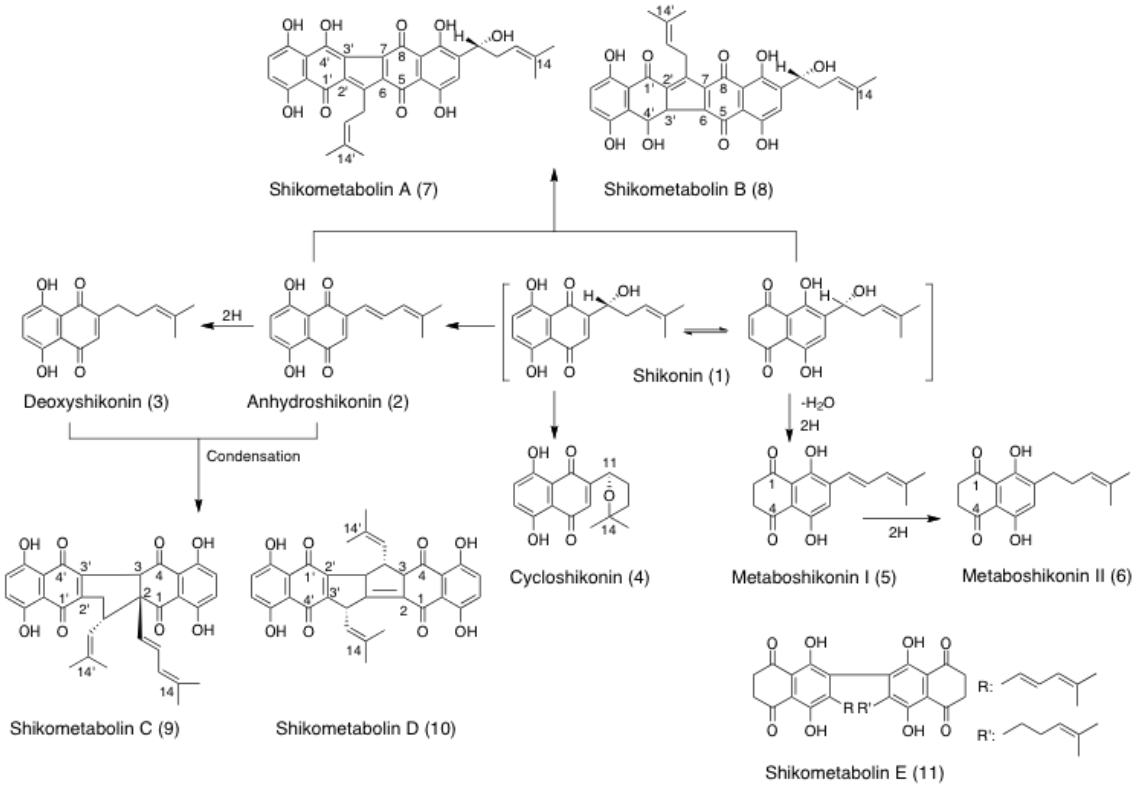
Shikonin



Metabolic processes of shikonin (**1**) by human intestinal bacteria

代謝実験

腸内細菌代謝 *Bacteroides fragilis* subsp. *thetaotus*、*Eubacterium* sp. A-44

単一化合物　shikonin

1. 腸内細菌による代謝

Transformation of shikonin (1) with *Bacteroides fragilis* subsp. *thetaotus*

Stock cultures of *B. fragilis* (2 1) were added to GAM broth (18 1) 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 1, 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% HC1, and extracted

with EtOAc (201 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. 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**. [Meselhy *et al*., *Tetrahedron*, **50**, 3081-3098 (1994)]

Compound 2 (Anhydroalkanin)

Red needles from $CHCl_3$, mp 134-135 °C, (2 mg, 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: 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. [Meselhy *et al*., *Tetrahedron*, **50**, 3081-3098 (1994)]

Compound 3 (Deoxyshikonin)

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}HNMR ($CDCl_3$) 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-l), 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. [Meselhy *et al*., *Tetrahedron*, **50**, 3081-3098 (1994)]

Compound 4 (Cycloshikonin)

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} ($CHC1_3$) nm (log ε): 280 (4.50), 480 (3.74), 520 (3.62). IR ν_{max} ($CHC1_3$) 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-l), 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. [Meselhy *et al*., *Tetrahedron*, **50**, 3081-3098 (1994)]

Compound 5 (Metaboshikonin I)

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 $C_{l6}H_{l6}O_4$, 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 in the following literature: [Meselhy *et al*., *Tetrahedron*, **50**, 3081-3098 (1994)].

Compound 6 (Metaboshikonin II)

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). EIMS *m/z*: 21A [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, l6-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 in the following literature: [Meselhy *et al*., *Tetrahedron*, **50**, 3081-3098 (1994)].

Compound 7 (Shikometabolin A)

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=0), 1460 (C=C). Negative ion FAB-MS *m/z*: 555 $[M\text{-}H]^-$, 457 $[(M–H) – C_6H_{14}O]^-$, 268 $[M^-$(partial structure I – $H_2O)]^-$, 193 [M–(partial structure II + 2H)]$^-$, 137, 93. HR-FAB-MS *m/z*: 555.1650 $[M\text{-}H]^-$(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-Hp), 2.54 (1H, dd, *J* = 12.0, 3.5 Hz, 12-Ha), 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 in the following literature: [Meselhy *et al*., *Tetrahedron*, **50**, 3081-3098 (1994)].

