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Biotransformation of Shikonin by Human Intestinal Bacteria[1]

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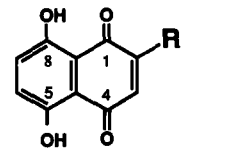
Abstract: The bacterial transformation of shikonin (**1**) in culture media has been investigated using human intestinal bacteria. Of 24 anaerobes tested, *Bacteroides fragilis* subsp. *thetaotus* showed an ability to transform **1** extensively to ten metabolites, five monomers; anhydroalkannin (**2**), deoxyshikonin (**3**), cycloshikonin (**4**), metaboshikonin I (**5**) and II (**6**), and five dimers; shikometabolins A (**7**), B (**8**), C (**9**), D (**10**) and E (**11**), after anaerobic incubation for 3 days. The structures of these metabolites were determined mainly by 2D-NMR including the INADEQUATE experiment.

Radix Lithospermi (Shikon in Japanese) is the dry root of *Lithospermum erythrorhizon* Sieb. et Zucc. (family Boraginaceae), one of three *Lithospermum* species indigenous to Japan.[2] It is officially listed in the Japanese and the Chinese Pharmacopeia and used as an anti-inflammatory and antipyretic agent in the treatment of measles and eczema.[3] Moreover, it has been used in combination with other herbs in the ancient Kampo medicine (Shikon-borei-to, in Japanese) as anti-tumor.[4] The red naphthoquinone pigment shikonin (**1**) and its derivatives are accumulated in the cork layers of radix, and are considered as potentially important compounds for medicinal use as anti-tumor,[5] antibacterial,[6] and anti-inflammatory.[7] They also showed a significant antiamebic activity in rats with induced intestinal amebiasis.[8] Tabata et al. have been investigated the biosynthesis and production of shikonin (**1**) in *Lithospermum* cell cultures.[9] In Japan, Fujita et al. received the cell line from Tabata et al. and developed a new cell lines from *L. erythrorhizon* protoplasts for commercial production of shikonin (**1**) which used for dyeing valuable clothes and as a coloring agent for cosmetics.[10] The chemistry of shikonin (**1**) and its enantiomer alkannin has been extensively studied by many workers.[11] Heretofore, the metabolic fate of shikonin (**1**, the main constituents in Radix Lithospermi) in the human intestinal tract has not been reported. A prospective approach to facilitate such studies has been performed by Hattori et al. using human intestinal bacteria, as predictive models to mimic the intestinal transformation of crude drugs and other xenobiotics in the gastrointestinal tract.[12] In the course of the studies on the transformation of natural pigments,[13] we report herein the isolation and structure determination of ten metabolites obtained after anaerobic incubation of shikonin (**1**) with *Bacteroides fragilis* subsp. *thetaotus*, a major bacterium from human feces.

RESULTS AND DISCUSSION

Screening of microbial transformation of shikonin (**1**) has been carried out using 24 anaerobic bacteria isolated from human feces. The bacterial strains are individually incubated with **1** for 3 days under anaerobic conditions. All the tested strains had the ability to transform **1** to four major metabolites (**5**, **6**, **7** and **8**) (Table



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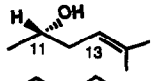

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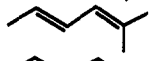

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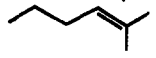

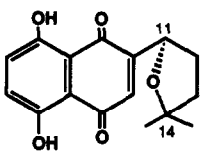

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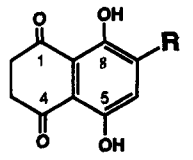

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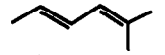

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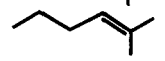

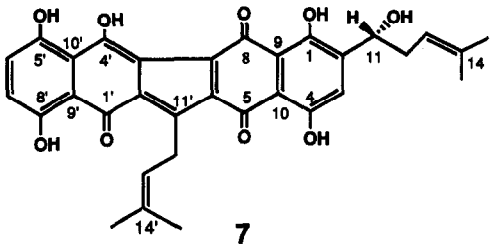

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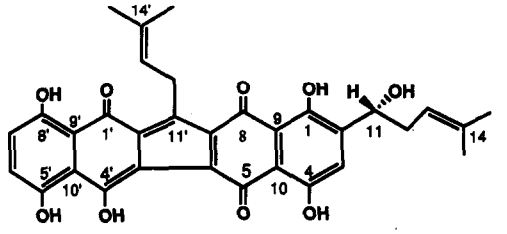

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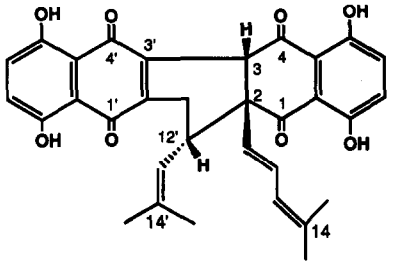

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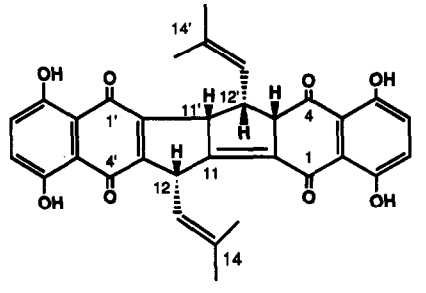

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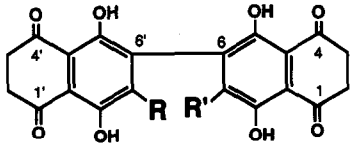

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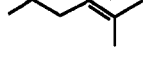

Chart 1

I), but in the absence of bacteria, **1** did not show any changes under the same experimental conditions. Of these, *Bacteroides fragilis* subsp. *thetaotus* reproducibly transformed **1** to ten metabolites, in relatively good yields (0.5 - 1.0% for **5**, **6**, **7** and **8**), as indicated by TLC and HPLC (Figure 1), and selected for a large scale preparation of the metabolites.

After anaerobic incubation with *B. fragilis* for 3 days, the metabolites were extracted with EtOAc[14] and rigorously chromatographed on Sephadex LH-20 and preparative TLC (Chart 1 and 2) to afford five naphthoquinone type monomers (**2**-**6**) and five dimers (**7**-**11**). The structures of these compounds were determined as follows.

Compound 2 was obtained as red needles from $CHCl_3$, mp 134-135 °C, (Rf 0.78, in solvent system A). The EI-MS spectrum showed a molecular ion peak at *m/z* 270 [M^+] with 18 mass units less than that of **1**, suggesting that **2** is a dehydrated compound of **1**. The ^{1}H-NMR spectra of **2** ($CDCl_3$) showed pattern similar to that of **1**, except for three olefinic protons (13-H, 11-H and 12-H) assignable to a transoid diene-type side chain in **2**. Accordingly, **2** was deduced as anhydroalkannin and finally confirmed by comparing its spectral data with those reported for the compound isolated from Nanshikon *Macrotomia euchroma* Pauls.[11f]

Compound 3 was obtained as red needles from $CHCl_3$, mp 93-94 °C, (Rf 0.76, in solvent system A). The EI-MS spectrum showed a molecular ion peak at *m/z* 272 [M^+] with two mass units higher than that of **2**. The ^{1}H-NMR spectrum of **3** ($CDCl_3$) was similar to that of **2**, except for the appearance of signals for two methylene groups, instead of signals due to the diene-type side chain in **2**. This led us to conclude that **3** is deoxyshikonin and the structure was further confirmed by comparison of the ^{1}H- and ^{13}C-NMR spectral data with those reported for deoxyshikonin isolated from *L. erythrorhizon* Sieb. et Zucc.[11b]

Table I. Screening of Bacterial Strains Capable of Metabolizing Shikonin (**1**)

Bacterial strain	Concentration (μmol/ml)					
	4	**5**	**6**	**7&8**	**9**	**1**
Bacteroides fragilis subsp. *thetaotus*	0.02	0.51	0.20	1.02	0.02	0.14
Bifidobacterium adolescentis	—	0.03	0.01	1.22	—	0.48
B. breve S-2 KZ 1287	—	0.18	0.08	1.21	0.04	0.26
B. bifidum aE319	—	0.05	0.01	0.34	0.02	0.04
B. longum IV-55	—	0.34	0.10	0.01	0.03	—
B. pseudolongum PNC-2-9-G	—	1.17	0.20	0.28	0.10	0.02
Clostridium butyricum	—	0.57	0.03	1.02	—	0.01
C. innocuum ES 24-06	0.01	0.01	0.05	0.60	0.01	1.03
C. innocuum KZ-633	0.01	0.02	0.02	1.21	0.02	0.65
C. perfringens To-23	—	0.20	0.05	0.17	0.11	—
Escherichia coli 0-127	0.09	0.63	0.58	0.50	0.20	0.01
Fusobacterium nucleatum G-470	0.01	0.01	0.97	0.46	0.06	0.03
Gaffkya anaerobia G-0608	0.01	0.01	0.01	1.34	0.01	0.50
Klebsiella pneumoniae ATCC 13883	—	0.04	0.02	0.25	—	1.04
Lactobacillus brevis II-46	0.01	1.08	0.23	0.21	0.10	0.01
L. acidophilus ATCC 4356	0.01	0.62	0.70	0.49	0.03	0.40
L. fermentum ATCC 9338	0.03	0.53	0.23	0.38	0.06	0.26
L. plantarum ATCC 14917	0.01	0.03	0.05	0.34	0.05	—
L. xylosus ATCC 155775	—	0.03	0.02	0.42	0.03	0.20
Peptostreptococcus anaerobius 0240	—	1.06	0.02	0.26	0.21	0.01
P. intermedius EBF 77/2	—	1.61	0.72	0.01	0.02	—
Proteus mirabillis S2	0.01	0.56	0.11	0.19	0.18	—
Streptococcus faecalis II-136	0.01	0.20	0.06	0.70	0.05	0.31
Veillonella parvula subsp. *parvula* ATCC 10790	0.01	0.62	0.01	0.17	0.04	—

Each bacterium was anaerobically incubated in 0.1 M phosphate buffer (pH 7.3) containing shikonin (1.73 μmol/ml, **1**) for 3 d at 37°. The incubation mixture was then acidified and extracted with EtOAc (10 ml x 3). The metabolites obtained were analyzed by HPLC.

