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Microbial transformation of mycophenolic acid Part II

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Abstract

Bioconversion of mycophenolic acid (MPA, **I**) having considerable immuno-suppressant activity was attempted with a great number of microorganisms. Four *Streptomyces* strains were selected, which transformed MPA to various bioconversion products. These derivatives of MPA (**II**–**VI**) were isolated and their structures were determined by IR, ^{1}H, ^{13}C NMR and mass spectroscopic methods. In the course of bioconversions, mycophenolic acid underwent one or more of the following transformations: hydroxylation on the side chain or on the lactone ring, amide and alcohol formation from the carboxylic acid group, oxidative cyclizations of the side chain and glycosylation.

Keywords: Mycophenolic acid; Microbial transformation; Hydroxylation; Glycosylation

1. Introduction

Mycophenolic acid (MPA), an antibiotic isolated from the fermentation broths of several *Penicillium* species, was found to exhibit antiproliferative activity on a variety of tumors in mice and rats by functioning as a potent uncompetitive, reversible IMPDH inhibitor [2]. *Mycophenolate mofetil* (MMF, CellCept), the morpholinoethyl ester prodrug of MPA, was approved some years ago for use as an immunosuppressive agent for acute kidney transplant rejection [3].

Much research has been carried out in order to obtain analogues of MPA with improved properties. Great number of derivatives and analogues of MPA were prepared by synthetic and microbial methods but only a few ones have considerably higher activity when compared with MPA (e.g. a derivative, which contains a methyl substituent at the position α to the COOH group in the side chain [4], and an analogue, where OCH_3 group of MPA is replaced by C_2H_5 [5]).

In the present study approximately 3000 strains, mainly belonging to the *Actinomycetales* genus were screened for their ability to transform mycophenolic acid. Taxonomical position of four *Streptomyces* strains producing bioconversion products was determined. Five bioconversion products were isolated from culture broths and their structures were elucidated.

2. Experimental

2.1. Screening of microorganisms for transformation of MPA

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

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† It is with great regret that we note the recent death of Dr. Antonia Jekkel.

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Approximately 3000 strains selected from the strain collection of our Institute or isolated from soil samples originating from natural habitats 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) MPA in ethanol was added to the shaken cultures to obtain a 100 mg/l final concentration and cultivation was continued for 7 days.

Microbial conversion of MPA was monitored by TLC (Merck, Alufoil DC_{254}). Acetone–*n*-hexane–acetic acid (4:6:0.5) or chloroform–methanol (8.5:1.5) solvent mixtures were used for developing the chromatogram. MPA and its bioconversion products were detected on the silica gel plate under UV lamp at 254 nm.

Bioconversion of mycophenolic acid was studied in detail with four *Streptomyces* strains. On the basis of taxonomic investigations the systemic position of these *Streptomyces* strains proved to be as follows: strain S-489, *Streptomyces griseus* according to Kämpfer et al. [6]; strain S-584, *Streptomyces* sp.; strain S-782, *Streptomyces albidoflavus* (cluster 1) according to Kämpfer et al. [6]; strain S-881, *Streptomyces omiyaensis* according to Umezawa et al. [7].

In the course of bioconversions, the changes of concentrations of MPA and its bioconversion products in the cultures of the selected strains were followed by HPLC measurements made under the following conditions: the broth samples were diluted two-fold with methanol, then centrifuged and the supernatants were used for assay (HPLC apparatus: Waters system with 996 diode array ultraviolet detector; guard column, Waters Novapack C_{18} 5 μm, 2 mm × 2.5 mm; analytical column, Waters Novapack C_{18} 5 μm, 150 mm × 3.9 mm; temperature, 20 °C; detection at 248 nm; eluent A, acetonitrile:water = 95:5; eluent B, acetonitrile:water = 5:95; pH adjusted with phosphoric acid; linear gradient; flow rate, 1 ml/min; injection volume, 10 μl).

Retention times of MPA (**I**) and its bioconversion products (**II**–**VI**): 35.0 min for compound **I**, 31.3 min for compound **II**, 29.2 min for compound **III**, 29.4 min for compound **IV**, 32.0 min for compound **V** and 32.4 min for compound **VI**.

