ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 11 (2001) 423–426

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Microbiological transformation of mycophenolic acid

A. Jekkel *, I. Barta, A. Kónya, J. Sütő, S. Boros, Gy. Horváth, G. Ambrus

Institute for Drug Research Ltd., Berlini u. 47-49, 1045 Budapest, Hungary

Abstract

In our microbial screening program, we have isolated a fungal strain which produced mycophenolic acid (MPA). This compound is a selective inhibitor of guanine synthesis and, therefore, it has antibacterial, antiviral, antitumor and selective immunosuppressive activities, too. This last effect was utilised by Roche-Syntex to develop a derivative of MPA to the immunosuppressive drug CellCept®.

In order to obtain novel derivatives of MPA with an enhanced activity, we applied bioconversion of MPA with various microorganisms. TLC with densitometric evaluation and HPLC methods were developed for measurement of MPA derivatives. In the course of the bioconversion of MPA by using various types of microorganisms amidation of the carboxyl group, hydroxylation of the C_4-methyl group and formation of glycoside derivatives from the hydroxyl group located on C_7 were observed as the most frequently occurring transformations. The structures of bioconversion products were determined by UV, IR, ^{1}H NMR, ^{13}C NMR and mass spectroscopic methods.

The taxonomic features of cultures of the species applied in the bioconversion were also determined.

Keywords: Mycophenolic acid; Microbial oxidation; Glycosylation

1. Introduction

Mycophenolic acid (MPA) was detected first by Gozio in the fermentation broth of *Penicillium glaucum* in 1896. This compound was also noticed in the culture of *P. stoloniferum* and named by Alsberg and Black in 1913 [1]. Subsequently, MPA was detected in several *Penicillium* strains belonging to *P. brevicompactum* and *P. stoloniferum* [2]. After many preliminary attempts, which lasted for decades, the complete structure of MPA was reported in 1952 [3]. MPA has a wide range of biological activities. It is effective against bacteria [4], fungi [5], viruses [6,7], tumours [7] and psoriasis [8] and it shows immunosuppressive effect, too [9]. Its morpholinoethyl ester derivative (MMF) [10] was introduced by Roche-Syntex into the therapy as an immunosuppressive drug in the transplantation of kidney.

Comparing MPA and MMF to other immunosuppressants, their immunosuppressive activity is more selective than those of the other recently used immunosuppressants.

Various kinds of microorganisms can catalyse the biotransformation reactions. In the preliminary screening procedure (monitored by TLC and HPLC), we have investigated the biotransformation products of MPA by strains of bacteria and fungi, to find derivatives which could be developed to an immunosuppressive drug for treatment of the following clini-

* Corresponding author. Fax: +36-1-399-3356.
E-mail address: h8773kon@ella.hu (A. Jekkel).

1381-1177/01/$ - see front matter
PII: S1381-1177(00)00146-6

cal entities: symptomatic treatment of psoriasis, atopic dermatitis, eczema, various allergic skin conditions, herpes, inflammatory ophthalmological diseases.

2. Experimental

2.1. Screening of microorganisms for transformation of MPA

The microorganisms were cultivated on agar slants containing meat extract–yeast extract (Difco).

Approximately 2000 strains selected from the strain collection of our Institute or isolated from soil samples originating from natural habitats (India, New Zealand, Israel, and Argentina) were screened.

Cultivation of microorganisms was carried out in 500-ml Erlenmeyer flasks containing 100 ml culture medium consisting of 1% glucose, 3% meat extract (Difco), 0.3% yeast extract (Difco). After cultivation at 28°C for 3 days on a rotary shaker (deflection 2.5 cm, 320 rotations min^{-1}) 100 mg/l of MPA in ethanol was added to the shaken cultures, and cultivation was continued for 7 days. The microbial conversion of MPA was monitored by TLC on silica gel chromatoplates (Merck, Alufoil DC_{254}) using acetone–*n*-hexane–acetic acid (4:6:0.5) or chloroform–methanol (8.5:1.5) as the developing solvent mixtures.

MPA and its derivatives were measured by HPLC under the following conditions. The broth samples were diluted twofold with methanol then centrifuged and the supernatants were used for assay [HPLC apparatus: LKB system: analytical column, Nucleosil C_{18} 10 μm (BST, Budapest, Hungary) — 250 × 4.6 mm; temperature: 20°C; detection at 240 nm; eluent A: 0.05% phosphoric acid; eluent B: 80% acetonitrile; linear gradient, flow rate: 1 ml/min; injection volume: 10 μl].