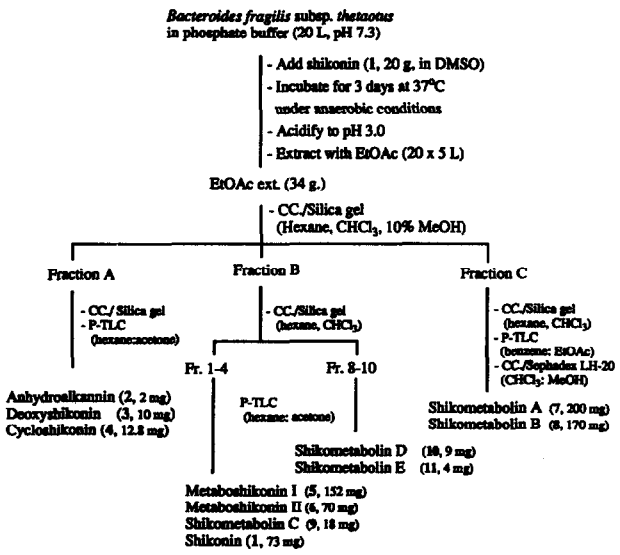

Chart 2. Flow chart for the isolation of shikonin (1) metabolites from the EtOAc extract.

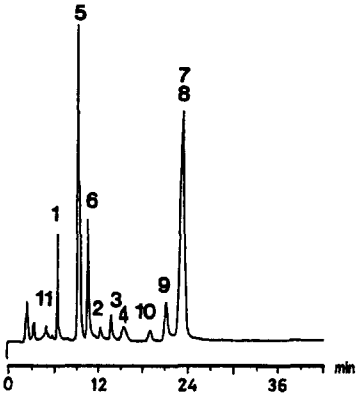

Figure 1. HPLC profile of the EtOAc extract.

Column: Finepak SIL C1-5 (Jasco); Mobile phase: MeCN: H_2O: H_3PO_4 (55: 45: 0.1); Detection: UV 280 nm. Flow rate: 1 ml/min; **1**, shikonin; **2**, anhydroalkannin; **3**, deoxyshikonin; **4**, cycloshikonin; **5**, metaboshikonin I; **6**, metaboshikonin II; **7**, shikometabolin A; **8**, shikometabolin B; **9**, shikometabolin C; **10**, shikometabolin D; **11**, shikometabolin E.

Compound 4 was obtained as red needles from hexane, mp 87-89 °C, (Rf 0.72 in solvent system A). The EI-MS spectrum of **4** showed a molecular ion peak at m/z 288 [M^+]. The ^{1}H-NMR ($CDCl_3$) spectrum exhibited signals characteristic for two tertiary methyl groups at δ_H 1.35 (15-CH_3 or 16-CH_3) and 1.38 (16-CH_3 or 15-CH_3), multiplets for two methylene groups at δ_H 1.80-2.63. Moreover, the ^{1}H-^{1}H COSY spectrum showed a lon-range coupling (J = 1.5 Hz) between 3-H and 11-H, suggesting intramolecular cyclization of the side chain in **1** to give **4**.[9a] The EI-MS spectrum displayed characteristic fragment ion peaks at m/z 230 [M^+- 58] and 219 [M^+- 69], attributable to the loss of an acetone molecule and an isopentylene side chain, respectively. The ^{1}H- and ^{13}C-NMR spectral data of **4** (see experimental) were in good agreement with those reported for cycloshikonin[9a] and cycloalkannin.[11c] A positive Cotton effect [θ]$_{330}$ + 205 ($CHCl_3$) in the CD spectrum of **4**, together with a long-range coupling observed between 3-H and 11-H, led us to identify **4** as cycloshikonin.

Compound 5, one of the main unstable metabolites, obtained as fine yellow needles from $CHCl_3$, mp 146-148 °C, (Rf 0.56, in solvent system A). The HRMS spectrum showed a molecular ion peak at m/z 272.1019 [M^+] corresponding to the molecular formula $C_{16}H_{16}O_4$ (Calcd 272.1047), indicating 9 degrees of unsaturation. The ^{1}H-NMR ($CDCl_3$) spectrum of **5** showed the presence of two methylene groups at δ_H 3.03 (4H, d, J = 3 Hz, 2-H_2 and 3-H_2), an aromatic ^{1}H signal at δ_H 7.30 assignable to 6-H, and two *peri*-hydroxyl protons at δ_H 12.01 (5-OH) and 12.63 (8-OH), along with a side chain pattern similar to that of **2** (δ_H 1.88, 1.90, 6.09, 6.61 and 7.19). The ^{13}C-NMR spectrum analyzed by the aid of DEPT and ^{1}H-^{13}C COSY, unequivocally indicated characteristic signals for two carbonyl carbons at δ_c 200.40 and 202.00 (C-1 and C-4), four sp^2 methine carbons at δ_c 131.80 (C-12), 125.90 (C-13), 122.00 (C-6) and 121.30 (C-11), and two methylene carbons at δ_c 36.00 (C-2 and C-3) (Table II). In the long-range ^{1}H-^{13}C COSY spectrum of **5** (Chart 3), the aromatic ^{1}H signal at δ_H 7.30 (6-H) was correlated with ^{13}C signals at δ_c 155.00 (C-5) and 153.50 (C-8) and 121.30 (C-11). Moreover, the ^{1}H signal at δ_H 6.61 (11-H) was correlated with the ^{13}C signals at

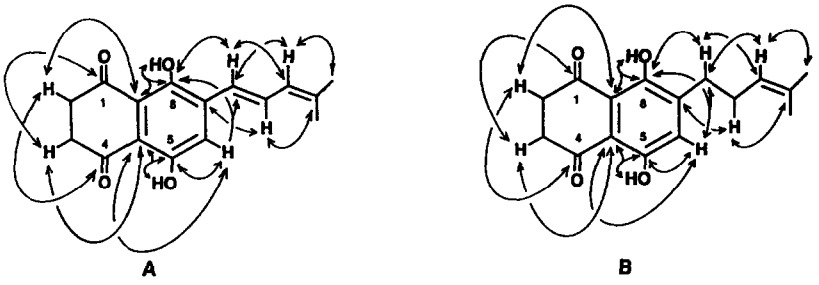

Chart 3. Significant correlations observed in ^{1}H-^{13}C long-range COSY.
A: Metaboshikonin I (5) B: Metaboshikonin II (6)

δ_C 153.50 (C-8) and 125.90 (C-13), indicating the connectivity of the side chain to C-7. Furthermore, the methylene ^{1}H signal at δ_H 3.03 (2-H_2 and 3-H_2) showed correlations with the carbonyl ^{13}C signals at δ_C 200.40 and 202.00 and with the^{13}C signals at δ_C 114.30 (C-9) and 113.00 (C-10). In addition, the ^{1}H signal at δ_H 12.63 (8-OH) was correlated with C-9, while C-10 was correlated with the ^{1}H signals at δ_H 7.30 (6-H) and 12.01 (5-OH). From the forgoing findings, **5** was concluded as 2,3-dihydro-5,8-dihydroxy-7-(4'-methylpenta-1',3'-dien-1'-yl)-1,4-naphthoquinone and named metaboshikonin I.

Compound 6, one of the main unstable metabolites, was obtained in relatively low yield as an orange amorphous powder (R_f 0.48, in solvent system A). The HRMS spectrum indicated a molecular ion peak at *m/z* 274.1174 [M^+] corresponding to the molecular formula $C_{16}H_{18}O_4$ (Calcd 274.1214). The ^{1}H-NMR (see experimental) and ^{13}C-NMR spectra (Table II) showed signals for a side chain similar to that of **3** and signals for a reduced 1,4-naphthoquinone moiety similar to that of **5**. The structure was further confirmed by the analysis of long-range ^{1}H-^{13}C COSY spectrum (Chart 3). Accordingly, **6** was concluded as 2,3-dihydro-(4'-methylpenta-3'-en-1'-yl)-1,4-naphthoquinone and named metaboshikonin II.

Compound 7, was a major metabolite obtained as dark violet needles from acetone-hexane, mp > 300 °C, (R_f 0.26, in solvent system B). The CD spectrum showed negative Cotton effect $[\theta]_{234}$ -4200 (MeOH) (Figure 2). The IR (KBr) spectrum showed absorption at 3350 and 1620 cm^{-1}. The UV spectrum (MeOH) showed λ_{max} (log ε) at 280 (2.97), 420 (2.52) and 575 (2.78) nm. The HR-FABMS spectrum (negative ion mode) displayed a molecular ion peak at *m/z* 555.1650 [M-H]$^-$ which was almost twice as much as the molecular weight of **1** (EI-MS*m/z* 288 [M^+]) and assigned to the molecular formula $C_{32}H_{27}O_9$ (Calcd, 555.1658) suggesting a dimeric form with 19 degrees of unsaturation.[15] The ^{1}H-NMR (DMSO-d_6) and the ^{1}H-^{1}H COSY spectra showed the presence of three distinct ^{1}H spin systems, one of which with a substitution pattern similar to that of **1**, as indicated by partial structure **I**, with a hydroxyl bearing methine proton at δ_H 4.95 (11-H) coupled to a methylene ^{1}H signal at δ_H 2.25 (12-H_β) which in turn coupled to an olefinic ^{1}H signal at δ_H 5.29 (13-H) (Chart 4a). AB-type ^{1}H signals at δ_H 6.98 (6'-H) and 7.03 (7'-H), and two *peri*-hydroxyl groups suggested partial structure **II**. A methylene ^{1}H signal at δ_H 4.15 (12'-H), coupled to the olefinic ^{1}H signal at δ_H 5.26 (13'-H) in another spin system suggested partial structure **III**. The ^{13}C-NMR spectrum of 7 analyzed by the application of DEPT, ^{1}H-^{13}C COSY and HMQC,[16] indicated 32 carbon atoms in the molecule with 20 sp^2 quaternary carbons (Table II). The sequences of most of the quaternary carbons were ultimately confirmed by 2D INADEQUATE[17] experiments which revealed the connectivities of carbon A (C-1', δ_C 186.63) with carbons X (C-9', δ_C 114.50) and M (C-2', δ_C 127.28), carbon B (C-5, δ_C 186.43) with carbons Y (C-10, δ_C 113.11)

