Journal of Industrial Microbiology, 13 (1994) 131–135

Published by The Macmillan Press Ltd

# Microbial transformation of immunosuppressive compounds III. Glucosylation of immunomycin (FR 900520) and FK 506 by *Bacillus subtilis* ATCC 55060

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(Received 5 May 1993; accepted 12 January 1994)

*Key words:* Microbial transformation; FK 506; *Bacillus subtilis*

## SUMMARY

The regiospecific glucosylation of FK 506 and immunomycin (FR 900520) at the 24-hydroxy position was performed using resting cells of *Bacillus subtilis* ATCC 55060. 24-Glucopyranosyl FK 506 and 24-glucopyranosyl immunomycin were isolated by methylene chloride extraction and purification using reverse phase HPLC. The metabolite structures were established using spectroscopic techniques including MS and NMR. The glucose conjugate was further confirmed by chemical degradation. Enzymatic glucosylation was demonstrated using cell-free extracts derived from *Bacillus subtilis* ATCC 55060. The 24-glucosyltransferase, which appears UDP-glucose dependent, was solubilized from cell membranes by treatment with 0.1% Nonidet P-40 detergent. The optimal conditions for assay of the enzyme have been determined.

## INTRODUCTION

FK 506 (**1**) and immunomycin (**2**) (Fig. 1) are a new class of polyhydroxylated macrolides, isolated from *Streptomyces tsukubaensis* [6–9,12] and *Streptomyces hygroscopicus* [1], respectively. Both compounds exhibit excellent immunosuppressive activity in the T-cell inhibition assay. As a result of our investigations on microbial modification of FK 506 and immunomycin, we recently reported the microbial desmethylation of FK 506 and immunomycin by *Actinoplanes* sp. ATCC 53771 [3] to yield various *O*-desmethylated products and the specific conversion of FK 506 and immunomycin to 13-desmethyl FK 506 and 13-desmethylimmunomycin by *Streptomyces* sp. ATCC 53828 [4]. The present communication describes the bioconversion of FK 506 and immunomycin to 24-glucosylated derivatives using resting cells and cell-free extracts of *Bacillus subtilis* ATCC 55060. Preliminary properties of the crude 24-glucosyltransferase are also described.

## MATERIALS AND METHODS

### *General*

All organic solvents (EM Science, Gibbstown, NJ, USA) were HPLC grade. Water was purified in a Millipore Milli-Q system (Bedford, MA, USA). Solvents for NMR analysis were purchased from MSD Isotopes, St Louis, MO, USA. [32-$^3$H] FK 506 was prepared at Merck Research Laboratories (Rahway, NJ, USA). Radioactivity measurements were by liquid scintillation counting in a Beckman (Fullerton, CA, USA) Model 171 Radioisotope flow detector using Redi Flow III solution. ADP-, GDP-, TDP-, and UDP-glucose were purchased from Sigma Chemical Co., St Louis, MO, USA.

### *NMR and MS analysis*

Proton NMR experiments were performed on a Varian Unity 400 NMR spectrometer. The spectra were measured in $CDCl_3$ and chemical shifts were referenced to solvent lines ($\delta_H$=7.26,$\delta_C$=77.0 p.p.m.). FAB-MS measurements were obtained on a Finnigan Mat TSQ 70 instrument.

### *Production of metabolite*

*Bacillus subtilis* ATCC 55060 was stored as a lyophilized culture in skim milk in the Merck Research Labs Culture Collection as MB 4974. A lyophile was used to inoculate a 250-ml Erlenmeyer flask containing 50 ml of an autoclaved seed medium consisting of (g $L^{-1}$) glucose 20.0, soybean meal (Sigma) 5, yeast extract (Difco, Detroit, MI, USA) 5, NaCl 5, and $KH_2PO_4$ 6.3. The pH of the seed medium was adjusted to 7.0 prior to autoclaving the medium. The seed flask was incubated on a rotary shaker (220 r.p.m.) at 30 °C for 18 h. A 2.5 ml portion of seed culture was used to inoculate a second-stage culture flask containing 50 ml of the same medium. Second-stage flasks were charged with

