VOL. 55 NO. 4, APR. 2002 THE JOURNAL OF ANTIBIOTICS pp. 437 — 441

NOTES

Microbial Hydroxylation of Zofimarin, a Sordarin-related Antibiotic

MASAHIRO TANAKA, TAKU MORIGUCHI[a], MASAAKI KIZUKA[a], YASUNORI ONO[a], SHUN-ICHI MIYAKOSHI[a] and TAKESHI OGITA[†,*]

Exploratory Chemistry Research Laboratories and
[a] Lead Discovery Research Laboratories,
Sankyo Co., Ltd.,
2-58, Hiromachi 1-chome, Shinagawa-ku, Tokyo 140, Japan

(Received for publication September 21, 2001)

The need for new antifungal agents is still growing due to the increasing number of immune-compromised patients. The present antifungal drugs suffer from several drawbacks such as resistance and toxicity. Therefore, continuous efforts to design novel drugs seem to be necessary. In this sense, the identification of new agents having mode of actions that are different from those currently used antifungal agents would be of great benefit.

Sordarin, a tetracyclic diterpene glycoside, and its derivatives are attracting much attention as a new class of antifungal agents. They selectively inhibit fungal protein synthesis by impairing the function of eukaryotic elongation factor 2.[1~4] We have discovered zofimarin,[5] a member of sordarin-related antibiotics, from a marine fungi, *Zopfiella marina*. The compound showed potent antifugal activity against a broad range of fungi including *Candida albicans*. An *in vivo* study of the compound, however, did not give the good result that was expected from its high *in vitro* potency, probably due to low bioavailability and metabolic instability. To overcome these problem, a modification of the compound would probably be required.

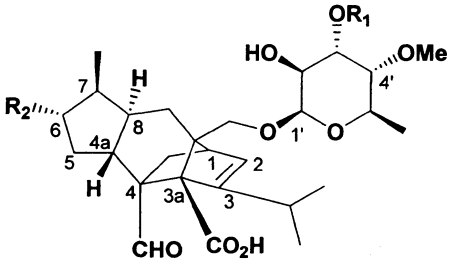
Microbial transformation systems have been very successful in the derivatization of natural products[6~9] and they can complement synthetic modification methods. Recently, the Glaxo group obtained a successful result in the derivatization study of sordarin using microbial transformation.[10] Therefore, we screened microorganisms that possessed zofimarin transformating activities, and discovered that *Streptomyces rimosus* SANK 60199(=IFO 12907) had the ability to hydroxylate zofimarin at the C-6 position. This finding will expand the scope of zofimarin derivatization since a broad range of compounds can be prepared by modification of the hydroxyl group. We report here the microbial transformation of zofimarin to 6-hydroxyzofimarin and the chemical modification thereof.

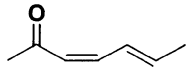
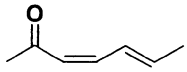
More than one hundred microorganisms, thought to have biotransformation activities, were tested. Bacteria and *Actinomyces* were cultured in a medium consisting of soy bean meal 2.5%, yeast extract 0.5%, KH_2PO_4 0.5%, and glucose 2%, at pH 7.0, while yeasts and fungi were in a medium, which consisting of glucose 2%, polypeptone 0.5%, yeast extract 0.3%, and molt extract 0.3%, at pH 7.0. Seeds were cultured in 20 ml of each medium in 100-ml Erlenmeyer flasks and incubated for 3 days before being used as inoculum (1%) in 20 ml of the medium in 100-ml Erlenmeyer flasks. All cultures were incubated with shaking at 200 rpm at the following suitable temperatures: 28°C for *Actinomyces*, 35°C for bacteria, 30°C for yeasts and 23°C for fungi. On day 3 of postinoculation, 10 mg of zofimarin dissolved in 50 μl of ethanol was added to each culture. On day 6 of postinoculation, cultivation was stopped by mixing with an equal volume of acetone.