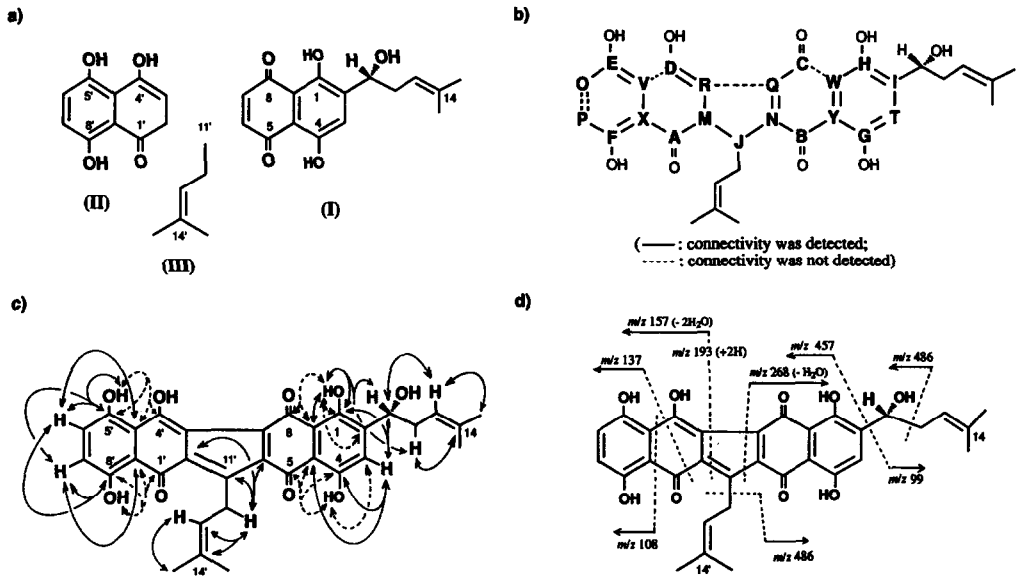

Chart 4. a) Deduced partial structures of 7.
b) ^{13}C-^{13}C connectivities of 7 confirmed by 2D INADEQUATE experiment.
c) Significant long-range correlations observed in HMBC and COLOC experiment of 7.
Long-range correlation observed in ^{13}C-{^1H} NOE experiment.
d) MS fragmentation of 7.

and N (C-6, δ_C 127.17), carbon M (C-2') with carbons J (C-11', δ_C 140.52) and R (C-3', δ_C 125.03), and carbon N (C-6) with carbons J (C-11') and Q (C-7, δ_C 125.13), as shown in Chart 4b. Moreover, long-range correlations observed in the HMBC[18], COLOC[19] and long-range ^1H-^{13}C COSY experiments (Chart 4c) revealed that the ^{13}C signal at δ_C 153.54 (C-1) was correlated to the ^1H signals at δ_H 4.95 (11-H), 14.21 (1-OH) and 7.21 (3-H), the latter of which showed also appreciable correlations to ^{13}C signals at δ_C 155.88 (C-4), δ_C 113.11 (C-10) and 66.76 (C-11). In addition, ^{13}C signals at δ_C 114.75 (C-9) and 113.11 (C-10) were correlated to ^1H signals at δ_H 14.21 (1-OH) and 13.79 (4-OH), respectively, which is consistent with the substitution pattern in **I**. Similarly, long-range correlations observed between C-5' (δ_C 156.04) and ^1H signals at δ_H 7.03 (7'-H) and 13.54 (5'-OH), and between C-8' (δ_C 155.94) and ^1H signals at δ_H 6.98 (H-6') and 13.77 (8'-OH) supported partial structure **II**. The COLOC spectrum showed significant correlations of a ^1H signal at δ_H 4.15 (12'-H) to a ^{13}C signal at δ_C 123.30 (C-13') and four sp^2 quaternary carbon signals, two of which were clearly assigned to C-11' (δ_C 140.52) and C-14' (δ_C 129.83), while the other two were assumed to be C-6 (δ_C 127.17) and C-2' (δ_C 127.28), leading a possible connection of partial structures **I** and **II** with **III** sharing at C-11'. In our experiments, neither 2D INADEQUATE, HMBC, nor COLOC pulse sequence experiments could elicit the connectivities between carbons C (C-8, δ_C 183.56) and W (C-9, δ_C 114.75), and carbons D (C-4', δ_C 183.41) and V (C-10') (Chart 4b). This problem was solved by measuring ^{13}C-{^1H} NOE[20]; ^{13}C signals of C (C-8, δ_C 183.56), H (C-1), I (C-2) and W (C-9) were enhanced after irradiation of the ^1H signal at δ_H 14.21 (1-OH), while those of B (C-5, δ_C 186.43), T (C-3), G (C-4), and Y (C-10), increased in signal intensity by irradiation of the ^1H signal at δ_H 13.79 (4-OH), which provided a conclusive evidence supporting the deduced structure for partial structure **I**. Similarly, the signal intensities of carbons D (C-4'), V

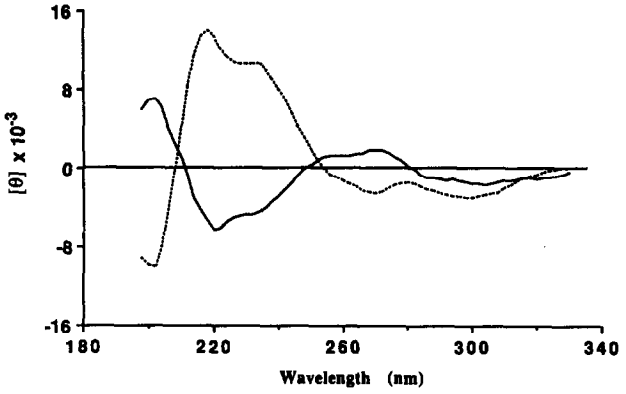

Figure 2. CD spectra (in MeOH) of: Shikometabolin A (**7**) (———)
Shikometabolin B (**8**) (·····)

(C-10') and E (C-5') were enhanced after irradiation at δ_H 13.54 (5'-OH), which supported partial structure **II**. In view of the molecular formula, a direct connectivity of carbons Q (C-7) and R (C-3') was deduced (Chart 4c).[21] From these findings, the gross structure of shikometaboline A was concluded as shown in formula **7**. In accordance with this conclusion, the FAB-MS spectrum of **7** revealed fragment ion peaks at *m/z* 268, 193 and 93, which could be interpreted in terms of the fragmentation shown in Chart 4d.

Compound 8 was obtained as black violet radiating plates from acetone-hexane, mp > 300 °C, (R_f 0.18, in solvent system B). The CD spectrum showed a positive Cotton effect $[\theta]_{234}$ + 11400 (MeOH) (Figure 2). Through tedious purification using Sephadex LH-20 and preparative TLC, the two metabolites (**7** and **8**) were obtained from the EtOAc extract in nearly equivalent amount (Chart 1). The IR, UV, ^{1}H-NMR and FAB-MS spectra of **7** and **8** were almost indistinguishable, and the ^{13}C-NMR spectra were also quite similar to each other except for ^{13}C signals at δ_C 183.00 (C-5), 124.72 (C-6), 126.83 (C-7), 187.04 (C-8) and 113.70 (C-9). Extensive analysis of these spectroscopic data suggested the same partial structures (**I-III**) for both **7** and **8** (Chart 4a). HMBC, COLOC and ^{13}C-{^{1}H} NOE experiments established the connectivities of C-11' to C-7 and C-2', which led us to conclude the formula **8** as shikometabolin B (Chart 5). The CD spectra of both **7** and **8** have mirror image shapes except for a band appearing at longer wavelength (ca. 300 nm) (Figure 2), reflecting their mirror image-like structures.

Compound 9 was obtained in a low yield as red needles from $CHCl_3$, mp 256-258°C, (R_f 0.42, in solvent system A). The CD spectrum showed a negative Cotton effect $[\theta]_{439}$ -295 (MeOH). The HRMS spectrum showed a molecular ion peak at *m/z* 540.1809 [M$^+$], indicating the molecular formula $C_{32}H_{28}O_8$ (Calcd 540.1784) which implies 19 degrees of unsaturation. The ^{1}H-NMR spectrum of **9** (see experimental) showed signals for four vinyl methyls at δ_H 1.19 (15'-CH_3), 1.31 (16'-CH_3), 1.58 (15-CH_3) and 1.73 (16-CH_3), a methylene at δ_H 2.68 and 2.88 (11'-H_β and 11'-H_α, respectively), two allylic protons at δ_H 3.35 (12'-H) and 4.94 (3-H), four olefinic protons at δ_H 4.73 (13'-H), 5.84 (13-H), 5.96 (11-H) and 6.12 (12-H) with the last three as ABX diene-type protons, a cluster of four aromatic protons at δ_H 7.19 (6-H), 7.24 (6'-H), 7.26 (7'-H) and 7.30 (7-H), and four ^{1}H signals at δ_H 11.20 (5-OH), 12.30 (8'-OH) and 12.50 (5'-OH and 8-OH). The ^{13}C-NMR, DEPT and ^{1}H-^{13}C COSY spectra indicated the presence of four carbonyls at δ_c 204.60 (C-1), 199.00 (C-4), 185.40 (C-1') and 184.70 (C-4'), eight sp^2 methine carbons, an sp^3 quaternary carbons at δ_c 55.70

