JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 96, No. 4, 344–348. 2003

# Microbial Glycosylation and Acetylation of Brefeldin A

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Received 14 May 2003/Accepted 20 June 2003

**The brefeldin A (BFA) derivatives 4-*O*-acetyl BFA, 7-*O*-acetyl BFA, and 4-*O*-D-glucosyl BFA were found by the microbial conversion of BFA. 4-*O*-D-Glucosyl BFA is a novel compound produced by the conversion of BFA with *Streptomyces* sp. Q53834. The cytotoxicity and antifungal activity of all of the compounds were markedly less than those of BFA.**

[**Key words:** brefeldin A (BFA), microbial conversion, glycosylation, acetylation, antifungal]

Brefeldin A (BFA), which is known to show antifungal, antiviral, and antitumor activities (1), has been isolated from the culture broth of a variety of fungal strains such as *Penicillium decumbens* (2), *P. brefeldianum* (3), *P. cyaneum* (4), and *Ascochyta imperfecta* (5). BFA is also known to inhibit the transport of proteins from the endoplasmic reticulum to the Golgi apparatus (6–8) and induce apoptosis in exposed cells (9). Though BFA has a wide range of biological activities, it does not exhibit a sufficient effect *in vivo* because of its negligible bioavailability, rapid clearance from the blood, and low solubility in aqueous solutions. Regarding its rapid clearance, it is reported that BFA is rapidly inactivated *in vivo* via conjugation with glutathione by glutathione *S*-transferase and subsequently transported out of the cell (10). In order to overcome these disadvantages, numerous studies on the chemical modification of BFA have been performed (Weigele *et al.*, WO95/21614, 1995) (11).

Microbial conversion has been applied to many kinds of natural products. It has sometimes brought innovative solutions to produce new useful compounds (12). In this study, we focused on searching for new improved derivatives obtained by the microbial conversion of BFA. We found several BFA derivatives, including a new compound. This paper deals with screening and taxonomy of the microorganisms, and the production, isolation, and biological activities of the microbial conversion products.

## MATERIALS AND METHODS

**Materials** BFA was obtained from the culture broth of *Penicillim* sp. AP322 isolated in our laboratory. Frozen mycelia of strain AP322 were inoculated into P6 medium consisting of glucose 2.0%, maltose 3.0%, soybean protein 1.5%, corn steep liquor 1.0%, Polypepton (Nihon Pharmaceutical, Tokyo) 0.3%, and NaCl 0.3%. The pH was adjusted to 6.0 before sterilization and the culture was incubated at 24°C for 5 d on a rotary shaker at 200 rpm. BFA was extracted with aqueous acetone from mycelia and purified by silica gel and ODS flash chromatography.

**Microorganisms** Microorganisms used for this study were derived from not only type culture collections but also freshly isolated strains in our laboratory.

The strains were examined according to the methods described by Goodfellow (13), Shirling and Gottlieb (14), and Waksman (15) to determine their taxonomy. The color of the aerial and substrate mycelia of the actinomycetes was determined with the ISCC-NBS centroid color charts (U.S. National Bureau of Standards, 1976). Cultural characterizations were observed on various media at 20°C–37°C for 14–21 d. Detailed observation of the microorganisms was performed using a scanning electron microscope (JSM-T220; JEOL, Tokyo). The chemical compositions of the cells were analyzed by the methods of Becker *et al.* (16) and Lechevalier and Lechevalier (17).

**Conditions of microbial conversion** For the screening of conversion products, each microorganism was inoculated into 500-ml Erlenmeyer flasks containing 100 ml of 108 medium consisting of glucose 1.0%, potato starch 2.0%, Polypepton 0.5%, yeast extract 0.5%, and $CaCO_3$ 0.4%, pH 7.0. The flasks were incubated at 28°C for 3 d on a rotary shaker at 220 rpm for actinomycetes and bacteria, and at 24°C for 3 d at 200 rpm for fungi. Cells were collected from 2.5 ml of culture broth by centrifugation and washed twice with 100 mM Tris·HCl, pH 7.5. The washed cells were suspended in 2.5 ml of conversion buffer (100 mM Tris·HCl, pH 7.5, 1% glucose, and 100 μg/ml BFA) and incubated at 28°C or 24°C for 20 h in a test tube (1.8×10 cm) on a reciprocal shaker.