2.2. Isolation and structural investigation of the biotransformation products

After fermentation, the cultures were extracted exhaustively in the presence of acetone with ethyl acetate and chloroform. The transformation products in the evaporation residues of extracts were separated by silica gel column chromatography and Sephadex gel filtration chromatography. In the case of compounds **II** and **IV**, the silica gel columns were eluted with chloroform–methanol–acetic acid (8.5:1.5:0.01) as well as with ethyl acetate–methylene-chloride mixtures with gradually increasing ethyl acetate content. For the purification of compounds **III** and **V** upon silica gel column chromatography, chloroform–methanol mixtures with gradually increasing methanol content were used and for Sephadex LH-20 gel filtration chromatography, methanol was applied.

*2.3. Characteristic spectral data of compounds **II** to **V***

IR spectra were recorded on a Bruker Vector-22 FT-IR spectrometer using OPUS software package in KBr pellets.

The NMR spectra were recorded on a Bruker AC-250 FT-NMR spectrometer using DISNMR software package. Data were processed by using WIN-NMR software package. The solvent is indicated before the spectral data. The reference was tetramethylsilane ($\delta_{TMS} = 0$ ppm).

Mass spectra were taken on a Finnigan MAT 8430 instrument under the following operating conditions: resolution, 1250; EI: ion accelarating voltage, 3 kV; ion source temperature, 250°C; electron energy, 70 eV; electron current, 500 μA; evaporation temperatures, **II**: 170°C; **IV**: 200°C, CI: ion accelerating voltage, 3 kV; ion source temperature, 250°C; reagent gas, *i*-butane; evaporation temperature, **II**: 200°C; + vs. −FAB: ion accelarating voltage, + vs. −3 kV; ion source temperature, 25°C; FAB gas: xenon; FAB gun accelarating voltage, 9 kV; matrix, *m*-nitrobenzylic alcohol.

Elemental compositions given for some ions were determined by high resolution ($R = 10{,}000$) mass measurements, using perfluorokerosene (PFK) as the reference standard.

*2.3.1. Compound **II***

IR [cm^{-1}]: 3430; 1735; 1665; 1620.

^1H NMR (δ [ppm] in $CDCl_3$): H_2-3, 5.19 (s, 2H); 4-Me, 2.31 (s, 3H); 5-OMe, 3.76 (s, 3H); H_2-1′, 3.38

(d, $J = 6.3$ Hz, 2H); H-2′, 5.24 (t, $J = 6.3$ Hz, 1H); 3′-Me, 1.80 (s, 3H); H_2-4′ and H_2-5′, 2.14 (br, 4H).

^{13}C NMR (δ [ppm]): C-1, 172.8; C-3, 70.0; C-3a, 144.1; C-4, 122.0; 4-Me, 11.4; C-5, 163.5; 5-OMe, 61.0; C-6, 116.7; C-7, 153.5; C-7a, 106.3; C-1′, 22.5; C-2′, 123.0; C-3′, 134.2; 3′-Me, 16.0; C-4′, 34.2; C-5′, 34.9; C-6′, 176.4.

MS (EI, 70 eV): $[M]^{+\cdot}$ 319, $C_{17}H_{21}NO_5$; *m/z* 302, $C_{17}H_{18}O_5$, $[M-NH_3]^{+\cdot}$; *m/z* 301, $C_{17}H_{19}NO_4$, $[M-H_2O]^{+\cdot}$; *m/z* 261, $C_{15}H_{17}O_4$, $[M-{}^{\cdot}CH_2CONH_2]^{+}$; *m/z* 207, $C_{11}H_{11}O_4$, $[M-{}^{\cdot}C_5H_8CONH_2]^{+}$; (CI, *i*-butane): $[M+H]^{+}$ 320; $[M]^{+\cdot}$ 319; *m/z* 303, $[M+H-NH_3]^{+}$; *m/z* 207, $[M-{}^{\cdot}C_5H_8CONH_2]^{+}$.

2.3.2. Compound *III*

IR [cm^{-1}]: 3340; 1735; 1655; 1615.

