MICROBIAL TRANSFORMATION OF IMMUNOSUPPRESSIVE COMPOUNDS

IV. HYDROXYLATION AND HEMIKETAL FORMATION OF ASCOMYCIN (IMMUNOMYCIN) BY *Streptomyçes* sp. MA 6970 (ATCC No. 55281)

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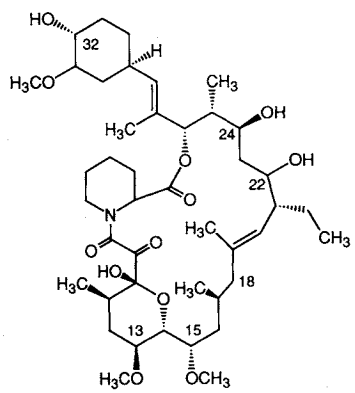
The important immunosuppressive agents FK 506[1,2] and ascomycin[3~5] (**1**) have been the target of intensive structure-activity relationship (SAR) studies[6]. FK 506 recently received FDA approval for liver organ transplantation in the United States. Because of the complexity of their structures, we initiated a microbial transformation program aimed at modification of chemically inaccessible sites. Previously, we disclosed the microbial desmethylation of FK 506/ascomycin to obtain 31-*O*-desmethyl, 13,31-*O*-bisdesmethyl, 15,31-*O*-bisdesmethyl, 13,15,31-*O*-tridesmethyl and 13-*O*-desmethyl derivatives[7,8]. We also carried out the specific methylation of C-31 hydroxyl group of 15,31-*O*-bisdesmethyl and 13,15,31-*O*-tridesmethyl ascomycins to prepare 15-*O*-desmethyl and 13,15-*O*-bisdesmethyl derivatives using 31-*O*-desmethylascomycin *O*: methyltransferase (DIMT) as catalyst and *S*-adenosylmethionine as the methyl donor[9]. In addition, we recently reported microbial glucosylation of FK 506/ascomycin to yield specific 24-glucosyl derivatives[10]. All these derivatives are difficult or tedious to obtain through synthetic chemical approach. The present communication describes the specific hydroxylation of 19-methyl group of ascomycin by *Streptomyces* sp. MA 6870 (ATCC No. 55281) leading to a novel hemiketal structure (**2**) that is formed by the addition of 19-hydroxy group to 22-carbonyl group.

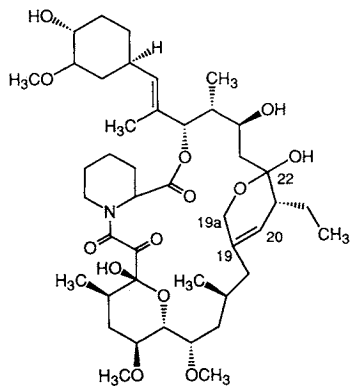
Fermentation Conditions for Conversion

Streptomyces sp. MA 6870 (ATCC No. 55281) was stored and maintained on skim milk in the culture collection of Merck Research Laboratories. The microorganism was inoculated into a seed medium that contained (in g/liter) glucose 1.0, dextrin 10.0, beef extract 3.0, Ardamine PH (Yeast Products, Inc.) 5.0, N-Z Amine type E 5.0, $MgSO_4 \cdot 7H_2O$ 0.05, KH_2PO_4 0.37, and $CaCO_3$ 0.5. The medium was adjusted to pH 7.1 with 1 N NaOH and autoclaved for 20 minutes. The inoculated 50 ml volumes of seed medium were shaken in 250-ml baffled flasks at 220 rpm for 24 hours at 27°C. The mature seed cultures were used as the source of inoculum (5% inoculum) for the fermentation medium. The fermentation medium consisted of (in g/liter) glucose 20.0, soya meal 5.0, yeast autolysate 5.0, and NaCl 5.0. After formulation the medium was adjusted to pH 7.0 with 1 N NaOH and sterilized by autoclaving. Ascomycin (**1**) was dissolved in dimethyl sulfoxide and added to the fermentation at 0 hour to achieve a final concentration of 50 μg/ml. The shake flask contents were subsequently incubated at 27°C on a rotary shaker for

Fig. 1. The structure of immunomycin (**1**) and its hemiketal derivative (**2**).



1



2

Fig. 2. ^1H NMR spectrum (400 MHz) of hemiketal derivative (2) in $CDCl_3$.