The culture broths extracted with acetone for 3 hours was centrifuged (3000 rpm, 4°C, 10 minutes). Each supernatant was concentrated *in vacuo* to remove the acetone, and the residue was extracted with 20 ml of ethyl acetate at pH 3.0. After washing with brine, the ethyl acetate extract was dried over Na_2SO_4 and concentrated. Each sample was dissolved in 4 ml of ethanol, and then analyzed by HPLC (column: Senshu Pak, PEGASIL 4.6 i.d.×150 mm, flow rate: 1 ml/minute, mobile phase: acetonitrile - 0.2% triethylamine phosphate buffer pH 3.2 (7 : 3), detection: UV absorption at 210 nm, injection volume: 10 μl). In the twenty samples, the desired decrease of the zofimarin peak, which showed strong UV absorption at 260 nm, and/or appearance of new zofimarin-related peaks were detected. In particular, in the culture of *S. rimosus* SANK 60199(=IFO 12907), zofimarin was completely metabolized and a product with a similar UV spectrum was clearly detected. Therefore, the scale of fermentation was

* Corresponding author: Ogitak@shina.sankyo.co.jp

Fig. 1. Structures of sordarin, zofimarin and the transformant (**1**).



	R_1	R_2
Sordarin	H	H
Zofimarin		H
Transformant (**1**)		H

increased so as to generate sufficient quantities of the transformant for characterization and structural analysis.

From two liters of the culture broth fed with 1 g of zofimarin and extracted as described above, 1.2 g of a yellow oil was obtained. The oil was applied onto a silica gel column equilibrated with a solution of hexane, ethyl acetate, and acetic acid (80 : 80 : 1). The column was eluted with the same solvent and the fractions contain the product were combined and concentrated. Further purification was accomplished by preparative HPLC (column: Senshu Pak, PEGASIL 20 i.d.×250 mm, flow rate: 6 ml/minute, mobile phase: acetonitrile - 0.2% triethylamine phosphate buffer pH 3.2 (7 : 3), detection: UV absorption at 210 nm) to give 600 mg of the product. The retention time under these conditions was 13 minutes, and the yield of the biotransformation was 60%.

The transformant (**1**) was isolated as a pale yellow powder and the molecular formula was determined to be $C_{33}H_{46}O_{10}$ (HR-FABMS $(M+Na)^+$, *m/z* 625.3012, Δ+2.3 mmu) showing an increment of one oxygen compared with that of zofimarin. The structural study was mainly carried out by interpretation of the NMR spectra taken in DMSO-d_6. When the NMR spectra of **1** were compared with those of zofimarin, signals belonging to an oxymethine (δ_C 79.7, δ_H 3.67) appeared with a concomitant loss in the signal corresponding to an aliphatic methylene. These findings suggested that **1** was a hydroxylated derivative of zofimarin. In the HMBC experiment, long-range correlations of the oxymethine proton (δ_H 3.67) with the C-5 (δ_C 37.9), C-7 (δ_C 42.9) and 7-CH_3 (δ_C 14.7) carbons were observed revealing that the hydroxylation occurred at the C-6 position. In the NOESY experiment, strong cross peaks between the 6-H oxymethine proton (δ_H 3.67) and the 7-H methine proton (δ_H 1.75), and between the 6-H oxymethine proton and the 7-substituted methyl proton (δ_H 0.74) were observed. Since the modeling study suggests that the only the proton located in 6-β position seems to show NOE correlation to both the 7-H methine proton and 7-substituted methyl proton, the absolute confinguration at the C-6 position was determined to be (S). Based on these results, the structure of **1** was determined as shown in Fig. 1.