Compound 8 (Shikometabolin B)

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_5) 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-Hp), 2.50 (1H, m, 12-Ha), 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 = 1.0, 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 in the following literature: [Meselhy *et al*., *Tetrahedron*, **50**, 3081-3098 (1994)].

Compound 9 (Shikometabolin C)

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, $[\theta]_{52O}$ −269. UV λ_{max} ($CHCl_3$) nm (log ε): 250 (3.20), 410 (2.73), 520 (2.66), 555 (2.43). IR ν_{max} ($CHC1_3$) 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, 1.0 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 in the following literature: [Meselhy *et al*., *Tetrahedron*, **50**, 3081-3098 (1994)].

Compound 10 (Shikometabolin D)

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 in the following literature: [Meselhy *et al*., *Tetrahedron*, **50**, 3081-3098 (1994)].

Compound 11 (Shikometabolin E)

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* = 6.5Hz, 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 in the following literature: [Meselhy *et al*., *Tetrahedron*, **50**, 3081-3098 (1994)].

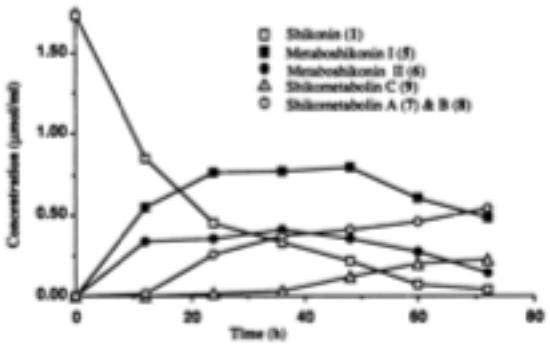


Fig. 1 Time course of the metabolism of shikonin (**1**) by *Bacteroides fragillis* subsp. *thetaotus*

Shikonin (**1**, 5 mg each) was 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. [Meselhy *et al*., *Tetrahedron*, **50**, 3081-3098 (1994)]

2. ヒト腸内細菌酵素による代謝

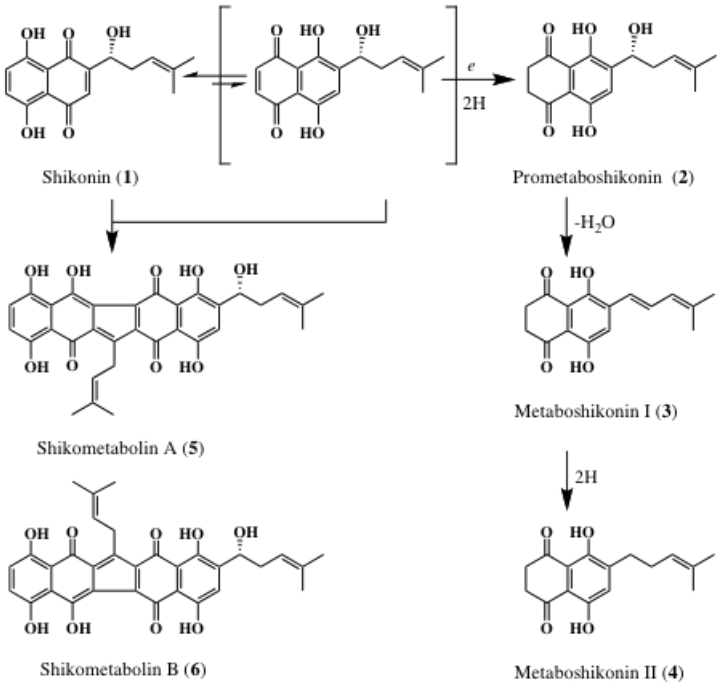


Chart 1. Possible metabolic pathways of shikonin (**1**) by a cell-free extract of *Eubacterium* sp. A-44. [Meselhy *et al.*, *J. Trad. Med.,* **18**, 58-63 (2001)]

Crude enzyme preparation and transformation of shikonin (1)

Eubacterium sp. A-44 has been previously isolated from human feces, and was maintained in GAM broth medium. 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 suspension (30 ml) was further centrifuged at 22500 × *g* for 30 min to obtain a bacterial cell-free extract, supernatant, which was kept on ice and used as the crude enzyme preparation. Ultracentrifugation of the supernatant (6 ml) at 3000 × *g* (for 45, 15 and 15 min) was carried out using Centriprep-10.

Shikonin (**1**, 50 mg in 1 ml 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 (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. Fr. 2-8 afforded **3** (6 mg) and **4** (4 mg), **2** (18 mg) was obtained from Fr. 17-21, while **5** (4 mg) and **6** (4 mg) were from Fr. 42-46. [Meselhy *et al.*, *J. Trad. Med.,* **18**, 58-63 (2001)]

Metabolite 2 (Premetaboshikonin)

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 x 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 (q, C-15), 26.2 (q, C-16), 36.4 (t, C-6 and C-7), 69.2 (d, C-11), 116.9 (s, C-10), 117.3 (s, C-9), 119.1 (d, C-13), 125.2 (d, C-3), 136.8 (s, C-14), 144.7 (s, C-2), 152.5 (s, C-4), 155.3 (s, C-1), 200.9 and 201.9 (s, C-5 and C-8). [Meselhy *et al.*, *J. Trad. Med.,* **18**, 58-63 (2001)]

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