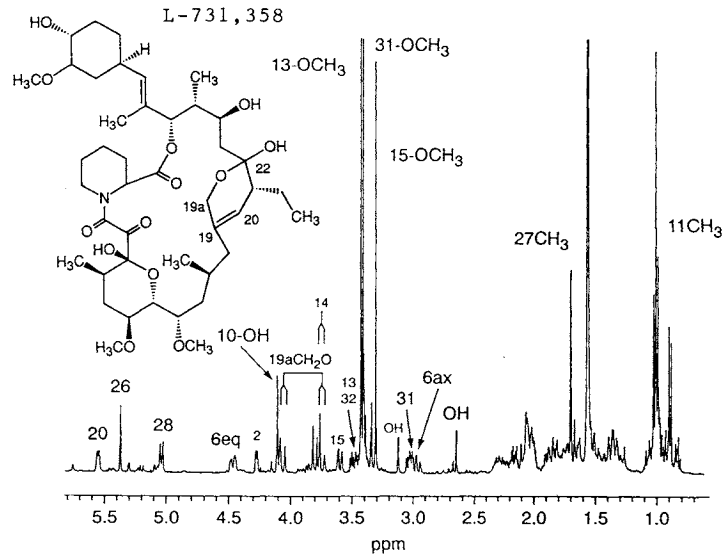
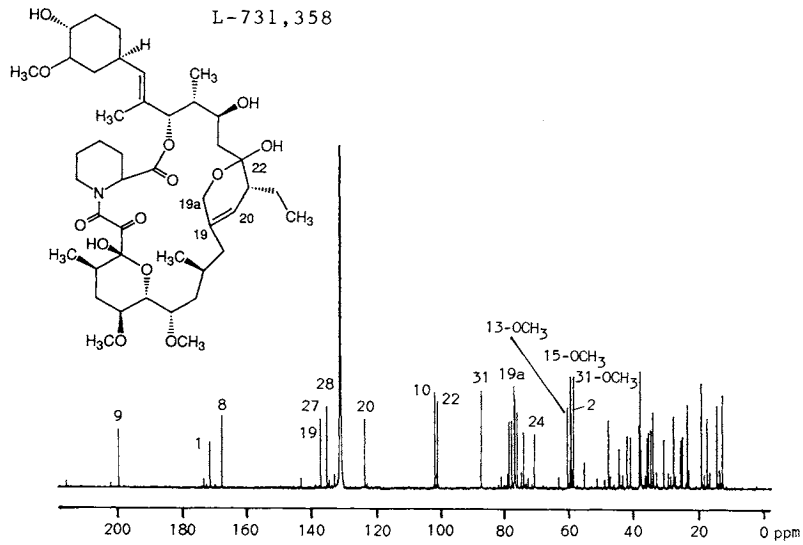


Fig. 3. ^{13}C NMR spectrum (125 MHz) of hemiketal derivative (2) in C_6D_6.



48 hours using the conditions described. These fermentations were the source of 19~22 hemiketal derivative of ascomycin.

Isolation and Characterization of Conversion Product

The whole broth (1 liter) was extracted three times with methylene chloride (3 × 500 ml). Methylene chloride extracts were combined, dried over sodium sulfate, and concentrated under vacuum to an oily residue. The residue was dissolved in methanol and subjected to high performance liquid chromatography (HPLC). HPLC was carried out on a Whatman Magnum 9 Partisil 10 ODS-3 column (9.6 mm × 25 cm) at room temperature and monitored at 205 nm. The column was developed at 4 ml/minute with a 25 minutes linear gradient of 40% to 80% acetonitrile in 0.1% phosphoric acid. The fraction at 22.4 minutes was collected. Concentration and desalting were accomplished by dilution with 3 volumes of distilled water, followed by absorption onto a C18 solid phase extraction column. After washing with excess water, the compound was eluted with methanol and eva-

porated to dryness of yield 6.8 mg of **2**.

Characterization of Conversion Product

The FAB mass spectrum of **2** gave a M+Li of 814 that corresponds to an increase of 16 mass units from ascomycin, indicating that it is a hydroxylated derivative. The compound was identified as a cyclized hemiketal structure based on its distinctive ^{1}H and ^{13}C NMR features. The ^{1}H NMR of **2** (Fig. 2) revealed the absence of H-19 methyl signal at 1.59 ppm, suggesting that 19-methyl group was hydroxylated. Further comparing the ^{1}H NMR data of **1** with that of **2** showed the following features: (1) the appearance of two novel proton signals at 3.75 and 4.65 ppm with a large coupling constant of 16.4 Hz, consistent with a $-CH_2O-$; (2) the absence of typical 23-methylene signals at 2.78 ppm; (3) the H-20 signal was shifted downfield by about 0.6 ppm and reduction of its vicinal coupling constant from 10.1 Hz to 6.0 Hz. The structure of **2** was further confirmed by the ^{13}C NMR data (Fig. 3) which, compared with that of the parent compound **1**, indicated (1) the loss of C-19 methyl signal at 15.8 ppm; (2) the loss of C-22 carbonyl signal at 212 ppm; (3) the appearance of a new signal at 98.7 ppm consistent with a hemiketal carbon C-22; (4) a new signal at 74.8 ppm that is assigned to C-19.