Bioconversion rates calculated on MPA: 90 % for compound **II** by strain S-489, 16% for compound **III** by strain S-584, 40% for compound **IV** by strain S-782, and 20% for compound **V** and 17% for compound **VI**, respectively, by strain S-881.

2.2. Isolation and structural investigation of the biotransformation products

After the bioconversions biotransformation products were isolated from 4 l of fermentation broth for compounds **II** and **IV,** and from 8 l of culture liquid for compounds **III**, **V** and **VI**. The fermentation broths were directly extracted with a solvent mixture of ethyl acetate and methanol (10:1, v/v). The transformation products in the evaporation residues of the extracts were separated by silica gel column chromatography and Sephadex gel filtration chromatography. For the purification of compounds **II** and **III**, two silica gel columns were used. The first column was eluted with a mixture of acetone–*n*-hexane–acetic acid (40:60:1 → 6, i.e. a stepwise acetic acid gradient proceeding from 1 to 6 vol.%). The fractions of interest were then further separated using the other silica gel column and an eluent mixture of chloroform–methanol–acetic acid (90:10:0.05).

The purification of compounds **IV**–**VI** was also carried out on two silica gel columns. With the first column, a chloroform–methanol mixture was used as eluent in such a way that the volume ratio of methanol was increased stepwise in the eluent. Fractions of interest were further purified on the second silica gel column using ethyl acetate–methylene chloride mixtures with increasing (stepwise) ethyl acetate volume ratios. Sephadex LH-20 gel filtration chromatography applying methanol as solvent was used for further purification of compound **VI**. As a result of purifications, 240 mg of compound **II**, 52 mg of compound **III**, 70 mg of compound **IV**, 40 mg of compound **V** and 50 mg of compound **VI** were obtained in pure form.

2.3. Characteristic spectral data of compounds II–VI

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

NMR spectra were recorded on a Bruker AC-250 NMR spectrometer (^{1}H frequency, 250 MHz; ^{13}C frequency, 62.1 MHz). The solvents applied are indicated at the respective spectral data. The internal reference was tetramethylsilane ($\delta_{TMS} = 0$ ppm).

Mass spectra were taken on a Finnigan MAT 8430 instrument at a resolution of 1250 under the following conditions. EI: ion accelerating voltage, 3 kV; ion source temperature, 250 °C; electron energy, 70 eV; electron current, 500 μA. CI: ion accelerating voltage, 3 kV; ion source temperature, 250 °C; reagent gas, *iso*-butane. +FAB versus −FAB: ion accelerating voltage, +3 kV versus −3 kV; ion source temperature, 25 °C; FAB gas, xenon; FAB gun accelerating voltage, 9 kV; matrix, *m*-nitrobenzylic alcohol.

*2.3.1. Compound **II***

Name: 6-(5-carboxy-3-methylpent-2-enyl)-5-methoxy-7-rhamnosyloxy-4-methylphthalan-1-one.

IR (cm^{-1}): 3430, 2936, 1752, 1141, 1061, 959.

^{1}H NMR (δ (ppm) in MeOH-d$_4$): H$_2$-3, 5.20 (s, 2H); 4-CH$_3$, 2.18 (s, 3H); 5-OCH$_3$, 3.76 (s, 3H); H$_2$-1′, 3.40 (d, J = 6.5 Hz, 2H); H-2′, 5.24 (t, J = 6.5 Hz, 1H); 3′-CH$_3$, 1.80 (s, 3H); H$_2$-4′ and H$_2$-5′, 2.22–2.42 (m, 4H); H-1″, 5.52 (d, J = 1.8 Hz, 1H); H-2″, 4.55 (dd, J = 3.2 and 1.8 Hz, 1H); H-3″, 3.88 (dd, J = 9.4 and 3.2 Hz, 1H); H-4″, 3.52 (dd, J = 9.4 and 9.4 Hz, 1H); H-5″, 3.90 (ovl., 1H); H$_3$-6″, 1.24 (d, J = 6.4 Hz, 3H).