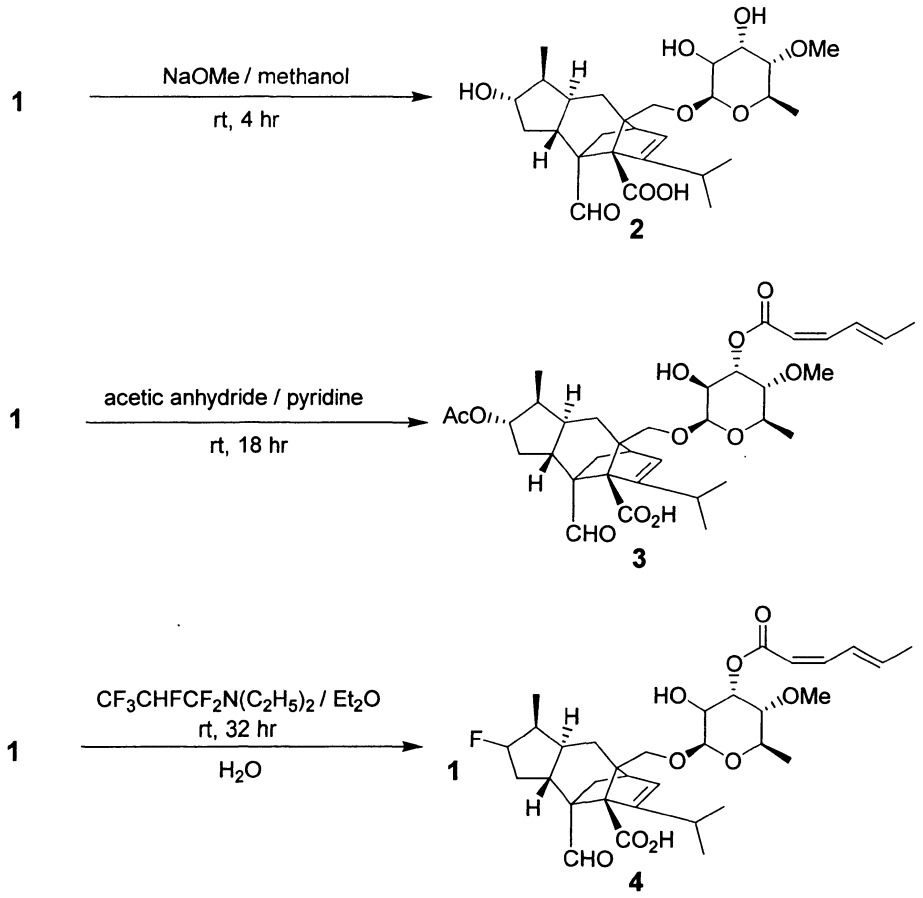
Next, we modified **1** chemically as shown in Fig. 2. First of all, **1** was hydrolyzed under basic conditions to give 6-hydroxysordarin (**2**). Reaction of **1** with acetic anhydride gave a regioselectively acetylated product, 6-acetoxy-zofimarin (**3**). Finally, **1** was converted to a fluoride, 6-fluorozofimarin (**4**), by the reaction with MEC-81(DAIKIN).

We were fortunate to find 6-hydroxyzofimarin because with this compound a series of zofimarin derivatives that are chemically inaccessible can be created. In the preliminary chemical modification studies, unfortunately, compounds that surpass the efficacy of zofimarin have not been found yet. Further derivatation studies will hopefully give us more promising derivatives in the future. In the presence of mouse serum and liver S-9 fraction, sordarin was metabolized to 6- and/or 7-hydroxysordarin[11] suggesting that this modification might be one of the causes for the reduced antifungal activity of sordarin *in vivo*. Therefore, the chemical modification of the C-6 hydroxyl group to enzymatically inert substituents may improve the pharmacokinetic stability and overcome the limitation of the diterpene skeleton of sordarin in its use as an antifungal agent.

Experimental

Spectral and physico-chemical data were obtained by the following instruments: UV, Shimadzu UV-265FW; IR, JASCO FT/IR-8300; NMR, Bruker AVANCE 500; HR-MS,

Fig. 2. Chemical modification of **1**.