^{1}H NMR (δ [ppm] in MeOH-d_4): H_2-3, 5.41 (s, 2H); 4-CH_2–OH, 4.68 (s, 2H); 5-OMe, 3.78 (s, 3H); H_2-1′, 3.38 (d, $J = 6.5$ Hz, 2H); H-2′, 5.26 (t, $J = 6.5$ Hz, 1H); 3′-Me, 1.80 (s, 3H); H_2-4′ and H_2-5′, 2.25 (br, 4H).

^{13}C NMR (δ [ppm]): C-1, 173.6; C-3, 71.5; C-3a, 146.8; C-4, 122.0*; 4-CH_2–OH, 57.8; C-5, 164.1; 5-OMe, 62.9; C-6, 123.6*; C-7, 156.1; C-7a, 108.2; C-1′, 23.4; C-2′, 124.2; C-3′, 135.3; 3′-Me, 16.2; C-4′, 35.3; C 5′, 36.5; C 6′, 178.7.

MS (+ and −FAB, xenon, 9 kV): $[M+H]^{+}$ 336; *m/z* 318, $[M+H-H_2O]^{+}$; $[M-H]^{-}$ 334.

2.3.3. Compound *IV*

IR [cm^{-1}]: 3415; 1740; 1710; 1625.

^{1}H NMR (δ [ppm] in MeOH-d_4): H_2-3, 5.40 (s, 2H); 4-CH_2–OH, 4.68 (s, 2H); 5-OMe, 3.78 (s, 3H); H_2-1′, 3.38 (d, $J = 6.6$ Hz, 2H); H-2′, 5.25 (t, $J = 6.6$ Hz, 1H); 3′-Me, 1.80 (s, 3H); H_2-4′, 2.25 (m, 2H); H_2-5′, 2.35 (m, 2H).

^{13}C NMR (δ [ppm]): C-1, 173.7; C-3, 71.5; C-3a, 146.8; C-4, 122.1*; 4-CH_2–OH, 57.8; C-5, 164.1; 5-OMe, 62.9; C-6, 123.6*; C-7, 156.0; C-7a, 108.2; C-1′, 23.4; C-2′, 124.1; C-3′, 135.2; 3′-Me, 16.3; C-4′, 33.7; C-5′, 35.7; C-6′, 177.2.

MS (EI, 70 eV): $[M]^{+\cdot}$ 336; *m/z* 318, $[M-H_2O]^{+\cdot}$; *m/z* 263, $[M-{}^{\cdot}CH_2CH_2COOH]^{+}$; *m/z* 245, $[263-H_2O]^{+}$; *m/z* 223, $[M-{}^{\cdot}C_5H_8COOH]^{+}$; *m/z* 205, $[223-H_2O]^{+}$.

2.3.4. Compound *V*

IR [cm^{-1}]: 3426; 1748; 1664; 1605; 1066.

^{1}H NMR (δ [ppm] in MeOH-d_4): H_2-3, 5.20 (s, 2H); 4-Me, 2.2.18 (s, 3H); 5-OMe, 3.74 (s, 3H); H_2-1′, 3.52 (d, $J = 6.5$ Hz, 2H); H-2′, 5.24 (t, $J = 6.5$ Hz, 1H); 3′-Me, 1.80 (s, 3H); H_2-4′ and H_2-5′, 2.28 (br, 4H); H-1″, 5.48 (br. S, 1H); H-2″, 4.62 (dd, 1H); H-3″, 3.75 (m, 1H); H-4″, 3.30 (m, 1H); H-5″, 3.88 (m, 1H); H3-6″, 1.22 (d, $J = 7$ Hz, 3H).

^{13}C NMR (δ [ppm]): C-1, 171.7; C-3, 69.7; C-3a, 148.9; C-4, 122.2; 4-Me, 11.6; C-5, 164.3; 5-OMe, 61.7; C-6, 130.2; C-7, 154.8; C-7a, 112.9; C-1′, 24.6; C-2′, 124.8; C-3′, 135.5; 3′-Me, 16.4; C-4′, 35.2; C-5′, 36.4; C-6′, 178.6; C-1″, 106.6; C-2″, 3″, 4″, 5″, 72.0, 72.1, 72.2, 53.3; C-6″, 18.2.

MS (+ and −FAB, xenon, 9 kV): $[M+H]^{+}$ 466; $[M+Na]^{+}$ 488; *m/z* 320, $[\text{aglycone}+H]^{+}$; *m/z* 318, $[\text{aglycone}-H]^{-}$.