**Detection of microbial conversion products** The conversion mixtures were centrifuged and the supernatants were extracted with EtOAc. The extracts were chromatographed on silica gel TLC plates (Merck 60F-254; Merck KGaA, Darmstadt, Germany) with $CHCl_3$–MeOH (9:1) and EtOAc–MeOH (4:1). Detection was carried out by spraying $H_2SO_4$ (10% in EtOH) on the plate and heating it at 120°C for 10 min. The analytical HPLC was performed on a Cosmosil 5C18-AR II (4.6×250 mm; Nacalai Tesque, Kyoto) column. The separation was achieved with MeOH–$H_2O$ (60:40) at a flow rate of 1.0 ml/min. UV detection was performed at 220 nm. Conversion products were detected by substrate utiliza-

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tion and the occurrence of new peaks on TLC and HPLC analysis, followed by further evaluation using HPLC/MS.

**Isolation and identification of conversion products** Screening cultures were scaled-up to support the isolation and identification of conversion products. Microorganisms were cultured in twelve 500-ml Erlenmeyer flasks containing 100 ml of 108 medium at 28°C for 3 d. Washed cells were prepared from the whole culture broth and suspended in the same volume of conversion buffer. After incubation at 28°C for 20 h, the supernatants were subjected to HP-20 extraction followed by aqueous MeOH elution. The eluates were concentrated *in vacuo*. The residues were applied to ODS flash chromatography (50×50 mm, YMC gel; YMC, Kyoto) and were eluted by the appropriate concentration of aqueous MeOH. Conversion products were finally purified using a preparative HPLC column (20×250 mm, Cosmosil 5C18-AR; Nacalai Tesque). Fast atom bombardment mass spectra (FAB-MS) were obtained with JEOL JMS-700T. $^{1}H$ and $^{13}C$ NMR spectra were recorded on a JEOL JNM-ALPHA500 FT NMR spectrometer.

**Biological activity** The MICs of BFA and its conversion products were evaluated by the micro dilution method described in National Committee for Clinical Laboratory Standards documents M27-A and M38-P. Cytotoxicities were determined using a cell counting kit (Wako Pure Chemical Industries, Osaka). HeLa S3 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and 20 mM HEPES buffer. The cells were incubated with compounds at 37°C for 48 h in a humidified atmosphere containing 5% $CO_2$.

## RESULTS AND DISCUSSION

**Microbial conversion of brefeldin A** The microbial conversion of BFA was examined using 3600 strains which included actinomycetes, bacteria, and fungi. Conversion products were detected by substrate utilization and the occurrence of new peaks on TLC and HPLC analysis, followed by HPLC/MS analysis as described in the Materials and Methods.

In the course of the examination, we found that many actinomycetes utilized BFA as a conversion substrate. Among the microorganisms, three strains, *Nocardia argentinesis* ATCC31306, strains Q50513, and Q53834 were selected for further work based on the conversion efficiency. The strains described above converted BFA to brefeldin derivatives, BFD-3, BFD-6, and BFD-16, respectively (Fig. 1). Chromatograms of the conversion products are summarized in Table 1.

The time course of the microbial conversions is shown in Fig. 2. The conversion efficiency of BFA to BFD-3 was 25% at 32 h after the start of incubation. In the case of BFD-6, the conversion efficiency was 20% at 24 h. BFA was converted to BFD-16 by strain Q53834 with about 100% conversion efficiency at 2 h. BFD-16 was almost stable in the conversion mixture after 48 h.

TABLE 1. TLC $R_f$ values and HPLC retention time of BFA and conversion products

| Compound | TLC $R_f$ value | | HPLC retention time (min) |
|---|---|---|---|
| | $CHCl_3$:MeOH (9:1) | EtOAc:MeOH (4:1) | |
| BFA | 0.29 | 0.63 | 8.17 |
| BFD-3 | 0.56 | 0.72 | 12.3 |
| BFD-6 | 0.56 | 0.75 | 16.5 |
| BFD-16 | 0.00 | 0.35 | 5.92 |