^{13}C NMR (δ, ppm): C-1, 171.2; C-3, 69.8; C-3a, 149.1; C-4, 122.3; 4-CH$_3$, 11.4; C-5, 164.4; 5-OCH$_3$, 61.7; C-6, 130.2; C-7, 154.8; C-7a, 113.0; C-1′, 24.6; C-2′, 124.8; C-3′, 135.4; 3′-CH$_3$, 16.4; C-4′, 35.8; C-5′, 33.8; C-6′, 177.2; C-1″, 106.7; C-2″, 72.0; C-3″, 72.2; C-4″, 72.3; C-5″, 73.4; C-6″, 18.2.

MS (EI): m/z 320 $[M - C_6H_{10}O_4]^{+\bullet}$; m/z 302 $[M - C_6H_{10}O_4 - H_2O]^{+\bullet}$; m/z 247 $[M - C_6H_{10}O_4 - {}^{\bullet}C_3H_5O_2]^+$; m/z 207 $[M - C_6H_{10}O_4 - {}^{\bullet}C_6H_9O_2]^+$.

MS (CI): m/z 321 $[M + H - C_6H_{10}O_4]^+$; m/z 320 $[M - C_6H_{10}O_4]^{+\bullet}$; m/z 303 $[M\text{-}C_6H_{10}O_4 - H_2O]^+$; m/z 247 $[M - C_6H_{10}O_4 - {}^{\bullet}C_3H_5O_2]^+$; m/z 207 $[M - C_6H_{10}O_4 - {}^{\bullet}C_6H_9O_2]^+$.

MS (+FAB): m/z 489 $[M + Na]^+$; m/z 343 $[M + Na - C_6H_{10}O_4]^+$; m/z 321 $[M + H - C_6H_{10}O_4]^+$; m/z 303 $[M + H - C_6H_{10}O_4 - H_2O]^+$.

MS (−FAB): m/z 465 $[M - H]^-$; m/z 319 $[M - H - C_6H_{10}O_4]^-$.

*2.3.2. Compound **III***

Name: 6-(6-hydroxy-3-methylhex-2-enyl)-5-methoxy-7-rhamnosyloxy-4-methylphthalan-1-one.

IR (cm^{-1}): 3388, 2930, 1735, 1072.

^{1}H NMR (δ (ppm) in DMSO-d$_6$): H$_2$-3, 5.28 (s, 2H); 4-CH$_3$, 2.14 (s, 3H); 5-OCH$_3$, 3.73 (s, 3H); H$_2$-1′, 3.33 (ovl., 2H); H-2′, 5.10 (t, J=6.5 Hz, 1H); 3′-CH$_3$, 1.72 (s, 3H); H$_2$-4′, 1.95 (t, J = 7.0 Hz, 2H); H$_2$-5′, 1.48 (qi, J = 7.0 Hz, 2H); H$_2$-6′, 3.32 (ovl., 2H); H-1″, 5.44 (s, 1H); H-2″, 4.25 (s, 1H); H-3″, 3.65 (dd, J = 9.5 and 2.9 Hz, 1H); H-4″, 3.32 (ovl., 1H); H-5″, 3.73 (ovl., 1H); H$_3$-6″, 1.10 (d, J = 6.6 Hz, 3H).

^{13}C NMR (δ, ppm): C-1, 168.8; C-3, 68.4; C-3a, 148.0; C-4, 120.6; 4-CH$_3$, 11.5; C-5, 162.6; 5-OCH$_3$, 60.9; C-6, 128.6; C-7, 152.8; C-7a, 111.7; C-1′, 23.3; C-2′, 122.4; C-3′, 135.1; 3′-CH$_3$, 16.3; C-4′, 31.1; C-5′, 35.7; C-6′, 60.9; C-1″, 105.5; C-2″, 70.3; C-3″, 70.7; C-4″, 71.3; C-5″, 71.5; C-6″, 18.2.

MS (EI): m/z 306 $[M - C_6H_{10}O_4]^{+\bullet}$; m/z 288 $[M - C_6H_{10}O_4 - H_2O]^{+\bullet}$; m/z 247 $[M - C_6H_{10}O_4 - {}^{\bullet}C_3H_7O]^+$; m/z 207 $[M - C_6H_{10}O_4 - {}^{\bullet}C_6H_{11}O]^+$.