JASCO VMS-HX110; Specific rotations, JASCO DIP-370 with a 10-cm cell.

Streptomyces rimosus SANK 60199 (=IFO 12907) was obtained from IFO.

6-Hydroxyzofimarin (1)

White powder. Molecular formula: $C_{33}H_{46}O_{10}$ (HR-FABMS $(M+Na)^+$, *m/z* 625.3012, Δ+2.3 mmu). UV spectrum: λ_{max} nm in methanol (ε) 262 nm (20000). IR spectrum: ν_{max} cm^{-1} (KBr) 3430, 2960, 2930, 2870, 2850, 1720, 1640, 1600, 1460, 1420, 1380, 1310, 1260, 1230, 1170, 1100, 1070, 1030, 1010, 970. Specific rotation: $[\alpha]_D^{26}$ −36° (*c* 0.05, methanol). ^1H NMR (500 MHz, DMSO-d_6): δ 9.55 (1H, s), 7.24 (1H, dd, *J*=15, 11.5 Hz), 6.72 (1H, t, *J*=11.5 Hz), 6.22 (1H, dd, *J*=15, 7 Hz), 6.02 (1H, d, *J*=3 Hz), 5.62 (1H, d, *J*=11.5 Hz), 5.25 (1H, dd, *J*=4, 3 Hz), 4.41 (1H, br s), 3.75 (1H, d, *J*=9.5 Hz), 3.67 (1H, br t, *J*=6 Hz), 3.64 (1H, dd, *J*=9, 6.5 Hz), 3.65~3.56 (2H, m), 3.23 (1H, m), 3.22 (3H, s), 2.72 (1H, br t, *J*=3.5 Hz), 2.27~2.17 (2H, m), 2.05~1.85 (4H, m), 1.84 (3H, d, *J*=7 Hz), 1.75 (1H, m), 1.57 (1H, m), 1.18 (3H, d, *J*=6 Hz), 1.13 (1H, d, *J*=12 Hz), 0.95 (3H, d, *J*=7 Hz), 0.88 (3H, d, *J*=6.5 Hz), 0.81 (1H, m), 0.74 (3H, d, *J*=7.5 Hz). ^{13}C NMR (125 MHz, DMSO-d_6): δ 204.7 (d), 174.3 (s), 165.5 (s), 148.9 (s), 146.5 (d), 142.0 (d), 131.1 (d), 128.9 (d), 115.7 (d), 99.8 (d), 79.7 (d), 78.7 (d), 74.8 (t), 72.8 (s), 69.8 (d), 68.8 (d), 68.8 (d), 65.8 (s), 58.7 (s), 57.6 (q), 46.3 (d), 42.9 (d), 41.0 (d), 38.9 (d), 37.9 (t), 29.6 (t), 29.5 (t), 28.0 (d), 23.2 (q), 21.8 (q), 19.5 (q), 19.1 (q), 14.7 (q).

6-Hydroxysordarin (2)

To a solution of 30 mg of **1** in 2 ml of methanol was added 2 equivalents of sodium methoxide (28% solution in methanol). The mixture was stirred at room temperature for 4 hours, and poured into 1 N HCl. The product was extracted with ethyl acetate. The organic layer was washed with water and brine, and then dried over Na_2SO_4. After

concentration *in vacuo*, the residue was purified by preparative HPLC (column: Nacalai tesque COSMOSIL 5C18-AR 20 i.d.×250 mm, flow rate: 6 ml/minute, mobile phase: acetonitrile - 0.2% triethylamine phosphate buffer pH 3.2 (3 : 7), detection: UV absorption at 210 nm) to give 16 mg of 6-hydroxysordarin (yield: 63%). The retention time under these conditions was 23 minutes.

