of **1**, which was given the name prometaboshikonin (**2**).

Most of the metabolic activity of *E.* sp. A-44 was recovered in a supernatant (designated as a crude enzyme preparation) obtained by centrifugation of the sonicated bacterial cell suspension and this preparation was used to investigate the reaction mechanism for the formation of **2-6**.

The effects of experimental conditions and cofactors on the transformation of **1** by the crude enzyme preparation were investigated. It was obvious that, under anaerobic conditions, the crude enzyme preparation effectively transformed **1** to **2-4** (Fig. 1A). Appreciable amount of **2** was produced 2 hr after the start of incubation, while **3-6** were formed later (after 4 hr) and gradually increased. Under the atmosphere of O_2, a significant amount of **1** was transformed to **5** and **6** after 2 hr of incubation, and a trace amount of **2** was detected, while **3** and **4** were not detected after prolonged incubation for 24 hr (Fig. 1C). However, the metabolites were not obtained when **1** was added to a crude enzyme preparation previously inactivated by boiling for 10 min.

In the absence of the crude enzyme preparation,

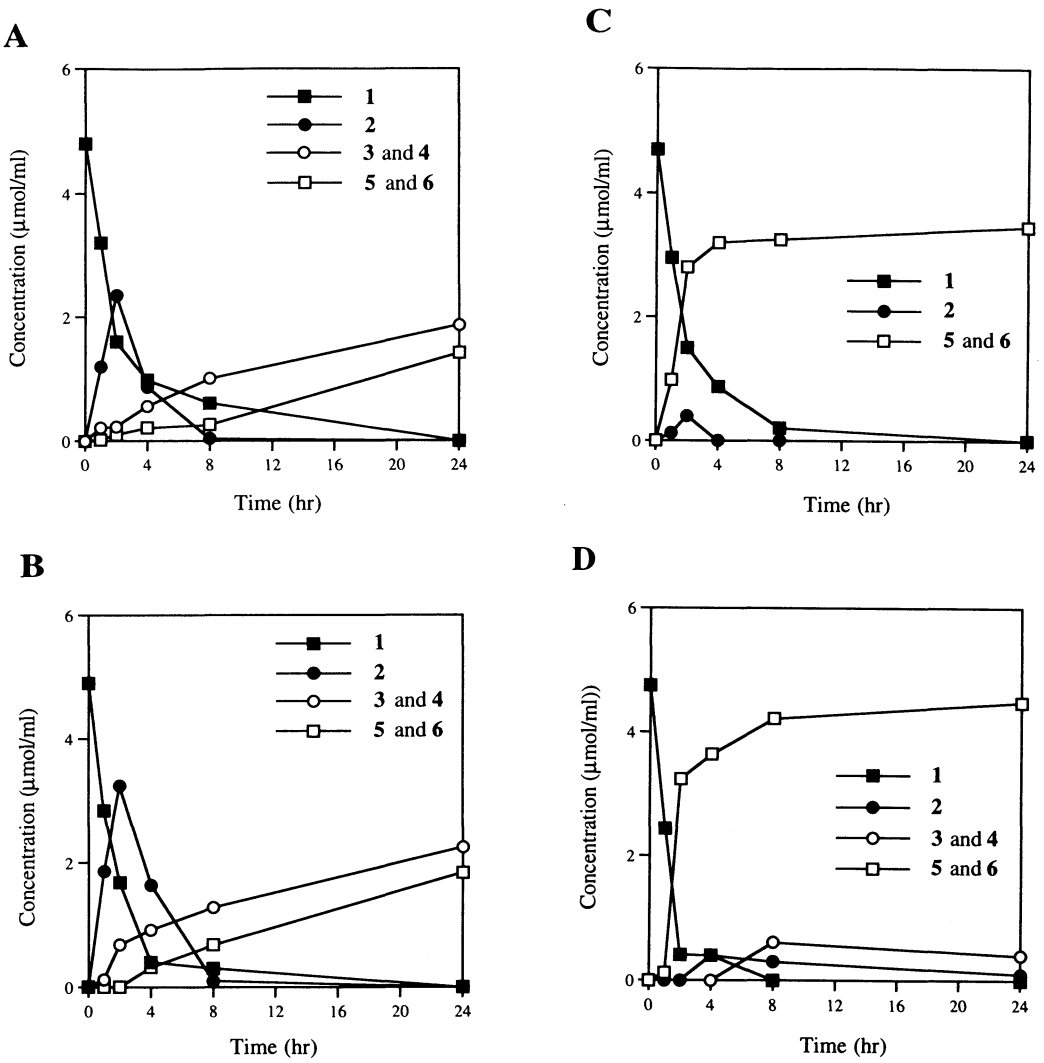

Fig. 1 Metabolism of shikonin (**1**) by a crude enzyme preparation from *Eubacterium* sp. A-44
A) under anaerobic conditions, B) under anaerobic conditions in the presence of NADH, C) under aerobic conditions, D) under aerobic conditions in the presence of NAD^+

NAD^+ or NADH could not convert **1** to any substance both in the presence and absence of O_2. However, when cofactors were added to reaction mixtures containing the crude enzyme, significant increases in yields of the metabolites were observed. Under anaerobic conditions in the presence of NADH, the crude enzyme showed an appreciable increase in a yield of **2** at the early stage of incubation (Fig. 1B). The similar increase in the formation of **2** was also observed when NADH was replaced by NADPH, while addition of FAD^+ and/or riboflavin did not show a significant increase of **2** in amount (data not shown). Under similar conditions, formation of the metabolites (in favor of **5** and **6**) was also enhanced by the addition of NAD^+ (data not shown). The similar increase in the formation of **5** and **6** was observed when a reaction mixture containing NAD^+ was used under the atmosphere of O_2, while trace amounts of **2**-**4** were only detected by HPLC (Fig. 1D).