Biological Activity

The immunosuppressive activity of **2** was determined in an *in vitro* T cell proliferation assay described previously[12]. Compound **2** gave an IC_{50} of 3 nM, compared to 0.8 nM of its parent compound **1**. In spite of structural and conformational changes caused by an oxygenated 19-methyl functionality which is attached to C-22 to form a hemiketal cyclic structure, compound **2** still retained 35% of biological activity of the parent **1**.

References

1) KINO, T.; H. HATANAKA, M. HASHIMOTO, M. NISHIYAMA, T. GOTO, M. OKUHARA, M. KOHSAKA, H. AOKI & H. IMANAKA: FK-506, a novel immunosuppressant isolated from a *Streptomyces*. J. Antibiotics 40: 1249~1255, 1987
2) TANAKA, H.; A. KURODA, H. MARUSAWA, H. HATANAKA, T. KINO, T. GOTO, M. HASHIMOTO & S. T. TAGA: Structure of FK 506: A novel immunosuppressant isolated from *Streptomyces*. J. Am. Chem. Soc. 109: 5031~5033, 1987
3) HATANAKA, H.; M. IWAMI, T. KINO, T. GOTO & M. OKUHARA: FR-900520 and FR-900523, novel immunosuppressants isolated from a *Streptomyces*. I. Taxonomy of the producing strain. J. Antibiotics 41: 1586~1591, 1988
4) HATANAKA, H.; T. KINO, S. MIYATA, N. INAMURA, A. KURODA, T. GOTO, H. TANAKA & M. OKUHARA: FR-900520 and FR-900523, novel immunosuppressants isolated from a *Streptomyces*. II. Fermentation, isolation and physico-chemical and biological characteristics. J. Antibiotics 41: 1592~1601, 1988
5) BORRIS, R. P.; K. M. BYRN, B. H. ARISON, D. L. ZINK, D. M. VESEY, F. DUMONT, G. M. GARRITY, R. L. MONAGHAN, L. KAPLAN & G. ALBER-SCHONBERG: Spectroscopic reinvestigation of the antibiotic, ascomycin. Thirtieth Annual Meeting of the American Society of Pharmacognosy, Poster Presentation 41. Aug. 6~10, Puerto Rico, 1989
6) PARSONS, W. H.; M. T. GOULET, K. RUPPRECHT, P. SINCLAIR & M. J. WYVRATT: Medicinal Chemistry of FK 506 to be published in Perspective in Drug Discovery & Design
7) CHEN, T. S.; B. H. ARISON, L. S. WICKER, E. S. INAMINE & R. L. MONAGHAN: Microbial transformation of immunosuppressive compound. I. Desmethylation of FK 506 and immunomycin (FR 900520) by *Actinoplanes* sp. ATCC 53771. J. Antibiotics 45: 118~123, 1992
8) CHEN, T. S.; B. H. ARISON, L. S. WICKER & E. S. INAMINE: Microbial transformation of immunosuppressive compounds II. Specific desmethylation of 13-methoxy group of FK 506 and FR 900520 by *Actinomycete* sp. ATCC 53828. J. Antibiotics 45: 577~580, 1992
9) SHAFIEE, A.; T. S. CHEN, B. H. ARISON, F. J. DUMONT, L. COLWELL & L. KAPLAN: Enzymatic synthesis and immunosuppressive activity of novel desmethylated immunomycins (ascomycins). J. Antibiotics 46: 1397~1405, 1993
10) PETUCH, B. R.; B. H. ARISON, A. HSU, R. L. MONAGHAN, F. J. DUMONT & T. S. CHEN: Microbial transformation of immunosuppressive compounds III. Glucosylation of immunomycin (FR 900520) and FK 506 by *Bacillus subtilis* ATCC 55060. J. Industrial Microbiology 13: 131~135, 1994
11) DUMONT, F. J.; M. J. STARUCH, S. L. KOPRAK, M. R. MELINO & N. H. SIGAL: Distinct mechanisms of suppression of murine T cell activation by the related macrolides FK-506 and rapamycin. J. Immunology 144: 251~258, 1990