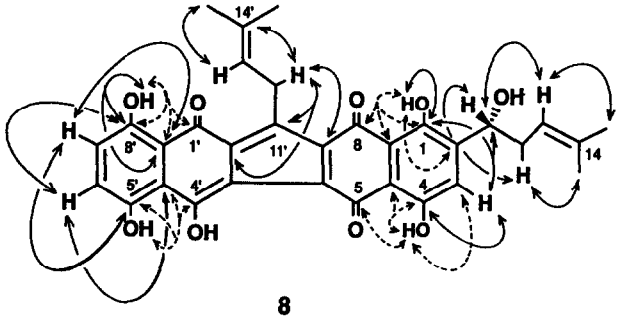

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Chart 5. Long-range correlations observed in HMBC and COLOC experiments of **8**.
Long-range correlations observed in ^{13}C-{^1H} NOE experiment

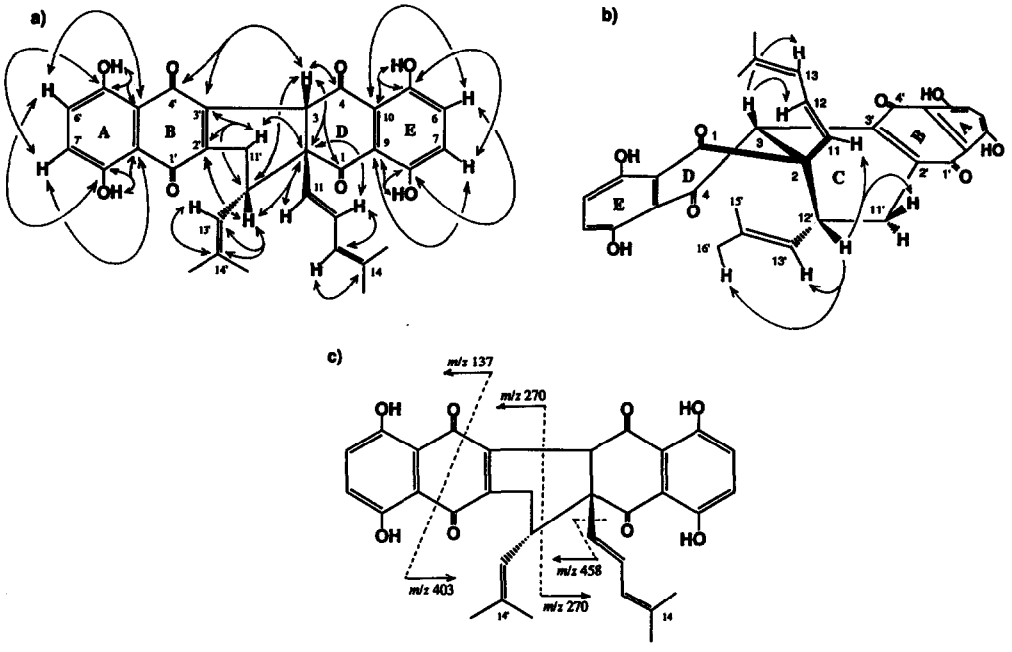

Chart 6. a) Significant long-range correlations observed in HMBC experiment of **9**.
b) NOE observed in difference NOE experiment of **9**.
c) MS fragmentation of **9**.

(C-2), two sp^3 allylic carbons at δ_c 45.65 (C-3) and 39.80 (C-12'), a methylene carbon at δ_c 27.30 (C-11'), and four vinyl methyl carbons at δ_c 18.04 (C-15'), 18.37 (C-15), 25.26 (C-16') and 25.96 (C-16) (Table II). The above mentioned data suggested that **9** was composed of two 1,4-naphthoquinone units as assumed by the formula **9**. The gross structure was verified by measuring the HMBC spectrum. The carbonyl ^{13}C signals at δ_C 204.60 (C-1) and 199.00 (C-4) and 184.70 (C-4') showed correlations with the ^1H signal at δ_H 4.94 (3-H)

which was further correlated to the ^{13}C signals at δ_c 140.17 (C-3'), 55.70 (C-2) and 39.80 (C-12'), suggesting the C_3—$C_{3'}$ linkage of the two naphthoquinone units. On the other hand, C_2—$C_{12'}$ linkage was confirmed by the correlations observed between C-2 and ^1H signals at δ_H 2.68 (11'-Hβ), 3.35 (12'-H), 5.96 (11-H) and 6.12 (12-H), in which the signals of 11'-Hβ and 12'-H were correlated to the ^{13}C signal at δ_c 144.8 (C-2'). Other pertinent long-range correlations observed are shown by arrows in Chart 6a. These data together with the fragment ion peaks at *m/z* 458, 403, 270, and 137 (Chart 6c) unequivocally confirmed the gross structure of this compound as shown by formula **9**. The relative stereochemistry of **9** (Chart 6) was predicted on the basis of the coupling constants and the results of difference NOE experiments. Irradiation at δ_H 3.35 (12'-H) increased the signals intensities of 11'-Hβ, 11-H and 13'-H, while irradiation at 4.94 (3-H) enhanced the signals intensities of 12-H and 13-H. In addition, a *W*-type long-range coupling between 3-H and 12'-H (J = 3.0 Hz) suggested that C-2 was a bridgehead carbon sharing C- and D-rings, with β-oriented neighboring protons at C-3 and C-12'. These data fit well with the half-chair conformation of the D-ring and the half boat for the C-ring (Chart 6b), which accounts for relative upfield shifts (δ_H 1.19 and 1.31 ppm) of the two vinyl methyl protons (15'-CH_3 and 16'-CH_3, respectively) caused by shielding effect of the benzene ring. These findings led us to conclude that **9** is a 1,4-naphthoquinone dimer named shikometabolin C.

Compound **10** was obtained as a minor red metabolite from $CHCl_3$ (Rf 0.38, in solvent system A). The CD spectrum showed negative Cotton effect $[\theta]_{500}$ -252 (MeOH). The HRMS spectrum suggested its dimeric nature with a molecular ion peak at *m/z* 538.1613 [M^+], assigned to the molecular formula $C_{32}H_{26}O_8$ (Calcd 538.1626). Extensive analysis of the ^1H- and ^{13}C-NMR spectra by the aid of ^1H-^1H COSY, ^1H-^{13}C COSY and DEPT, indicated a pattern similar in part to that of **9** with four vinyl methyl carbons, two olefinic carbons, a cluster of four aromatic protons assignable to two 1,2,3,4-tetrasubstituted benzene rings, two carbonyl carbons, and four *peri*-hydroxyl groups. However, characteristic signals for four sp^3 allylic groups were observed at δ_c 37.56 (C-12'; δ_H 3.35), 38.08 (C-11'; δ_H 3.88), 40.66 (C-12; δ_H 3.82), and 45.30 (C-3; δ_H 2.68) (Table II). From the above mentioned data the structure of this metabolite was assumed to be formula **10**. Long-range correlations observed in the HMBC spectrum confirmed the proposed structure (Chart 7a). A ^1H signal at δ_H 3.88 (11'-H) was correlated to ^{13}C signals at δ_c 182.25 (C-1'), 144.27 (C-3'), 137.54 (C-2) and 45.30 (C-3), and a ^1H signal at δ_H 3.82 (12-H) showed the correlation with ^{13}C signals at δ_C 182.61 (C-4'), 137.80 (C-2') and 38.08 (C-11'), indicating the connectivity between $C_{3'}$—C_{12}, which inturn led to the connectivity of C-ring to D-ring through C-11 and C-11'. The observed three bond correlations of 3-H (δ_H 2.68) to C-1 (δ_C 193.10), C-13' (δ_C 117.43) and C-11' revealed that C-3 was assigned as an α-keto allylic carbon connected to C-12'. The above mentioned data together with characteristic fragment ions peak at *m/z* 457, 352, 199, 153 and 137 (Chart 7c) suggested the plain structure of this compound as shown by formula **10**. The relative stereochemistry of **10** was deduced by consideration of the coupling constants and the NOE difference spectra (Chart 7b). Irradiation at δ_H 2.68 (3-H) enhanced the signals intensities of 12'-H and 13'-H, and measurable NOE effects of 3-H, 13'-H, 15'-H, 12-H and 11'-H were observed by irradiation at δ_H 3.35 (12'-H). On the other hand, irradiation at δ_H 3.82 (12-H) intensified the signals intensities of 11'-H, 12'-H, 13-H and 15-H, and pulsing at δ_H 3.88 (11'-H) increased the signals intensities of 12-H, 12'-H and 15'-H. Coupling constants observed between 11'-H and 12'-H (J = 4.5 Hz), and between 3-H and 12'-H (J = 3.0 Hz) implied dihedral angles of near 110 ^{o} and 70 ^{o}, respectively. An observation that clearly defined the usual half boat conformation of D-ring with the protons (3-H, 12'-H, 11'-H and 12-H) *cis* disposed to the β face of the five membered-rings (ring C and D) was also inspected with the Dreiding model. On these grounds, **10** was concluded as shikometabolin D.

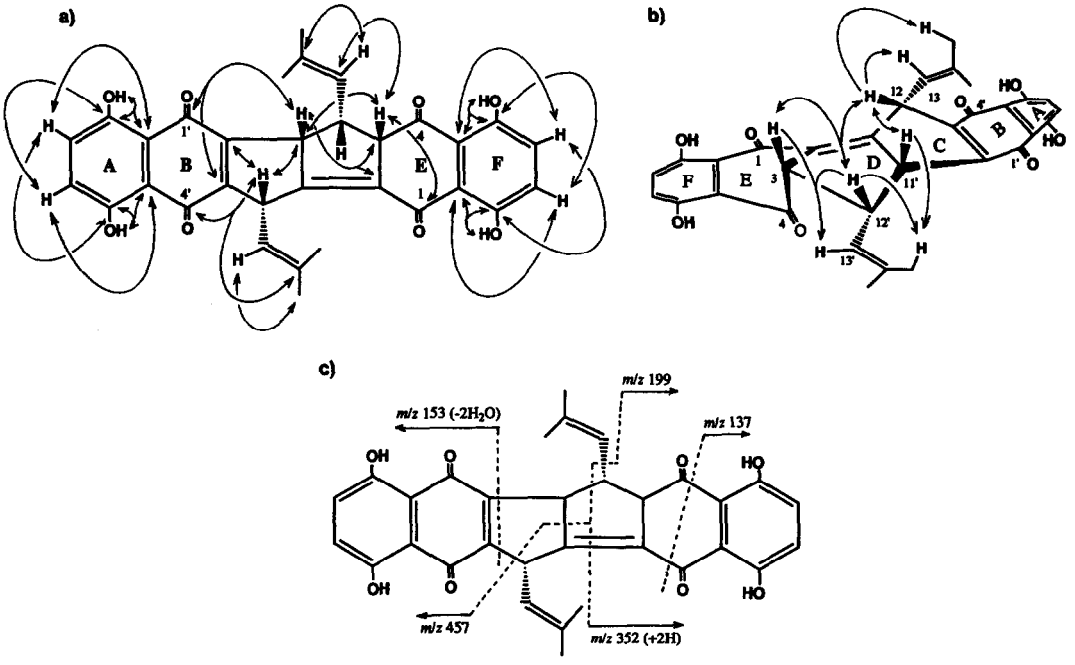

Chart 7. a) Significant long-range correlations observed in HMBC experiment of **10**.
b) NOE observed in difference NOE experiment of **10**.
c) MS fragmentation of **10**.