MS (CI): m/z 307 $[M + H - C_6H_{10}O_4]^+$; m/z 289 $[M + H - C_6H_{10}O_4 - H_2O]^+$; m/z 247 $[M - C_6H_{10}O_4 - {}^{\bullet}C_3H_7O]^+$; m/z 207 $[M - C_6H_{10}O_4 - {}^{\bullet}C_6H_{11}O]^+$.

*2.3.3. Compound **IV***

Name: 6-(5-carboxamido-4-hydroxy-3-methylpent-2-enyl)-5-methoxy-7-hydroxy-4-methylphthalan-1-one.

IR (cm^{-1}): 3420, 1748, 1660, 1080.

^{1}H NMR (δ (ppm) in DMSO-d$_6$): H$_2$-3, 5.24 (s, 2H); 4-CH$_3$, 2.08 (s, 3H); 5-OCH$_3$, 3.69 (s, 3H); 7-OH, 9.41 (br, 1H); H$_2$-1′, 3.32 (d, J=6.8 Hz, 2H); H-2′, 5.35 (t, J=6.8 Hz, 1H); 3′-CH$_3$, 1.70 (s, 3H); H-4′, 4.17 (m, 1H); 4′-OH, 4.81 (d, J=4.4 Hz, 1H); H$_2$-5′, 2.14 (m, 2H); 6′-NH$_2$, 6.75 (s, 1H) and 7.21 (s, 1H).

^{13}C NMR (δ, ppm): C-1, 172.9$^{\#}$; C-3, 68.8; C-3a, 146.0; C-4, 116.1; 4-CH$_3$, 11.3; C-5, 162.8; 5-OCH$_3$, 60.8; C-6, 122.5; C-7, 152.9; C-7a, 107.1; C-1′, 22.2; C-2′, 122.5; C-3′, 137.5; 3′-CH$_3$, 12.2; C-4′, 72.8; C-5′, 42.1; C-6′, 170.3$^{\#}$ (where # represents tentative NMR assignment).

MS (EI): m/z 335 $[M]^{+\bullet}$; m/z 317 $[M - H_2O]^{+\bullet}$; m/z 273 $[M - H_2O - {}^{\bullet}CONH_2]^+$; m/z 259 $[M - H_2O - {}^{\bullet}CH_2CONH_2]^+$; m/z 207 $[M - {}^{\bullet}C_6H_{10}NO_2]^+$.

MS (CI): m/z 336 $[M + H]^+$; m/z 318 $[M + H - H_2O]^+$.

2.3.4. Compound ***V***

Name: 6-hydroxy-4-methoxy-5-methyl-2-(2-methyl-5-oxotetrahydro-2-furyl)-2,3-dihydrobenzo[2, 1-b: 3,4-c′]difuran-8(6*H*)-one

^{1}H NMR (δ (ppm) in MeOH-d_4): H-3, 6.54 (s, 1H); 4-CH_3, 2.27 (s, 3H); 5-OCH_3, 3.82 (s, 3H); H_2-1′, 2.86 (dd, J = 13.8 and 9.5 Hz, 1H) and 2.93 (dd, J = 13.8 and 3.2 Hz, 1H); H-2′, 3.98 (dd, J = 9.5 and 3.2 Hz, 1H); 3′-CH_3, 1.50 (s, 3H); H_2-4′, 1.98 (ddd, J = 11.7, 8.0 and 8.0 Hz, 1H) and 2.51 (ddd, J = 11.7, 8.0 and 8.0 Hz, 1H); H_2-5′, 2.66 (t, J = 8.0 Hz, 2H).

^{13}C NMR (δ, ppm): C-1, 171.1; C-3, 99.2; C-3a, 146.8; C-4, 123.3; 4-CH_3, 11.2; C-5, 165.6; 5-OCH_3, 61.5; C-6, 120.4; C-7, 155.1; C-7a, 109.1; C-1′, 27.5; C-2′, 76.3; C-3′, 90.6; 3′-CH_3, 22.8; C-4′, 29.5; C-5′, 30.3; C-6′, 179.8.

MS (EI): *m/z* 334 $[M]^{+\bullet}$; *m/z* 236 $[M-C_5H_6O_2]^{+\bullet}$; *m/z* 99 $[C_5H_7O_2]^+$.