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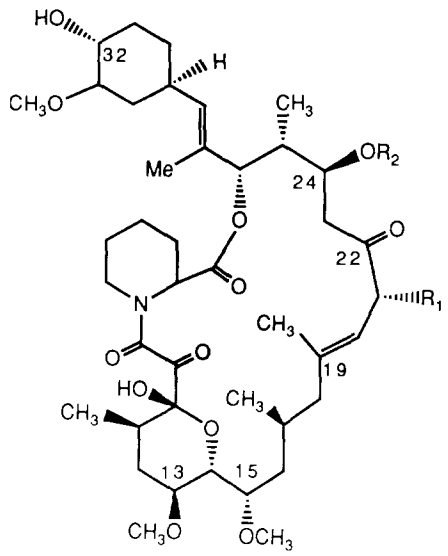


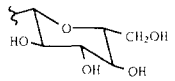
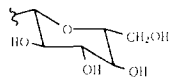

| Compound | $R_1$ | $R_2$ |
|---|---|---|
| 1 | $CH_2CH{=}CH_2$ | H |
| 2 | $CH_2CH_3$ | H |
| 3 | $CH_2CH{=}CH_2$ |  |
| 4 | $CH_2CH_3$ |  |

Fig. 1. The structure of FK 506/immunomycin and their 24-glucopyranosyl derivatives produced by *Bacillus subtilis* ATCC 55060.

10 μg $ml^{-1}$ substrate in DMSO solution as a possible inducer of the glycosylation reaction, then incubated at 30 °C on a rotary shaker for 18 h. Following incubation, each flask was harvested by centrifugation, washed once with sterile saline, and resuspended in 50 ml of 100 mM MES buffer at pH 6.0 containing 2% glucose. Substrate (FK 506 or immunomycin) was added as a solution in DMSO to achieve a final concentration of 0.2 mg $ml^{-1}$. The flasks were then incubated on a rotary shaker (220 r.p.m.) at 30 °C for 24 h.

*Isolation and purification of 24-glucopyranosyl FK 506/immunomycin*

The whole broth (150 ml) was extracted with three 75-ml portions of methylene chloride. The methylene chloride extracts were combined, dried over anhydrous sodium sulfate, filtered and concentrated under vacuum to yield a brown oil. The oil residue was defatted by extracting it twice with cold petroleum ether. The remaining material was dissolved in 45% aqueous acetonitrile, filtered and subjected to HPLC. The preparative HPLC was carried out on a Whatman Partisil 10 ODS-3 column (9.4 mm × 250 mm) at 60 °C and monitored at 205 nm. The column was developed at 3 ml $min^{-1}$ using a linear gradient of 0.1% aqueous $H_3PO_4$-$CH_3CN$ (55:45) to 0.1% aqueous $H_3PO_4$-$CH_3CN$ (20:80) over 40 min. The compound was collected during repeated injections of the extract. Fractions eluting at Rt 15 min were collected, pooled, and adjusted to pH 6.5. The pooled fractions were concentrated to remove acetonitrile. The aqueous solution was applied to a water equilibrated $C_{18}$ solid phase extraction column, washed with water and eluted with methanol. The methanol extract was evaporated to yield 5 mg of 24-glucopyranosyl FK 506 (**3**). The whole broth obtained from 2 flasks was processed as described above yielding 6 mg of 24-glucopyranosyl immunomycin (**4**, Fig. 1).

*Chemical degradation*

To 13 mg of 24-glucopyranosyl immunomycin (**4**, Fig. 1) was added 2 ml of anhydrous trifluoroacetic acid. After 15 min at 37 °C, the reaction mixture was evaporated to dryness, dissolved in chloroform and washed twice with water. The water washes were pooled and evaporated to dryness at 50 °C under vacuum. The residue was dissolved in 2 ml of water and passed through a $C_{18}$ solid phase extraction column. The eluate was concentrated, dissolved in a small amount of water and purified by HPLC. The HPLC was performed using a Brownlee Spheri-5 Amino column (4.6 × 250 mm) at room temperature and developed at 2 ml $min^{-1}$ with 90% aqueous acetonitrile. Glucose was detected by a refractive index detector (Altex, Fullerton, CA, USA). The peaks at retention time 8.6 min were pooled and evaporated to dryness to yield 0.5 mg of glucose.