Compound 11 was obtained as minor unstable orange amorphous powder from $CHCl_3$ (Rf 0.20, in solvent system A). The HRMS spectrum displayed a molecular ion peak at m/z 544.1211 [M^+] corresponding to the molecular formula $C_{32}H_{32}O_8$ (Calcd 544.1273), implying that **11** is a dimer with 17 degrees of unsaturation. The ^1H- and ^{13}C-NMR spectra (Table II) showed a pattern similar in part to those of **5** and in the other part to those of **6**, with signals for four vinyl methyl groups, four methylene groups at δ_H 3.05 (δ_C 36.13 and 36.32), four carbonyl carbons at δ_C 200.90, 200.95, 201.83 and 201.89, and four *peri*-hydroxyl protons. Moreover, no aromatic protons were detected in the ^1H-NMR spectrum (see experimental) and a characteristic fragment ion peak was observed at m/z 271 attributable to **5**, (the loss of **6** from **11**). All these data showed that **11** was a dimer composed of **5** and **6**, which was formed by bonding at C_6 with $C_{6'}$. Accordingly, the structure of this compound was concluded to be as shown by formula **11** in Chart 1 and we named it shikometabolin E.

Figure 3 showed the time course for the metabolism of shikonin (**1**), which revealed that **1** was completely metabolized by *B. fragilis* 72 h after anaerobic incubation. Of the major metabolites, **5** and **6** were produced firstly and reached its maximum after 24 h. However, these metabolites seem to be unstable and were gradually condensed each other after prolonged incubation. The slow change in color from red to green and finally to dark blue in the culture medium showed gradual formation of the major dark violet metabolites (**7** and **8**) to reach its maximum after 72 h. The red dimer **9** was produced in a relatively low yield after 24 h and reached its maximum after 72 h.

Table II. ^{13}C-NMR (100 MHz) Spectral Data of Metabolites **5** - **11**.

Carbon no.	**5** [a)]	**6** [a)]	**7** [b)]	**8** [b)]	**9** [a)]	**10** [a)]	**11** [a)]
1	202.00 (s) [c]	201.30 (s) [c]	153.54 (s)	153.28 (s)	204.60 (s)	193.11 (s)	200.95 (s)[c]
2	36.00 (t) [d]	36.10 (t) [d]	144.97 (s)	144.23 (s)	55.70 (s)	137.54 (s)	36.13 (t)[d]
3	36.00 (t) [d]	36.30 (t) [d]	122.60 (s)	122.90 (d)	45.65 (d)	45.30 (d)	36.13 (t)[d]
4	200.40 (s) [c]	200.90 (s) [c]	155.88 (s)	155.85 (s)	199.00 (s)	195.48 (s)	200.90 (s)[c]
5	155.00 (s)	154.10 (s)	186.43 (s)	183.00 (s)	153.56 (s)	158.96 (s)	153.92 (s)
6	122.00 (d)	127.50 (d)	127.17 (s)	124.72 (s)	127.84 (d)	129.27 (d)	127.54 (s)
7	138.20 (s)	143.70 (s)	125.13 (s)	126.83 (s)	129.97 (d)	129.09 (d)	136.56 (s)
8	153.50 (s)	155.00 (s)	183.56 (s)	187.04 (s)	159.05 (s)	156.29 (s)	153.71 (s)
9	114.30 (s)	113.60 (s)	114.75 (s)	113.70 (s)	111.29 (s)	112.42 (s)	114.67 (s)
10	113.00 (s)	112.70 (s)	113.11 (s)	113.98 (s)	115.19 (s)	112.67 (s)	113.88 (s)
11	121.30 (d)	30.40 (t)	66.76 (d)	66.50 (d)	128.68 (d)	117.43 (s)	122.80 (d)
12	131.80 (d)	27.70 (t)	35.66 (t)	35.52 (t)	127.45 (d)	40.66 (d)	127.69 (d)
13	125.90 (d)	122.90 (d)	121.14 (d)	120.97 (d)	123.90 (d)	116.82 (d)	127.15 (d)
14	141.30 (s)	133.00 (s)	132.24 (s)	132.06 (s)	137.80 (s)	140.03 (s)	141.41 (s)
15	18.80 (q)	17.60 (q)	17.75 (q)	17.51 (q)	18.37 (q)	18.68 (q)	17.43 (q)
16	26.40 (q)	25.60 (q)	25.59 (q)	25.32 (q)	25.96 (q)	25.99 (q)	24.20 (q)
1'			186.63 (s)	186.70 (s)	185.40 (s)	182.25 (s)	201.83 (s)[e]
2'			127.28 (s)	127.04 (s)	144.80 (s)	137.80 (s)	36.32 (t)[f]
3'			125.03 (s)	125.15 (s)	140.17 (s)	144.27 (s)	36.32 (t)[f]
4'			183.41 (s)	183.15 (s)	184.70 (s)	182.61 (s)	201.89 (s)[e]
5'			156.04 (s)	155.85 (s)	159.17 (s)	157.35 (s)	143.15 (s)
6'			126.25 (d)	126.49 (d)	128.42 (d)	128.94 (d)	127.54 (s)
7'			126.77 (d)	125.97 (d)	129.73 (d)	129.94 (d)	142.90 (s)
8'			155.94 (s)	156.30 (s)	158.17 (s)	158.51 (s)	155.05 (s)
9'			114.50 (s)	114.44 (s)	111.85 (s)	111.42 (s)	113.88 (s)
10'			115.46 (s)	115.38 (s)	114.43 (s)	111.66 (s)	111.26 (s)
11'			140.52 (s)	140.58 (s)	27.30 (t)	38.08 (d)	29.67 (t)
12'			26.25 (t)	26.12 (t)	39.80 (d)	37.56 (d)	26.66 (t)
13'			123.30 (d)	123.08 (d)	122.20 (d)	117.43 (s)	114.67 (d)
14'			129.83 (s)	129.62 (s)	138.17 (s)	138.65 (s)	127.49 (s)
15'			18.20 (q)	17.94 (q)	18.04 (q)	18.68 (q)	17.43 (q)
16'			25.65 (q)	25.39 (q)	25.26 (q)	25.78 (q)	24.20 (q)

[a,b] Chemical shifts in δ ppm, measured in: [a] $CDCl_3$, [b] DMSO-d_6.; [a,b] The assignments of carbon signals were verified by C-H correlation experiments (^{13}C-^{1}H COSY, HMBC, and [b] COLOC), and multiplicities (in parentheses) indicated as singlet (s), douplet (d), triplet (t), and quartet (q); [c-f] Assignments with the same superstrock may be interchanged within column.

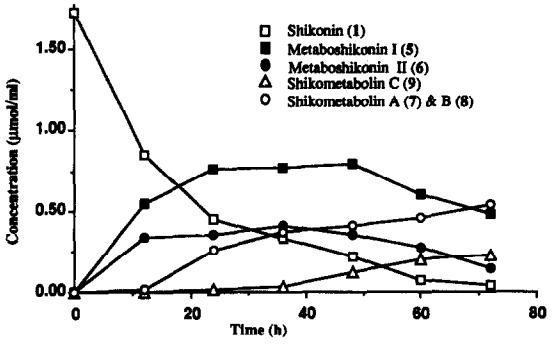

Figure 3. Time course of metabolism of shikonin by *B. fragilis* subsp. *thetaotus*

Ten metabolites, anhydroalkannin (**2**), deoxyshikonin (**3**), cycloshikonin (**4**), metaboshikonin I (**5**), II (**6**), shikometabolins A (**7**), B (**8**), C (**9**), D (**10**) and E (**11**) were isolated after anaerobic incubation of shikonin (**1**) with *B. fragilis* for 3 days. The production of these metabolites (Chart 8) seems to be mainly through dehydration and reduction of **1** or its tautomer (in one route) to yield the monomeric metabolites (**2**, **3**, **5** and **6**), followed by condensation (in the other) to yield the dimeric ones (**7** - **11**). Cyclization of **1** was also considered to produce **4**. Tautomerization of **1** seems to be a key step in the biotransformation of **1**, since dehydration and reduction of this tautomer by bacterial enzymes afforded major metabolites (**5** and **6**) in one of the main pathways, followed by condensation of them through C_6— $C_{6'}$ bonding to produce **11**. On the other hand, condensation of a shikonin tautomer with **2** seems to be the other main pathway to produce the violet dimers (**7** and **8**). The minor dimeric metabolites (**9** and **10**) were produced slowly by condensation of **2** and **3**. Coupling of C_3—$C_{3'}$ and C_2—$C_{12'}$ resulted in the formation of **9**, while that of C_3—$C_{12'}$ and C_{11}—$C_{11'}$ afforded **10**. Repeated experiments on the metabolism of **1** by intestinal bacterial strains or the human feces showed no appreciable change in the metabolites under the conditions used, which suggested that common bacterial species including *B. fragilis*, as demonstrated above, may take part in the metabolic processes in the intestinal tract.