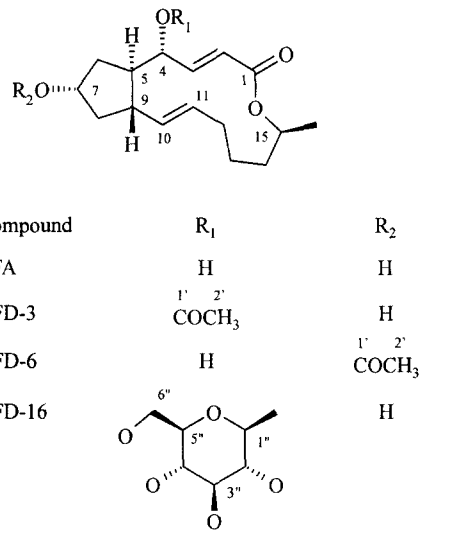


FIG. 1. The structures of BFA and conversion products.

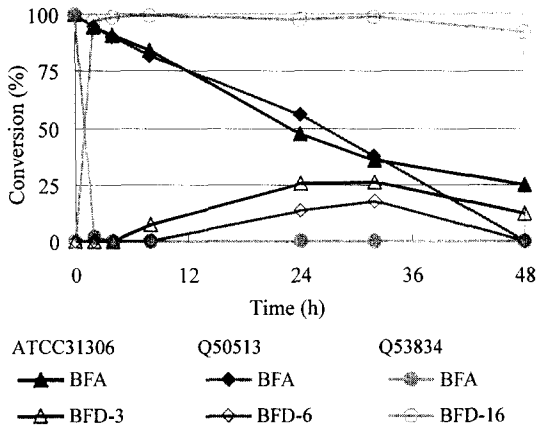


FIG. 2. Typical time course of microbial conversion of BFA. The closed symbols represent consumption of BFA. The open symbols represent production of conversion products.

**Characteristics of the microorganisms** Strain Q50513 was isolated from a soil sample collected in Taisyo-cho, Kochi, Japan. Morphologically, this strain grows abundantly in complex and synthetic media. Spores are oval in shape and 0.6×1.0~1.2 μm in diameter with a smooth surface. The aerial mass color is brownish white to reddish brown on ISP-4 and ISP-5 media. The reverse color of growth is yellowish brown. The strain produces several extracellular enzymes such as amylases and proteases. LL-Diaminopimelic acid was detected in the whole-cell hydrolysates of the strain. Strain Q50513 was classified on the basis of its 16S rDNA sequence. Comparison of the sequence obtained with those found in the databases showed 99.87% identity to *Streptomyces lavendulae*. Thus, we tentatively classified this strain as *S. lavendulae* Q50513. An electron micrograph of the spores is shown in Fig. 3.

Strain Q53834 was isolated from a soil sample collected in Tsukuba, Ibaraki, Japan. Based on morphological and chemotaxonomic studies, strain Q53834 was considered to belong to the genus *Streptomyces*. A detailed examination is

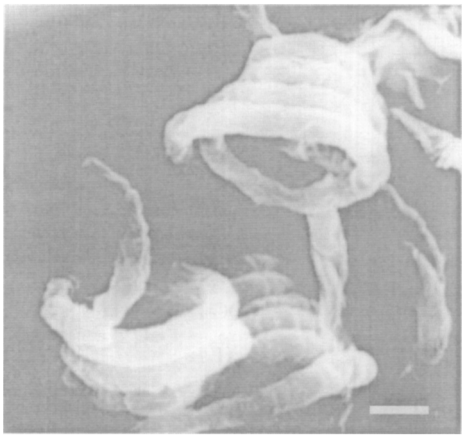

FIG. 3. Electron micrograph of *S. lavendulae* Q50513. The bar represents 1 μm.

in progress.

*N. argentinesis* ATCC31306 is a type culture strain deposited in ATCC.