*Assessment of immunosuppressive activity*

The immunosuppressive activities of the isolated glucopyranosyl derivatives were determined in an in vitro T-cell proliferation assay described previously [5]. Briefly, nylon wool-purified splenic T cells were prepared from C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME, USA). Microcultures were performed in flat-bottom 96-well plates containing 5 × $10^4$ T cells per well. Proliferation was induced by the addition of 250 ng $ml^{-1}$ ionomycin and 10 ng $ml^{-1}$ phorbol PMA (phorbol 12-myristate 13-acetate). Cell division was assessed by the addition of 2 μCi $well^{-1}$ of tritiated thymidine for the last 4 h of a 48-h culture period. Test compounds were added at culture inoculation. The $IC_{50}$ for inhibition by each compound was calculated using a 4-parameter algorithm.

*Solubilization of the membrane-bound 24-glucosyltransferase*

Cells prepared using the procedure described previously were centrifuged. Sedimented cellular mass (22 g) contained in an ice bath was resuspended in 10 ml of 100 mM MES buffer at pH 6.0. The cells were ruptured by sonicating the

suspension twice for 30 s, with one cooling period of 30 s. The homogenates were centrifuged at 20 000 × *g* in a Sorvall SS-34 rotor for 20 min at 5 °C. The supernatant fluid was decanted and the cell pellet suspended in buffer containing detergent (0.1% Nonidet P-40 detergent in 100 mM MES buffer pH 6.0) and stirred for 30 min at 5 °C. The resulting suspension was centrifuged for 20 min at 49 500 × *g* at 5 °C and the supernatant fluid used as the crude enzyme. Crude enzyme protein was quantitated by the Pierce BCA protein assay, using BSA as standard [2]. The protein concentrations of the crude enzymes averaged 9.5 mg per ml.

*Assay of glucosyltransferase activity*

For routine assay, incubation mixtures contained the following components in a final volume of 50 μl: 2.7 nmol [32-$^3$H] FK 506 (2700 mCi mmol$^{-1}$), 10 mM UDP-glucose, 15% glycerol, 10 mM $MgCl_2$, 100 mM MES buffer, pH 7.5 and enzyme in 100 mM MES buffer. The reaction was incubated for 60 min at 40 °C, then terminated with an equal volume of methanol. The reaction mixture was diluted with water and applied to SEP-PAK (Waters) cartridge. The cartridge was washed with water, then eluted with acetonitrile. The eluate was concentrated and examined for the presence of 24-glucopyranosyl FK 506 (**3**, Fig. 1) by HPLC using a Beckman Model 171 Radioisotope flow detector. Analysis was performed using a Whatman Partisil 10 ODS-3 column (4.6 mm × 250 mm) maintained at 60 °C and developed at 1.0 ml min$^{-1}$ using a linear gradient of 0.1% aqueous $H_3PO_4$-$CH_3CN$ (55:45) to 0.1% aqueous $H_3PO_4$-$CH_3CN$ (20:80) over 40 min. Scintillation cocktail flow rate was 1.0 ml min$^{-1}$.

## RESULTS AND DISCUSSION

*Biotransformation*

Microbial transformation is one principal approach which is widely practiced for structural modifications of complex natural products. We have used the approach in an attempt to improve clinical characteristics and as a source for mammalian metabolites. Over 1000 cultures were screened to test their abilities to carry out biotransformation of FK 506 and immunomycin. Among these cultures, *Bacillus subtilis* ATCC 55060 was able to convert FK 506 and immunomycin to its corresponding 24-glucopyranosyl derivative in good yields after 18 h of incubation. The metabolites were purified by solvent extraction and HPLC.

*Structure determination*

Positive ion FAB-MS of **3** and **4** (Fig. 1) gave $[M+Na]^+$ signal at m/z 988 and 976, respectively, 162 more atomic mass units than their corresponding parent compounds FK 506 (**1**, Fig. 1) and immunomycin (**2**, Fig. 1), suggesting that they are glycosylated derivatives. To confirm the presence of the glucose moiety, the metabolites were hydrolyzed by trifluoroacetic acid to yield a polar sugar moiety which was purified and identified as glucose by proton NMR analysis. The conjugates remained recalcitrant to hydrolysis with various glycolytic enzymes. Instability of **1** and **2** (Fig. 1) in the presence of strong acid and base prevented their isolation during hydrolysis. The proton NMR spectra of **3** (Fig. 2) and **4** (Fig. 3) showed the presence of at least four novel methines in the region characteristic for CH attached to oxygen. The spectral perturbations relative to the parent compounds are most apparent in the $C_{22}$–$C_{26}$ region. This is evidenced by an increase in the $H_{26}$–$H_{25}$ coupling constant from 2.5 Hz to 6.0 Hz and a 0.3–0.4 p.p.m. downfield displacement of the higher field signal of one of the $H_{23}$ protons. The changes can be rationalized by alterations in the $C_{25}$–$C_{26}$ and $C_{24}$–$C_{23}$ bond angles suggesting that the site of attachment of glucose is probably the hydroxyl group attached to $C_{24}$. The attachment of glucose to the $C_{24}$ hydroxyl group is further strengthened by 2D NOSEY spectra (Fig. 4). Cross peaks show the proximity between $H_{24}$ and the anomeric proton of glucose. The stereochemistry of 24-glucose was determined as β-configuration based on the proton–proton coupling constant of J = 7.5 Hz of the anomeric proton.