Obtaining 10 metabolites of **1** from one culture, coupled with the selectivity and mild conditions associated with biologically catalyzed reactions, is a good indication that transformation reactions carried out in the gut can be used as a useful adjust in assessing a drug safety and efficacy, and for production of novel derivatives. Obviously, such information can rarely be obtained initially using humans. Therefore, studies on drug metabolism have traditionally relied on the use of micro-organisms as models. Smith and Rosazza have summarized the many advantages of using micro-organisms as models.[22] The major advantage cited is the ability of micro-organisms to produce significant quantities of metabolites that would be difficult to obtain from either animal systems or from chemical synthesis. Other advantages include the greatly reduced cost of carrying out microbial metabolism studies as compared to animal metabolism studies. Microbial cultures are relatively easy to maintain at a very low cost. The authors demonstrated also that several aromatic compounds were metabolized by micro-organisms in a manner analogous to human biotransformations. In addition, the total number of research animals necessary for the evaluation of a drug's metabolic profile can be significantly reduced when micro-organisms are used as predictive models for initial studies.

Since shikonin (**1**) was reported to have potent anti-inflammatory[7] and anti-tumor[5] activities, the metabolites obtained in the present experiment were screened for their biological activities. However, these metabolites showed no suppressive effects on the carragenin-enduced hind-paw edema in mice (data not shown),

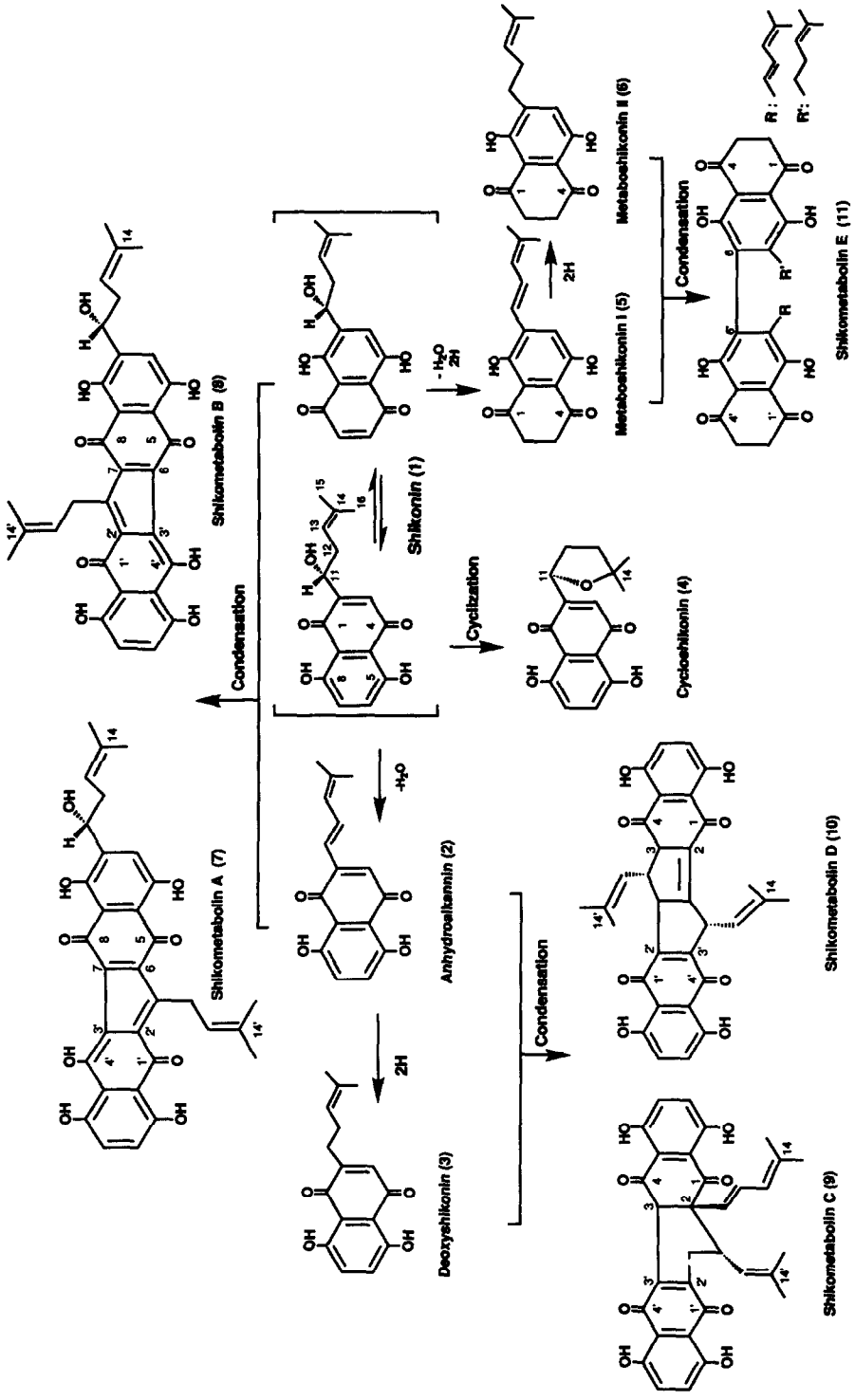

Chart 8. Possible Metabolic Pathways of Shikonin (1)

which indicated that the anti-inflammatory effect of **1** was abolished after anaerobic incubation with human intestinal bacteria. Of the 10 metabolites, violet dimers (**7** and **8**) showed inhibitory effects (IC_{50}: 0.71 and 1.29 mM, respectively) on reverse transcriptase of human immunodefficency virus but their inhibitory potencies were less than that shown by **1** (IC_{50}: 0.63 mM). With regard to the structures of these metabolites, it is of interest to note that anaerobic reduction and/or condensation of **1** resulted in the reduction of biological activities. However, we are screening the biological activities of these metabolites using other bio-assay systems.

EXPERIMENTAL

General Experimental Procedures. Melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. CD spectra were recorded with a JASCO J-720 automatic recording spectropolarimeter in MeOH and $CHCl_3$. IR spectra were measured on a Hitachi 260-10 infrared spectrometer. UV spectra were taken on a Shimadzu UV-2200 UV-VIS spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were measured with a JEOL GX- 400 spectrometer with TMS as an internal standard and chemical shifts were recorded in δ values. ^{1}H-^{1}H shift correlation spectroscopy (COSY), ^{1}H-^{13}C COSY, HMQC[16], long-range ^{1}H-^{13}C COSY, HMBC[18] COLOC[19] and 2D INADEQUATE ($J_{C\text{-}C}$ = 60 Hz, 36 h)[17] experiments were performed with the usual pulse sequence and data processing was obtained with the standard JEOL software. Difference NOE spectra were obtained by the use of JEOL standard pulse sequence with irradiation for 5 sec. EI-MS and FAB-MS spectra were measured with a JEOL JMS DX-300 mass spectrometer. HPLC was performed on a Tri-Rotar-V (JASCO Co. Ltd., Tokyo, Japan) equipped with a JASCO UVIDEC-100 UV spectrophotometer and a Shimadzu C-R 6A chromatopac (Shimadzu Co. Ltd., Kyoto, Japan). Kieselgel 400 (Wako pure Chemical Co., Osaka, Japan), Merck Kieselgel 60 (E. Merck, Darmestadt, Germany) and Sephadex LH-20 (Pharmacia LKB, Sweden) were used for column chromatography. Merck Kieselgel 60 F_{254} (layer thickness 0.25 mm and 0.5 mm) were used for TLC and preparative-TLC, respectively, with solvent systems A, hexane-Me_2CO (8 : 3); and B, benzene-EtOAc (25 : 30). Spots were detected in day light, under a UV lamp and after spraying with $Ce(SO_4)_2$-H_2SO_4.

Chemicals. Shikonin was purchased from Mitsui Petrochemical Industries, Ltd. (Tokyo, Japan) and its purity was confirmed by means of TLC, HPLC, CD, and ^{1}H- and ^{13}C-NMR spectroscopy before use. General anaerobic medium (GAM) was purchased from Nissui Co. (Tokyo, Japan).

Micro-organisms. Human intestinal bacterial strains were provided by Professor T. Mitsuoka, Tokyo University.

Screening of Bacterial Strains for Their Abilities to Metabolize Shikonin (1). Precultured bacterial strains (0.2 ml each) were individually incubated for 24 h at 37 °C in general anaerobic medium (GAM; 10 ml) under anaerobic conditions in an anaerobic jar in which the air had been replaced with oxygen-free CO_2. The culture was diluted 10-fold with the same medium and incubated for further 24 h. The pellets obtained by centrifugation at 7,800 x g for 10 min were washed twice with a saline solution and the bacterial cells were suspended in 0.1 M phosphate buffer (10 ml, pH 7.3). Initial screening was carried out in 30-ml test tubes containing 10 ml of each bacterial suspension. Shikonin (10 mg, **1** in 0.1 ml DMSO) was added and the mixture was incubated under the same anaerobic conditions for 3 days at 37 °C. The incubation mixture was adjusted to pH ca. 3.0 with 5% HCl and then extracted with EtOAc (10 ml x 3). The extract was evaporated *in vacuo* to give a residue. The residue was dissolved in MeOH (1 ml) and the metabolites were analyzed by HPLC using a

Finepack SIL C1-5 column (JASCO Co. Ltd., Tokyo, Japan, 250 mm x 4.5 mm i.d.); mobile phase, MeCN-H_2O-H_3PO_4 (55 : 45 : 0.1), flow rate, 1.0 ml/min; injection volume, 1 ml; detection, UV 280 nm. The metabolites were quantitatively determined by using calibration lines obtained with the isolated compounds.