White powder. Molecular formula: $C_{27}H_{40}O_9$ (HR-FABMS $(M+Na)^+$, *m/z* 531.2549, Δ −2.1 mmu). IR spectrum: ν_{max} cm^{-1} (KBr) 3440, 2960, 2930, 2870, 1720, 1460, 1380, 1370, 1300, 1250, 1180, 1140, 1090, 1080, 1030, 1000 cm^{-1}. ^1H NMR (500 MHz, CD_3OD): δ 9.63 (1H, s), 6.14 (1H, dd, *J*=3, 1 Hz), 4.58 (1H, d, *J*=1 Hz), 4.13 (1H, dd, *J*=3, 1 Hz), 3.91 (1H, d, *J*=9.5 Hz), 3.84 (1H, m), 3.75~3.70 (3H, m), 3.38 (3H, s), 3.14 (1H, dd, *J*=9, 3 Hz), 2.84 (1H, br t, *J*=3.5 Hz), 2.45~2.25 (2H, m), 2.20~1.95 (4H, m), 1.86 (1H, m), 1.76 (1H, m), 1.25 (1H, m), 1.25 (3H, d, *J*=7 Hz), 1.05 (3H, d, *J*=7 Hz), 0.98 (3H, d, *J*=7 Hz), 0.92 (1H, m), 0.86 (3H, d, *J*=7.5 Hz). ^{13}C NMR (125 MHz, CD_3OD): δ 206.1 (d), 175.4 (s), 150.0 (s), 132.3 (d), 100.3 (d), 81.3 (d), 81.2 (d), 76.0 (t), 73.7 (s), 72.4 (d), 70.1 (d), 68.2 (d), 67.0 (s), 59.9 (s), 57.3 (q), 47.7 (d), 43.7 (d), 42.0 (d), 40.3 (d), 38.1 (t), 30.3 (t), 30.2 (t), 29.1 (d), 23.2 (q), 21.0 (q), 18.6 (q), 14.3 (q).

6-Acetoxyzofimarin (**3**)

To a solution of 30 mg of **1** in 1.5 ml of pyridine was added 1.2 equivalents of acetic anhydride. The mixture was stirred at room temperature for 18 hours, and poured into 1 N HCl. The product was extracted with ethyl acetate. The organic layer was washed with water and brine, and then dried over Na_2SO_4. After concentration *in vacuo*, the residue was purified by HPLC (column: Nacalai tesque COSMOSIL 5C18-AR 20 i.d.×250 mm, flow rate: 6 ml/minute, mobile phase: acetonitrile - 0.2% triethylamine phosphate buffer pH 3.2 (7 : 3), detection: UV absorption at 210 nm) to give 10 mg of a regioselectively acetylated product, 6-acetoxyzofimarin (yield: 31%). The retention time under these conditions was 18 minutes.

White powder. Molecular formula: $C_{35}H_{48}O_{11}$ (HR-FABMS $(M+Na)^+$, *m/z* 667.3098, Δ +0.3 mmu). IR spectrum: ν_{max} cm^{-1} ($CHCl_3$) 2970, 2930, 1720, 1640, 1600, 1450, 1420, 1380, 1310, 1250, 1160, 1100, 1060, 1030, 1010, 910. ^1H NMR (500 MHz, $CDCl_3$): δ 9.61 (1H, s), 7.39 (1H, dd, *J*=14, 12 Hz), 6.61 (1H, t, *J*=11 Hz), 6.18~6.10 (2H, m), 5.61 (1H, d, *J*=11 Hz), 5.51 (1H, br t, *J*=3.5 Hz), 4.74 (1H, t, *J*=6 Hz), 4.61 (1H, s), 4.07 (1H, d, *J*=9.5 Hz), 3.86 (1H, d, *J*=4.5 Hz), 3.79 (1H, m), 3.67 (1H, d, *J*=9.5 Hz), 3.38 (3H, s), 3.32 (1H, dd, *J*=8.5, 3 Hz), 2.75 (1H, br t, *J*=3 Hz), 2.54 (1H, m), 2.37 (1H, d t, *J*=13.5, 6.5 Hz), 2.15~2.03 (3H, m), 2.02 (3H, s), 2.00 (1H, m), 1.96 (1H, dd, *J*=13, 4.5 Hz), 1.90 (3H, d, *J*=6.5 Hz), 1.81 (1H, m), 1.32 (1H, m), 1.31 (3H, d, *J*=6.5 Hz), 1.15 (1H, m), 1.06 (3H, d, *J*=6.5 Hz), 0.99 (3H, d, *J*=6.5 Hz), 0.92 (3H, d, *J*=7 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ 203.9 (d), 175.0 (s), 171.1 (s), 165.4 (s), 148.7 (s), 146.7 (d), 141.5 (d), 131.3 (d), 128.5 (d), 114.6 (d), 98.6 (d), 82.1 (d), 78.4 (d), 74.2 (t), 72.5 (s), 70.1 (d), 69.4 (d), 67.3 (d), 65.6 (s), 59.2 (s), 57.9 (q), 46.7 (d), 40.8 (d), 40.3 (d), 39.3 (d), 34.3 (t), 30.0 (t), 28.9 (t), 28.0 (d), 22.8 (q), 21.5 (q), 21.3 (q), 19.0 (q), 18.4 (q), 13.4 (q).