*Biological activity*

The 24-glucopyranosyl derivatives were not active up to 100 μM in the T-cell inhibition assay, suggesting that the conformation in the region of $C_{22}$–$C_{26}$ may play an important

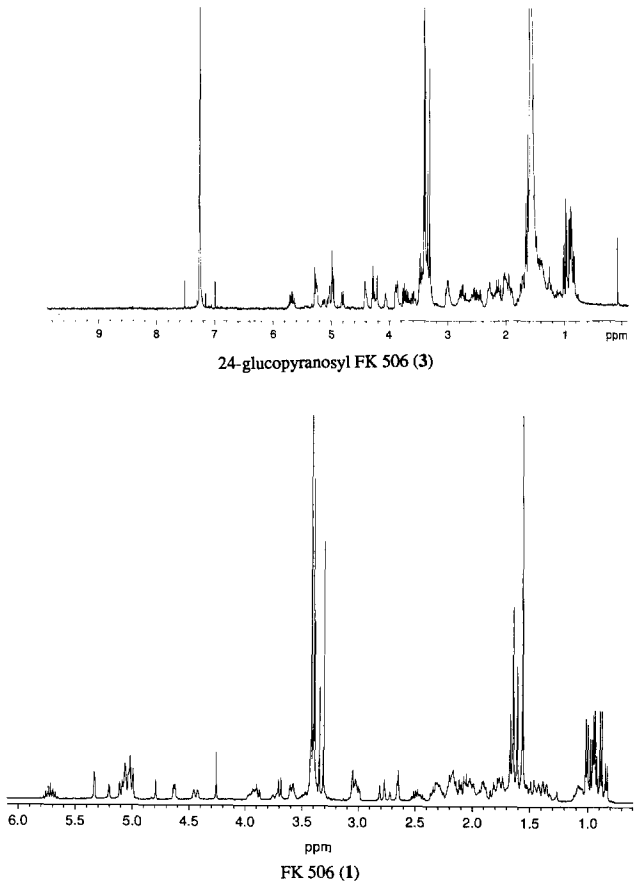



Fig. 2. $^1$H NMR spectra (400 MHz) of 24-glucopyranosyl FK 506 (**3**) and FK 506 (**1**) in $CDCl_3$.