Inoue *et al.*[11] reported that shikonin (**1**) exists in solution as a 2-substituted 5,8-dihydroxy-1,4-naphthoquinone form rather than its tautomeric form. However, the results obtained in the present experiment suggested that under anaerobic conditions, transformation of **1** to **2** proceeded through the tautomeric form of **1** followed by reduction. Furthermore, reduction of

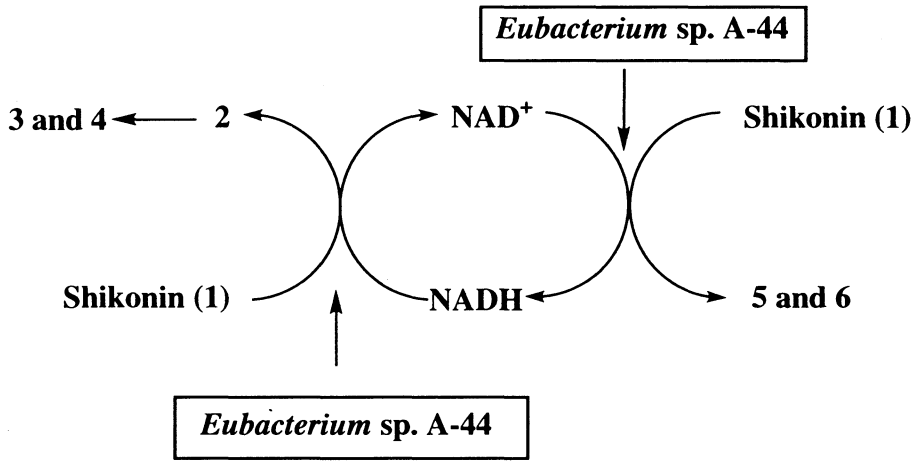

Chart 2. Reduction and condensation of shikonin (**1**) by a cell-free extract of *Eubacterium* sp. A-44 in the presence of NADH and NAD^+

2 resulted in the formation of **4** *via* **3** (Chart 1), as confirmed by further incubation of **2** with the crude enzyme preparation under anaerobic conditions (data not shown). Under the atmosphere of O_2, formation of **5** and **6** was due to the oxidative condensation of **1** and its tautomer (Chart 1) as previously speculated,[9] and increased probably by the oxidized form of cofactors generated in the bacterial cytosol at neutral pH (Fig. 1C). Further enhancement was also observed after addition of NAD^+ to the reaction mixture (Fig. 1D).

The finding that small amounts of **5** and **6** were detected after prolonged incubation of **1** with the crude enzyme preparation under anaerobic conditions, which were more evident in the presence of NAD^+, can be explained as follows: reduced cofactors, e.g. NADH, in the bacterial cytosol enhance the reduction of the tautomeric form of **1** to give an intermediate **2**, which is subsequently led to **3** and **4**. As a result, NAD^+ regenerated from NADH may catalyze the oxidative condensation of **1** and its tautomer to give **5** and **6** (Chart 2). The turnover of this process seemed to be catalyzed by the addition of NADH as shown in Fig. 1B since the yields of metabolites were significantly increased within 8 hr, when compared with those shown in Fig. 1A. Under anaerobic conditions, this turnover may shift towards the tautomerization and reduction of **1**, while the condensation will be in favor under aerobic conditions (enhanced by NAD^+) to yield **5** and **6**.

In conclusion, shikonin (**1**) was effectively transformed to **2**-**6** in the presence of a cell-free extract of *E.* sp. A-44 under anaerobic conditions. The formation of **2**-**4** and the dimers **5** and **6** may proceed two different processes as shown in Chart 1. In the reductive process, formation of **2**-**4** was significantly accelerated by the addition of NAD(P)H as an electron donor and was completely inhibited by oxygen. In the oxidative process, formation of the dimers **5** and **6** was stimulated by the addition of NAD^+.

和文抄録

ヒト腸内細菌による紫根成分 shikonin の代謝過程を研究する目的で shikonin (**1**) を種々の代謝物に変換する腸内細菌 *Eubacterium* sp. A-44 の細胞破砕懸濁液及び粗酵素標品を調製した。この粗酵素標品と shikonin (**1**) を嫌気条件下で1時間インキュベーションすると，shikonin (**1**) のほとんどは prometaboshikonin (**2**) および metaboshikonin I (**3**), II (**4**) に変換された。しかしながら，好気条件下では shikometabolin A (**5**), B (**6**) などの二量体が生成した。化合物 **2**-**4** の生成は，酸素によって阻害され，NADH の添加により増強した。一方，化合物 **5**, **6** の生成は NAD^+ の添加により増加した。加熱処理した場合と同様，粗酵素標品を除去した場合は，NADH, NAD^+ を添加しても shikonin の変換は起こらなかった。したがって，NADH や NAD^+ の存在下での化合物 **2**-**4** や化合物 **5**, **6** への変換過程は酵素反応によって触媒されることを示している。

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