**Isolation and identification of BFD-3, BFD-6, and BFD-16** Microbial conversion products were isolated as described in the Materials and Methods. The amounts of BFD-3, BFD-6, and BFD-16 obtained from 1.2 *l* conversion mixtures containing 120 mg of BFA were 2.6 mg, 4.5 mg and 88.4 mg, respectively. The structures of these compounds were determined by comparison of their MS and NMR spectra with those of BFA. The positive FAB mass spectra of BFD-3 and BFD-6 gave an $(M+H)^+$ ion at *m/z* 323, indicating a monoacetylated derivative. The changes in their NMR spectra indicated their acetylated positions. BFD-3 and BFD-6 were identified as 4-*O*-acetyl BFA and 7-*O*-acetyl BFA, respectively. The assignment of the NMR spectra is given in Table 2. Though both of the acetyl BFAs were easily synthesized by chemical modification (11), acetylation by the microbial conversion occurred more site-specifically. *N. argentinesis* ATCC31306 acetylated BFA at the 4-hydroxyl. On the other hand, *S. lavendulae* Q50513 acetylated BFA at the 7-hydroxyl. Each of the microorganisms acetylated the other hydroxyl group with negligibly low efficiency.

The positive FAB mass spectrum of BFD-16 gave an $(M+H)^+$ ion at *m/z* 443. The NMR spectra displayed by BFD-16 indicated that it is 4-*O*-D-glucosyl BFA. 4-*O*-D-Glucosyl BFA is a novel compound. It is presumed that the glycosylation reaction occurs more easily by microorganisms than by chemical modification. In fact, microbial glycosylation has been reported regarding some microbial metabolites, such as immunomycin, FK506 (18), and erythromy-

TABLE 2. $^{13}C$ and $^{1}H$ NMR chemical shifts (ppm) of BFA and conversion products

| Position | BFA | | 4-*O*-Acetyl BFA | | 7-*O*-Acetyl BFA | | 4-*O*-Glucosyl BFA | |
|---|---|---|---|---|---|---|---|---|
| | $^{13}C$[a] | $^{1}H$[b] | $^{13}C$ | $^{1}H$ | $^{13}C$ | $^{1}H$ | $^{13}C$ | $^{1}H$ |
| 1 | 165.6 | | 164.9 | | 165.5 | | 165.5 | |
| 2 | 116.2 | 5.71 | 116.9 | 5.61 | 116.4 | 5.72 | 118.4 | 6.13 |
| 3 | 154.3 | 7.34 | 148.7 | 7.24 | 153.9 | 7.33 | 150.4 | 7.13 |
| 4 | 74.3 | 3.93 | 76.0 | 5.20 | 73.9 | 4.00 | 79.3 | 4.28 |
| 4-OH | | 5.08 | | | | 5.19 | | |
| 5 | 51.7 | 1.71 | 49.0 | 1.94 | 51.7 | 1.70 | 50.1 | 1.78 |
| 6 | 40.9 | 1.83 | 40.3 | 1.74 | 38.0 | 1.99 | 40.8 | 1.78 |
| | | 1.64 | | 1.47 | | 1.79 | | 1.50 |
| 7 | 70.5 | 4.04 | 70.3 | 4.06 | 74.7 | 4.99 | 70.5 | 4.05 |
| 7-OH | | 4.46 | | 4.55 | | | | 4.47 |
| 8 | 43.1 | 1.97 | 42.8 | 2.00 | 39.1 | 2.18 | 43.2 | 1.98 |
| | | 1.30 | | 1.32 | | 1.44 | | 1.31 |
| 9 | 43.3 | 2.31 | 42.9 | 2.51 | 42.6 | 2.44 | 43.4 | 2.34 |
| 10 | 137.1 | 5.19 | 136.7 | 5.23 | 136.2 | 5.18 | 136.9 | 5.19 |
| 11 | 129.2 | 5.66 | 129.7 | 5.70 | 130.1 | 5.72 | 129.4 | 5.65 |
| 12 | 31.4 | 1.92 | 31.3 | 1.93 | 31.3 | 1.94 | 31.4 | 1.92 |
| | | 1.77 | | 1.78 | | 1.78 | | 1.78 |
| 13 | 26.4 | 1.77 | 26.3 | 1.79 | 26.3 | 1.77 | 26.5 | 1.78 |
| | | 0.75 | | 0.73 | | 0.76 | | 0.74 |
| 14 | 33.4 | 1.72 | 33.4 | 1.74 | 33.4 | 1.72 | 33.5 | 1.75 |
| | | 1.48 | | 1.49 | | 1.47 | | 1.48 |
| 15 | 70.8 | 4.70 | 71.2 | 4.73 | 70.8 | 4.71 | 70.8 | 4.76 |
| 16 | 20.7 | 1.18 | 20.6 | 1.18 | 20.6 | 1.19 | 20.7 | 1.18 |
| 1′ | | | 169.5 | | 170.0 | | | |
| 2′ | | | 20.6 | 2.10 | 20.9 | 1.97 | | |
| 1″ | | | | | | | 100.4 | 4.07 |
| 2″ | | | | | | | 73.7 | 3.01 |
| 3″ | | | | | | | 76.9 | 3.12 |
| 4″ | | | | | | | 70.4 | 3.05 |
| 5″ | | | | | | | 77.0 | 3.05 |
| 6″ | | | | | | | 61.4 | 3.78 |
| | | | | | | | | 3.44 |