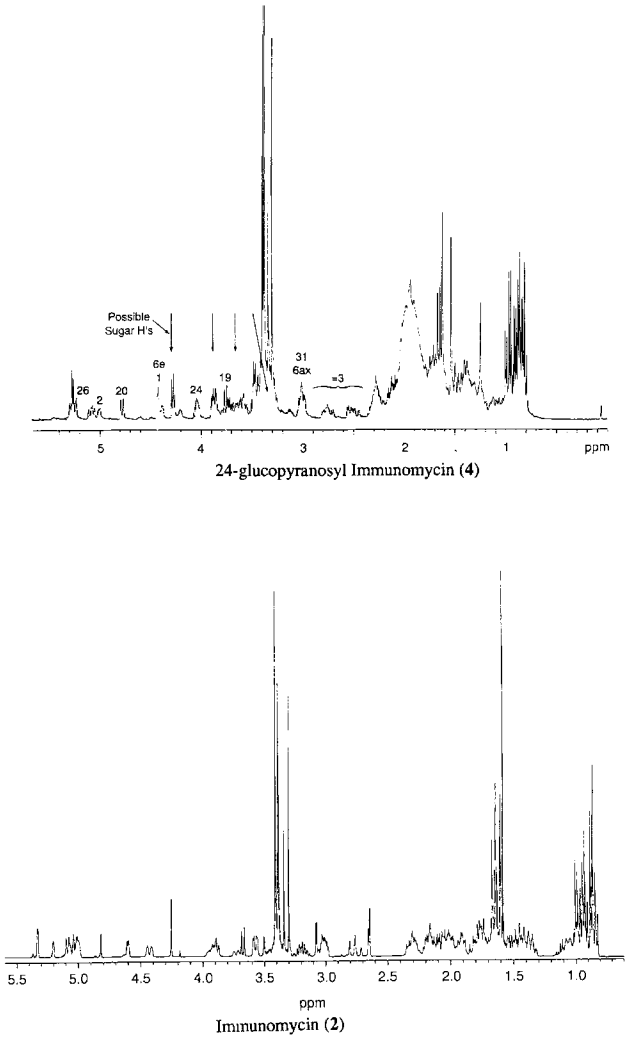



Fig. 3. $^1$H NMR spectra (400 MHz) of 24-glucopyranosyl immunomycin (**4**) and immunomycin (**2**) in $CDCl_3$.

role for T-cell activity. The 24-OH can form a hydrogen bond with the $C_{22}$ ketone. Glucosylation at 24-OH results in loss of hydrogen bonding and the change in the conformation of the molecules. It is possible that the steric bulk of the glucosyl group imparts a negative effect on activity. Recent studies indicate that the complex of FK-506-FKBP interacts with calcineurin [10], a calcium–calmodulin dependent protein phosphatase. The loss of T-cell inhibition activity of 24-glucopyranosyl derivatives is presumably due to some specific calcineurin-binding feature of the region of $C_{22}$–$C_{26}$.

*Time course studies*

A time course study of transformation of FK 506 into the 24-glucopyranosyl derivatives by growing cells in Soy–Glucose medium was carried out using vegetative cells as an inoculum. The 24-glucopyranosyl derivatives appeared at 2 h post incubation. The maximum concentration of 24-glucopyranosyl derivative was reached at approximately 18 h.

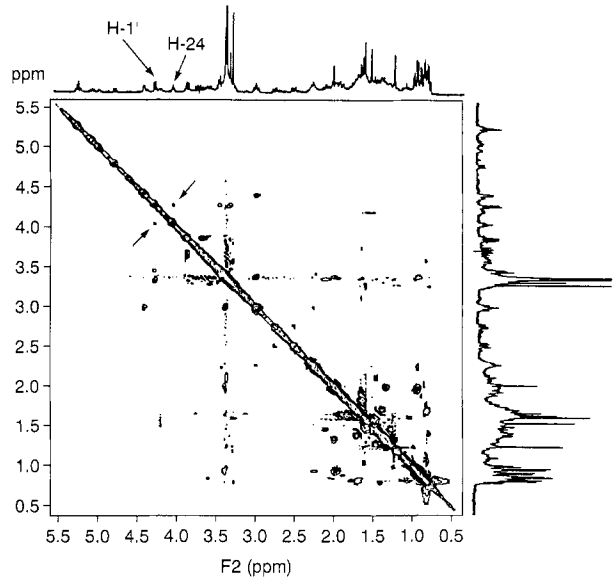



Fig. 4. NOSEY spectrum of 24-glucopyranosyl immunomycin.

Cells used for enzyme isolation were harvested 18 h after inoculation to achieve best cellular activity.

*Solubilization of 24-glucosyltransferase*

24-Glucosyltransferase activity was found repeatedly in the cell pellets. No activity was found in the 20 000 × *g* supernatant fraction. Based on this observation, attempts to solubilize the membrane-bound 24-glucosyltransferase were made using a published procedure [11]. Nonidet P-40 at the final concentration of 0.1% gave best results for preferential solubilization of 24-glucosyltransferase.

*Properties of 24-glucosyltransferase*

The membrane-bound 24-glucosyltransferase appeared to have a good pH profile in the basic region, whereas activity drastically decreased below pH 6.5. Therefore, pH 7.5 was chosen for subsequent experiments due to substrate instability above pH 8.0. The enzyme has a temperature optimum at 40 °C. Addition of various metal ions showed that 10 mM magnesium stimulated activity tenfold. In contrast to another glucosyltransferase [11], 0.6 M potassium chloride gave 30% inhibition. The activated donors ADP-, GDP-, TDP- and UDP-glucose were tested under these optimum reaction conditions. UDP-glucose was the only activated donor acceptable to the *Bacillus subtilis* 24-glucosyltransferase.

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