Transformation of Shikonin (1) with *Bacteroides fragilis* subsp. *thetaotus*. Stock cultures of *B. fragilis* (2 l) were added to GAM broth (18 l) and cultured overnight at 37 °C under anaerobic conditions. The bacterial culture was centrifuged at 7,800 x g for 10 min and the pellets were washed twice with saline and suspended in 0.1 M phosphate buffer (20 l, pH 7.3). A total of 20 g of shikonin (**1**, in 200 ml DMSO) was added to the bacterial suspension and the mixture was anaerobically incubated for 3 d at 37 °C. The mixture was pooled, adjusted to pH ca. 3.0 with 5% HCl, and extracted with EtOAc (20 l x 5). The EtOAc layer was washed with H_2O and evaporated *in vacuo* to give a dark-brown residue (34 g). The residue was applied to a silica gel column (50 x 10 cm), which was then gradiently eluted with hexane, $CHCl_3$ and $CHCl_3$-MeOH (9 : 1), successively. Fractions (1500 ml each) were collected and monitored by TLC. Fraction A was concentrated and applied to a Kieselgel 60 (E. Merck) column. Elution with hexane: Me_2CO (9:1) gave three compounds **2, 3** and **4**. Fraction B was subjected to column chromatography [Kieselgel 60, hexane-$CHCl_3$ (7 : 3 and 1 : 9)] and subsequent preparative-TLC (solvent system A) to afford compounds **5** and **6** as main yellow and orange powder, respectively, with minor compounds **9-11**. Successive elution of the EtOAc extract with $CHCl_3$-MeOH (9 :1) afforded a mixture of dark blue compounds in fraction C which could not be separated by HPLC (Figure 1). However, repeated column chromatography over Kieselgel 60 [hexane-$CHCl_3$ (1 : 9) and $CHCl_3$-MeOH (9 : 1)], preparative-TLC and subsequent Sephadex LH-20 [$CHCl_3$-MeOH (7 : 3)] column chromatography afforded two main dark blue compounds **7** and **8**. The fractionation procedures of the EtOAc extract are illustrated in Chart 2.

Compound 2. Red needles from $CHCl_3$, mp 134-135 °C, (2mg, 0.01 %). UV λ_{max} ($CHCl_3$) nm (log ε): 290 (2.89), 500 (2.51). IR ν_{max} ($CHCl_3$) cm^{-1}: 3450 (OH), 1620 (C=O), 1520 (C=C). EI-MS *m/z*: 270 [M^+]. ^{1}H-NMR ($CDCl_3$) δ_H: 1.96 (3H, d, *J* = 1.5 Hz, 15-CH_3), 2.29 (3H, s, 16-CH_3), 6.09 (1H, dd, *J* = 11, 1.5 Hz, 13-H), 6.47 (1H, d, *J* = 15.5 Hz, 11-H), 7.21 (1H, s, 3-H), 7.26 (2H, s, 6-H and 7-H), 7.63 (1H, dd, *J* = 15.5, 11 Hz, 12-H), 12.78 (1H, s, 5- or 8-OH), 12.85 (1H, s, 8- or 5-OH). This compound was identified as anhydroalkannin by comparison of the spectral data with the published ones.[11f]

Compound 3. Red needles from $CHCl_3$, mp 93-94 °C, (10 mg, 0.05%). UV λ_{max} ($CHCl_3$) nm (log ε): 280 (2.79), 520 (2.59). IR ν_{max} ($CHCl_3$) cm^{-1}: 3450 (OH), 1620 (C=O), 1520 (C=C). EI-MS *m/z*: 272 [M^+]. ^{1}H-NMR ($CDCl_3$) δ_H: 1.60 (3H, s, 16-CH_3), 1.69 (3H, d, *J* = 1.5 Hz, 15-CH_3), 2.31 (2H, q, *J* = 7.3 Hz, 12-H), 2.63 (2H, t, *J* = 7.3 Hz, 11-H), 5.15 (1H, t, *J* = 7.3 Hz, 13-H), 6.85 (1H, s, 3-H), 7.21 (2H, s, 6-H and 7-H), 12.47 (1H, s, 5- or 8-OH), 12.63 (1H, s, 8- or 5-OH). ^{13}C-NMR ($CDCl_3$) δ_C: 17.80 (q, C-15), 25.66 (q, C-16), 26.54 (t, C-11), 29.70 (t, C-12), 111.69 (s, C-10), 111.96 (s, C-9), 122.29 (d, C-13), 130.82 (d, C-7), 131.15 (d, C-6), 133.61 (s, C-14), 134.55 (d, C-3), 151.49 (s, C-2), 162.21 (s, C-5), 162.88 (s, C-8), 183.04 (s, C-1), 183.10 (s, C-4). Compound **3** was identified by comparison of the ^{1}H- and ^{13}C-NMR spectra with those reported for deoxyshikonin.[11b]

Compound 4. Red needles from hexane, mp 87-89 °C, (12.8 mg, 0.064%). CD (*c* = 3 mM, $CHCl_3$): $[\theta]_{330}$ +205. UV λ_{max} ($CHCl_3$) nm (log ε): 280 (4.50), 480 (3.74), 520 (3.62). IR ν_{max} ($CHCl_3$) cm^{-1}: 3350, 1610, 1265. EI-MS *m/z* : 288 [M^+], 230 [M^+-C_3H_6], 219 [M^+-C_5H_9]. ^{1}H-NMR ($CDCl_3$) δ_H: 1.35 (3H, d, *J* = 1.0 Hz, 15-CH_3 or 16-CH_3), 1.38 (3H, s, 16-CH_3 or 15-CH_3), 1.80 (1H, m, 13-H), 1.82 (1H, m, 12-H), 1.90 (1H, m, 13-H), 2.63 (1H, m, 12-H), 5.15 (1H, dd, *J* = 7.5, 1.5 Hz, 11-H), 7.18 (1H, d, *J* = 9.0 Hz, 6-H or 7-

H), 7.20 (1H, d, J = 9.0 Hz, 7-H or 6-H), 7.21 (1H, d, J = 1.5 Hz, 3-H), 12.51 (1H, s, 5-OH or 8-OH), 12.53 (1H, s, 8-OH or 5-OH). ^{13}C-NMR ($CDCl_3$) δ_C: 27.82 (q, C-15 or C-16), 28.70 (q, C-16 or C-15), 33.43 (t, C-13), 38.44 (t, C-12), 74.35 (d, C-11), 82.13 (s, C-14), 111.63 (s, C-9 or C-10), 112.10 (s, C-10 or C-9), 131.21 (d, C-6), 131.34 (d, C-7), 131.70 (d, C-3), 152.95 (s, C-2), 163.36 (s, C-5), 163.88 (s, C-8), 181.46 (s, C-1), 182.40 (s, C-4). This compound was identified as cycloshikonin by comparing the ^{1}H- and ^{13}C-NMR spectra with the reported data.[9a]

Compound 5. Unstable major metabolite obtained as fine yellow needles from $CHCl_3$, mp 146-148 °C, (152 mg, 0.76%). UV λ_{max} ($CHCl_3$) nm (log ε): 275 (2.96), 440 (2.88). IR ν_{max} ($CHCl_3$) cm^{-1}: 3450 (OH), 1520 (C=C). EI-MS *m/z*: 272[M$^+$], 257 [M$^+$-CH_3], 229 [M$^+$-$COCH_3$] and 136. HRMS *m/z* : 272.1019 (Calcd for $C_{16}H_{16}O_4$, 272.1047). ^{1}H-NMR ($CDCl_3$) δ_H : 1.88 (3H, d, J = 1.5 Hz, 15-CH_3), 1.90 (3H, s, 16-CH_3), 3.03 (4H, t, J = 3.0 Hz, 2-H and 3-H), 6.09 (1H, dd, J = 11.0, 1.5 Hz, 13-H), 6.61 (1H, d, J = 15.5 Hz, 11-H), 7.19 (1H, dd, J = 15.5, 11.0 Hz, 12-H), 7.30 (1H, s, 6-H), 12.01 (1H, s, 5-OH), and 12.63 (1H, s, 8-OH). ^{13}C-NMR spectral data: see Table II.

Compound 6. Unstable major metabolite obtained as an amorphous powder (70 mg, 0.35%). UV λ_{max} ($CHCl_3$) nm (log ε): 240 (2.77), 265 (2.87), 440 (2.58). IR ν_{max} ($CHCl_3$) cm^{-1}: 3450 (OH), 1520 (C=C). EI-MS *m/z*: 274 [M$^+$], 256 [M$^+$-H_2O], 218 [M$^+$-CH=C(CH_3)$_2$], 206 and 96. HRMS *m/z* : 274.1174 (Calcd for $C_{16}H_{18}O_4$, 274.1214). ^{1}H- NMR ($CDCl_3$) δ_H: 1.51 (3H, d, J = 1.5 Hz, 15-CH_3), 1.61 (3H, s, 16-CH_3), 2.24 (2H, q, J = 7.5 Hz, 12-H), 2.63 (2H, t, J = 7.5 Hz, 11-H), 2.95 (4H, s, 2-H and 3-H), 5.09 (1H, dd, J = 7.5, 1.5 Hz, 13-H), 7.10 (1H, s, 6-H), 11.94 (1H, s, 5-OH), and 12.30 (1H, s, 8-OH). ^{13}C-NMR spectral data: see Table II.