3. Results and discussion

As a part of our research program aiming to develop a new immunosuppressive drug, we have investigated the microbial transformation of MPA. In the course of the screening experiments, the bioconversion of MPA was attempted with a great number of microorganisms of different types.

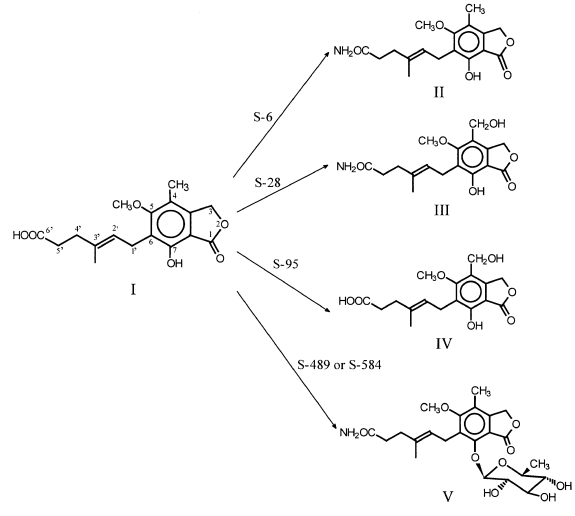

Fig. 1. Bioconversion of MPA by various microorganisms.

In this paper, we discussed the results of biotransformations carried out with five *Streptomyces* strains designated as S-6, S-28, S-95, S-489 and S-584, which were selected from the microbial isolates tested in the screening program.

The selected strains produced four different derivatives (see Fig. 1). The structures of the isolated transformation products were determined mainly by NMR- and mass spectroscopic methods. It was found that a *Streptomyces resistomycificus* sp. strain (S-28) was able to produce hydroxy-mycophenolamide (**III**), while a *Streptomyces* sp. S-6 transformed the MPA (**I**) into mycophenolamide (**II**). Bioconversion of MPA by a *Streptomyces* sp. strain (S-95) resulted in compound **IV**, which has been described and thoroughly investigated earlier [11]. The site of hydroxylation in compound **IV** was corroborated by NOESY experiment, where the signal of the 4-CH_2–OH group gave crosspeaks with the H_2-3 and the 5-OCH_3 signals. The similar experiment proved the site of hydroxylation in the case of compound **III**. The same glucoside derivative (**V**) of mycophenolamide (**II**) was formed by *Streptomyces* sp. S-489 and S-584. The amide formation in the cases of compounds **II**, **III** and **V** was indicated by the masses of their (quasi)molecular-ions. In the case of compound **II**, the mass spectral fragmentation pattern was confirmed by accurate mass measurements (see Experimental). The structure of the sugar moiety of compound **V** was found to be rhamnose on the basis of the NMR spectra, the assignment of the ^{13}C spectrum was achieved by using the INEPT technique.

References

[1] E.L. Jones, W.W. Epinette, V.C. Hackney, L. Menendez, P. Frost, J. Invest. Dermatol. 65 (1975) 537–542.
[2] P.W. Clutterbuck, A.E. Oxford, H. Raistrick, G. Smith, Biochem. J. 26 (1932) 1441–1458.
[3] J.H. Birkinshaw, H. Raistrick, D.J. Ross, Biochem. J. 50 (1952) 630–634.
[4] E.P. Abraham, Biochem. J. 39 (1945) 398–408.
[5] K. Gilliver, Ann. Bot. (Kibdib) 10 (1946) 271–282.
[6] K. Ando, S. Suzuki, G. Tamura, K. Amura, J. Antibiot. 21 (1968) 649–652.
[7] R.H. Williams, D.H. Lively, D.C. Delong, J.C. Cline, J. Sweeney, G.A. Poore, S.H. Parson, J. Antibiot. 21 (1968) 463–464.
[8] I.S. Johnson, Ger. Offen. (1972), 2, 158,824.
[9] A. Mitsui, S. Suzuki, J. Antibiot. 22 (1969) 358–363.
[10] W.H. Lee, I. Gu, A.R. Miksztel, N. Chu, K. Leung, P.H. Nelson, Pharm. Res. 7 (1990) 161–166.
[11] D.F. Jones, R.H. Moore, G.C. Crawley, J. Chem. Soc. C (1970) 1725–1737.