6-Fluorozofimarin (**4**)

To a solution of 20 mg of **1** in 2 ml of ethyl ether was added 60 μl of MEC-81 (DAIKIN). The mixture was stirred at room temperature for 32 hours. After concentration *in vacuo*, the residue was purified by preparative TLC developed with a solution of hexane, ethyl acetate, and acetic acid, 80 : 80 : 1 (Rf.: 0.5). Further purification was performed by HPLC (column: Nacalai tesque COSMOSIL 5C18-AR 20 i.d.×250 mm, flow rate: 6 ml/minute, mobile phase: acetonitrile - 0.2% triethylamine phosphate buffer pH 3.2 (7 : 3), detection: UV absorption at 210 nm) to give 3 mg of 6-fluorozofimarin (yield: 15%). The retention time under these conditions was 18 minutes.

White powder. Molecular formula: $C_{33}H_{45}O_9F$. ^1H NMR (500 MHz, $CDCl_3$): δ 9.66 (1H, s), 7.39 (1H, dd, *J*=14, 12 Hz), 6.61 (1H, t, *J*=11.5 Hz), 6.18~6.10 (2H, m), 5.61 (1H, d, *J*=11 Hz), 5.51 (1H, br t, *J*=3.5 Hz), 5.16 (1H, dt, $J_{H\text{-}F}$=54 Hz, $J_{H\text{-}H}$=7 Hz), 4.61 (1H, s), 4.04 (1H, d, *J*=9 Hz), 3.85 (1H, d, *J*=4.5 Hz), 3.79 (1H, m), 3.70 (1H, d, *J*=9.5 Hz), 3.38 (3H, s), 3.32 (1H, dd, *J*=8.5, 3 Hz), 2.73 (1H, br t, *J*=3.5 Hz), 2.44 (1H, m), 2.37 (1H, m), 2.30 (1H, m), 2.18 (1H, m), 1.96~1.84 (2H, m), 1.90 (3H, d, *J*=6 Hz), 1.79 (1H, m), 1.43 (1H, m), 1.32 (3H, d, *J*=6.5 Hz), 1.32~1.22 (2H, m), 1.06 (3H, d, *J*=6.5 Hz), 1.00 (3H, d, *J*=7 Hz), 0.87 (3H, m). ^{13}C NMR (125 MHz, $CDCl_3$): δ 203.8 (d), 175.0 (s), 165.4 (s), 148.8 (s), 146.7 (d), 141.5 (d), 131.3 (d), 128.6 (d), 114.7 (d), 98.6 (d), 95.3* (d) $J_{C\text{-}F}$=184 Hz, 78.5 (d), 74.2 (t), 72.3 (s), 70.0 (d), 69.4 (d), 67.3 (d), 65.7 (s), 58.8 (s), 57.9 (q), 46.6 (d), 40.7 (d), 39.5 (d), 37.0* (d) $J_{C\text{-}F}$=19 Hz, 36.1* (t) $J_{C\text{-}F}$=25 Hz, 29.8* (t) $J_{C\text{-}F}$=24 Hz, 28.9 (t), 28.0 (d), 22.8 (q), 21.4 (q), 19.0 (q), 18.4 (q), 8.6* (q) $J_{C\text{-}F}$=19 Hz. *: coupled with fluorine.