Compound 7. Obtained as dark violet needles from hexane-acetone, mp > 300 °C, (200 mg, 1.0%). CD (c = 0.67 mM, MeOH): $[\theta]_{234}$ - 4200, $[\theta]_{276}$ +1800, $[\theta]_{304}$ -1800. UV λ_{max} (MeOH) nm (log ε): 280 (2.97), 420 (2.52), 575 (2.78). IR ν_{max} (KBr) cm^{-1}: 3350 (OH), 1620 (C=O), 1460 (C=C). Negative ion FAB-MS *m/z* : 555 [M-H]$^-$, 457 [(M-H) - $C_6H_{14}O$]$^-$, 268 [M-(partial structure **I** - H_2O)]$^-$, 193 [M-(partial structure **II** + 2H)]$^-$, 137, 93 (Chart 4d). HR-FAB-MS *m/z* : 555.1650 [M-H]$^-$ (Calcd for $C_{32}H_{27}O_9$: 555.1658). ^{1}H-NMR (DMSO-d_6) δ_H: 1.56 (3H, d, J = 2.0 Hz, 15-CH_3), 1.64 (3H, s, 16'-CH_3), 1.67 (3H, s, 16-CH_3), 1.84 (3H, d, J = 2.0 Hz, 15'-CH_3), 2.25 (1H, dt, J = 12.0, 7.0 Hz, 12-H$_\beta$), 2.54 (1H, dd, J = 12.0, 3.5 Hz, 12-H$_\alpha$), 4.15 (2H, d, J = 7.0 Hz, 12'-H), 4.95 (1H, m, 11-H), 5.16 (1H, s, 11-OH), 5.26 (1H, dd, J = 7.0, 2.0 Hz, 13'-H), 5.29 (1H, dd, J = 7.0, 2.0 Hz, 13-H), 6.98 (1H, d, J = 9.0 Hz, 6'-H), 7.03 (1H, d, J = 9.0 Hz, 7'-H), 7.21 (1H, s, 3-H), 13.54 (1H, s, 5'-OH), 13.77 (1H, s, 8'-OH), 13.79 (1H, s, 4-OH), 14.21 (1H, s, 1-OH). ^{13}C-NMR spectral data: see Table II.[21]

Compound 8. Obtained as dark violet radiating plates from hexane-acetone (170 mg, 0.85 %). CD (c = 0.67 mM, MeOH): $[\theta]_{218}$ + 15600, $[\theta]_{234}$ +11400, $[\theta]_{270}$ -3000, $[\theta]_{302}$ -3000. UV λ_{max} (MeOH) nm (log ε): 280 (2.97), 420 (2.52), 575 (2.78). IR ν_{max} (KBr) cm^{-1}: 3350 (OH), 1620 (C=O), 1460 (C=C). Negative ion FAB-MS *m/z* : 555 [M-H]$^-$. HR-FAB-MS *m/z:* 555.1650 [M-H]$^-$ (Calcd for $C_{32}H_{27}O_9$: 555.1658). ^{1}H-NMR (DMSO-d_6) δ_H: 1.55 (3H, d, J = 1.5 Hz, 15-CH_3), 1.63 (3H, d, J = 1.5 Hz, 15'-CH_3), 1.68 (3H, s, 16-CH_3), 1.82 (3H, s, 16'-CH_3), 2.20 (1H, dt, J = 13.0, 7.0 Hz, 12-H$_\beta$), 2.50 (1H, m, 12-H$_\alpha$), 4.14 (2H, d, J = 7.0 Hz, 12'-H), 4.91 (1H, m, 11-H), 5.25 (1H, dd, J = 7.0, 1.5 Hz, 13'-H), 5.29 (1H, dd, J = 7, 1.5 Hz, 13-H), 7.06 (1H, d, J = 9.0 Hz, 7'-H), 7.09 (1H, d, J = 9.0 Hz, 6'-H), 7.17 (1H, s, 3-H), 13.76 (2H, s, 4-OH and 5'-OH), 13.80 (1H, s, 8'-OH), 14.34 (1H, s, 1-OH). ^{13}C-NMR spectral data: see Table II.[21]

Compound 9. Red needles from $CHCl_3$, mp 256-258 °C, (18 mg, 0.09 %). CD (c = 3.71 mM, MeOH): $[\theta]_{439}$ - 295, $[\theta]_{486}$ +269, $[\theta]_{520}$ -269. UV λ_{max} ($CHCl_3$) nm (log ε): 250 (3.20), 410 (2.73), 520 (2.66), 555 (2.43). IR ν_{max} ($CHCl_3$) cm^{-1}: 3450 (OH), 1620 (C=O), 1520 (C=C). EI-MS *m/z:* 540 [M^+], 471 [M^+-C_5H_9], 458 [M^+-C_6H_{11}], 403, 270, 149 and 69. HRMS *m/z:* 540.1809 (Calcd for $C_{32}H_{28}O_8$, 540.1784). ^{1}H-NMR ($CDCl_3$) δ_H: 1.19 (3H, d, J = 1.5 Hz, 15'-CH_3), 1.31 (3H, d, J = 1.0 Hz, 16'-CH_3), 1.58 (3H, d, J = 1.5 Hz, 15-CH_3), 1.73 (3H, d, J = 1.0 Hz, 16-CH_3), 2.68 (1H, ddd, J = 20.0, 5.5, 1 Hz, 11'-Hβ), 2.88 (1H, dd, J = 20.0, 1.5 Hz, 11'-Hα), 3.35 (1H, dd, J = 10.5, 5.5 Hz, 12'-H), 4.73 (1H, ddt, J = 10.5, 1.5, 1.0 Hz, 13'-H), 4.94 (1H, d, J = 1.0 Hz, 3-H), 5.84 (1H, ddt, J = 10.5, 1.5, 1.0 Hz, 13-H), 5.96 (1H, d, J = 15.5 Hz, 11-H), 6.12 (1H, dd, J = 15.5, 10.5 Hz, 12-H), 7.19 (1H, d, J = 9.0 Hz, 6-H), 7.24 (1H, d, J = 9.0 Hz, 6'-H), 7.26 (1H, d, J = 9.0 Hz, 7'-H), 7.30 (1H, d, J = 9.0 Hz, 7-H), 11.20 (1H, s, 5-OH), 12.30 (1H, s, 8'-OH), 12.50 (2H, s, 8-OH and 5'-OH). ^{13}C-NMR spectral data: see Table II.

Compound 10. Red amorphous powder (9 mg, 0.045 %). CD (c = 1 mM, MeOH): $[\theta]_{280}$ - 252, $[\theta]_{500}$ -252. UV λ_{max} ($CHCl_3$) nm (log ε): 220 (2.92), 420 (2.57), 520 (2.47). IR ν_{max} ($CHCl_3$) cm^{-1}: 3450 (OH), 1620 (C=O), 1540 (C=C). EI-MS *m/z:* 538 [M^+], 457 [M^+-C_5H_8], 403 [M^+-$C_7H_4O_3$], 352, 199, 153, 137. HRMS *m/z:* 538.1613 (Calcd for $C_{32}H_{26}O_8$, 538.1626). ^{1}H-NMR ($CDCl_3$) δ_H: 1.56 (3H, d, J = 1.0 Hz, 16-CH_3), 1.61 (3H, d, J = 1.0 Hz, 16'-CH_3), 1.73 (3H, d, J = 2.0 Hz, 15'-CH_3), 1.83 (3H, d, J = 2.0 Hz, 15-CH_3), 2.68 (1H, dd, J = 3.0, 1.0 Hz, 3-H), 3.35 (1H, ddd, J = 7.5, 4.5, 3.0 Hz, 12'-H), 3.82 (1H, dd, J = 9.0, 4.5 Hz, 12-H), 3.88 (1H, td, J = 4.5, 1.0 Hz, 11'-H), 4.38 (1H, ddd, J = 9.0, 2.0, 1.0 Hz, 13-H), 4.63 (1H, ddd, J = 7.5, 2.0, 1.0 Hz, 13'-H), 7.23 (1H, d, J = 9.0 Hz, 6'-H), 7.25 (1H, d, J = 9.0 Hz, 7-H), 7.29 (1H, d, J = 9.0 Hz, 6-H), 7.30 (1H, d, J = 9.0 Hz, 7'-H), 12.08 (1H, s, 8'-OH), 12.30 (1H, s, 8-OH), 12.38 (1H, s, 5-OH), 12.50 (1H, s, 5'-OH). ^{13}C-NMR spectral data: see Table II.

Compound 11. Orange amorphous powder (4 mg, 0.02%). UV λ_{max} ($CHCl_3$) nm (log ε): 240 (2.50), 260 (2.62), 420 (2.40). IR ν_{max} ($CHCl_3$) cm^{-1}: 3450 (OH), 1610 (C=O), 1520 (C=C). EI-MS *m/z*: 544 [M^+], 271 [M^+- $C_{16}H_{17}O_4$]. HRMS *m/z:* 544.1211 (Calcd for $C_{32}H_{32}O_8$, 544.1263). ^{1}H- NMR ($CDCl_3$) δ_H: 1.35 (6H, d, J = 1.5 Hz, 15-CH_3 and 15'-CH_3), 1.75 (6H, s, 16-CH_3 and 16'-CH_3), 1.87 (2H, q, J = 7.5 Hz, 12'-H), 2.77 (2H, t, J = 7.5 Hz, 11'-H), 3.05 (8H, d, J = 2.5 Hz, 2-H, 3-H, 2'-H and 3'-H), 4.37 (1H, dd, J = 7.5, 1.5 Hz, 13'-H), 5.69 (1H, d, J = 16.0 Hz, 11-H), 5.84 (1H, dd, J = 16.0, 6.5 Hz, 12-H), 7.13 (1H, d, J = 6.5 Hz, 13-H), 11.99 (1H, s, 5'-OH), 12.00 (1H, s, 5-OH), 12.38 (1H, s, 8'-OH), and 12.40 (1H, s, 8-OH). ^{13}C-NMR spectral data: see Table II.

Incubation of Shikonin (1) with an Intestinal Bacterial Mixture from Human Feces. Shikonin (**1**, 10 mg, in 0.1 ml DMSO) was incubated with an intestinal bacterial mixture (10 ml) prepared as described in the previous report[13] for 3 days at 37 °C in an anaerobic jar in which the air has been replaced by O_2-free CO_2. The incubation mixture was acidified with 5% HCl and extracted with EtOAc (10 ml x 3). The EtOAc extract was evaporated *in vacuo* to give a residue. The residue was dissolved in MeOH (1ml) and analyzed by HPLC as mentioned above .

Time Course of the Metabolism of Shikonin (1). Shikonin (**1**, 5 mg each) was separately incubated with a precultured bacterial suspension of *B. fragillis* (10 ml each) for 3 days under anaerobic conditions. The incubation mixtures were taken at 12 h intervals, adjusted to pH ca. 3 and extracted with EtOAc (10 ml x 3). The EtOAc extract was evaporated *in vacuo* to give a residue. The residue was dissolved in MeOH (1 ml) and analyzed by HPLC.

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