[a] 125 MHz, DMSO-$d_6$ as solvent.
[b] 500 MHz, DMSO-$d_6$ as solvent.

TABLE 3. Antifungal activities of brefeldin A and conversion products

| Microorganism | MIC (μg/ml) | | | |
|---|---|---|---|---|
| | BFA | 4-*O*-Acetyl BFA | 7-*O*-Acetyl BFA | 4-*O*-Glucosyl BFA |
| *Candida albicans* TIMM1768 | 16 | >32 | >32 | >32 |
| *C. glabrata* TIMM1064 | >32 | >32 | >32 | >32 |
| *C. krusei* IFO1396 | >32 | >32 | >32 | >32 |
| *C. tropicalis* IFO0006 | 8 | >32 | >32 | >32 |
| *C. kefyr* TIMM0298 | 16 | >32 | >32 | >32 |
| *C. parapsilosis* IFO1396 | 16 | >32 | >32 | >32 |
| *Saccharomyces cerevisiae* ATCC26108 | >32 | >32 | >32 | >32 |
| *Trichosporon cutaneum* TIMM0526 | 16 | >32 | 16 | >32 |
| *Aspergillus fumigatus* TIMM1776 | >32 | >32 | >32 | >32 |

cin A (19). In the case of erythromycin A, *Streptomyces hygroscopicus* ATCC31080, which produces a polyether antibiotic carriomycin, converted erythromycin A to 2′-*O*-glucosyl erythromycin A. Many *Streptomyces* strains which produce polyketides are known to have macrolide glycosyl transferase (MGT) activity (19). MGT modifies the desosamine sugar moiety of macrolides by glucosylation of the 2′-hydroxyl. *Streptomyces* sp. Q53834, though it has not been confirmed to be a polyketide producer, was shown to have glycosyl transferase activity in this study. But the glycosyl transferase of *Streptomyces* sp. Q53834 directly glucosylated the macrolide skeleton of BFA at the 4-hydroxyl.

Studies on the enzymes involved in these microbial conversions should provide useful information on substrate specificity. Detailed characterization of the enzymes is currently in progress.

**Biological activity** The MICs of the conversion products against fungal species including *Candida albicans* and *Aspergillus fumigatus* were determined by the micro dilution method. As shown in Table 3, BFA showed antifungal activity against *C. albicans* with an MIC of 16 μg/ml. However, the antifungal activities of the conversion products decreased markedly compared with those of BFA. Cytotoxicity ($IC_{50}$) was also measured against HeLa S3. The cytotoxicities of 4-*O*-acetyl BFA, 7-*O*-acetyl BFA, 4-*O*-D-glucosyl BFA, and BFA were 0.09 μg/ml, 0.14 μg/ml, 0.14 μg/ml, and 0.003 μg/ml, respectively. The cytotoxicities of the conversion products were lower than that of BFA. These results suggest that the hydroxy groups of BFA are important for the antifungal activity as well as the cytotoxicity in HeLa S3. Zhu *et al.* reported that acetyl BFAs exhibited nearly equivalent activities with BFA with respect to cytotoxicity and induction of apoptosis in the human colon cancer cell HCT116 (11). The difference in the effect of acetylation might depend on the cells used.

In this study we found three conversion products of BFA; two acetyl BFAs and a new glucosyl BFA. It was suggested that the conversion based on microbial specific reactions was useful for the production of new BFA derivatives. Further studies are being performed to find candidates which have strong bioactivity with low cytotoxicity and favorable pharmacokinetic properties.

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