References

1) DOMINGUEZ, J. M. & J. J. MARTIN: Identification of elongation factor 2 as the essential protein targeted by sordarins in *Candida albicans*. Antimicrob. Agents

Chemother. 42: 2279~2283, 1998

2) JUSTICE, M. C.; M. J. HSU, B. TSE, T. KU, J. BALKOVEC, D. SCHMATZ & J. NIELSEN: Elongation factor 2 as a novel target for selective inhibition of fungal protein synthesis. J. Biol. Chem. 273: 3148~3151, 1998
3) DOMINGUEZ, J. M.; V. A. KELLY, O. S. KINSMAN, M. S. MARRIOTT, F. GOMEZ DE LAS HERAS & J. J. MARTIN: Sordarins: A new class of antifungals with selective inhibition of the protein synthesis elongation cycle in yeasts. Antimicrob. Agents Chemother. 42: 2274~2278, 1998
4) CAPA, L.; A. MENDOZA, J. L. LAVANDERA, F. GOMEZ DE LAS HERAS & J. F. GARCIA-BUSTOS: Translation elongation factor 2 is part of the target for a new family of antifungals. Antimicrob. Agents Chemother. 42: 2694~2699, 1998
5) SATO, A.; S. TAKAHASHI, T. OGITA, M. SUGANO & K. KODAMA: Marine Natural Product. Annu. Rep. Sankyo Res. Lab. 47: 1~58, 1995
6) SERIZAWA, N.; S. SERIZAWA, K. NAKAGAWA, K. FURUYA, T. OKAZAKI & A. TERAHARA: Microbial hydroxylation of ML-236B (compactin). Studies on microorganisms capable of 3 beta-hydroxylation of ML-236B. J. Antibiotics 36: 887~891, 1983
7) SERIZAWA, N.; K. NAKAGAWA, Y. TSUJITA, A. TERAHARA, H. KUWANO & M. TANAKA: 6 alpha-Hydroxy-iso-ML-236B (6 alpha-hydroxy-iso-compactin) and ML-236A, microbial transformation products of ML-236B. J. Antibiotics 36: 918~920, 1983
8) SERIZAWA, N.; K. NAKAGAWA, Y. TSUJITA, A. TERAHARA & H. KUWANO: 3 alpha-Hydroxy-ML-236B (3 alpha-hydroxycompactin), microbial transformation product of ML-236B (compactin). J. Antibiotics 36: 608~610, 1983
9) SERIZAWA, N.; K. NAKAGAWA, K. HAMANO, Y. TSUJITA, A. TERAHARA & H. KUWANO: Microbial hydroxylation of ML-236B (compactin) and monacolin K (MB-530B). J. Antibiotics 36: 604~607, 1983
10) BUENO, J. M.; J. CHICHARRO, S. I. HUSS, J. M. FIANDOR & F. GOMEZ DE LAS HERAS: Synthesis of the antifungal GM237354. 37th Intersci. Conf. Antimicrob. Agents Chemother., Sept. 28~Oct. 1, Toronto, 1997, Abst F54.
11) CUEVAS, J. C.; J. L. LAVANDERA & J. L. MARTOS: Design and synthesis of simplified sordaricin derivatives as inhibitors of fungal protein synthesis. Bioorg. Med. Chem. Lett. 9: 103~108, 1999