MS (CI): *m/z* 335 $[M+H]^+$; *m/z* 334 $[M]^{+\bullet}$; *m/z* 236 $[M-C_5H_6O_2]^{+\bullet}$; *m/z* 99 $[C_5H_7O_2]^+$.

2.3.5. Compound ***VI***

Name: 6-(5-carboxy-3-methylpent-2-enyl)-3,7-dihydroxy-5-methoxy-4-methylphthalan-1-one.

IR (cm^{-1}): 3446, 3425, 1707, 1632, 1090.

^{1}H NMR (δ (ppm) in DMSO-d_6 at ambient temperature): H-3, 6.54 (br, $W_{1/2}$=12 Hz, 1H); 3-OH, 7.90 (br, s, 1H); 4-CH_3, 2.17 (s, 3H); 5-OCH_3, 3.69 (s, 3H); 7-OH, 7.37 (br, s, 1H); H_2-1′, 3.30 (d, J = 6.5 Hz, 2H); H-2′, 5.12 (t, J = 6.5 Hz, 1H); 3′-CH_3, 1.73 (s, 3H); H_2-4′ and H_2-5′, 2.18–2.28 (m, 4H); 6-OH, 12.00 (br, s, 1H).

^{13}C NMR (δ, ppm): C-1, 168.2; C-3, 97.4 (br, $W_{1/2}$=31 Hz,); C-3a, 145.2; C-4, 118.4; 4-CH_3, 10.9; C-5, 163.1; 5-OCH_3, 60.7; C-6, 124.3; C-7, 152.5; C-7a, 108.2; C-1′, 22.7; C-2′, 122.6; C-3′, 134.0; 3′-CH_3, 16.2; C-4′, 34.3; C-5′, 32.6; C-6′, 174.2.

MS (EI): *m/z* 336 $[M]^{+\bullet}$; *m/z* 318 $[M-H_2O]^{+\bullet}$; *m/z* 300 $[M-2H_2O]^{+\bullet}$; *m/z* 292 $[M-CO_2]^{+\bullet}$.

MS (CI): *m/z* 336 $[M]^{+\bullet}$; *m/z* 319 $[M+H-H_2O]^+$; *m/z* 292 $[M-CO_2]^{+\bullet}$.

3. Results and discussion

In the bioconversion experiments four strains which produce derivatives from MPA have been selected and designated as S-489, S-584, S-782 and S-881.

The structures of the biotransformation products of MPA (**I**) produced by these *Streptomyces* strains are shown in Fig. 1. The selected strains produced five different derivatives (see Fig. 1). Two *Streptomyces* strains, S-489 and S-584, were found earlier to produce a glycoside derivative of mycophenolamide from MPA [1]. The fermentation broths of these prokaryote strains also contained compounds **II** and **III**, which were isolated in this study.

The structures of the isolated transformation products were determined mainly by NMR and mass spectroscopic methods. The structure of the sugar moiety of the compounds **II** and **III** was found to be rhamnose on the basis of the NMR spectra. The configuration at the anomeric carbon was proven by the heteronuclear coupling constant determined from the ^{1}H coupled ^{13}C NMR spectrum. The value ^1J(C-1″, H-1″) = 175 Hz indicates, that the anomeric H is in equatorial position [8]. It was found that the main fragmentation of the rhamnosyl derivatives (**II** and **III**) under mass spectrometric conditions is the elimination of the rhamnose as 6-deoxy-L-arabino-hex-1-enitol ($C_6H_{10}O_4$) and the mass spectrum reflects that of the aglycone.

In the case of bioconversion of MPA by *Streptomyces* sp. S-782 strain, the place of the hydroxyl group of the compound obtained (**IV**) was established by using long range H–H COSY spectrum, which indicated an allylic coupling between H-2′ and H-4′. It was found that the *Streptomyces* sp. S-881 strain was able to produce compounds **V** and **VI** from MPA.

In the case of compound **VI**, we observed opening and closure of the hydroxylactone ring in the ^{1}H and ^{13}C NMR spectra recorded in DMSO solution (see Fig. 2) [9]. The signals of the atoms H-3 and C-3 are broad at ambient temperature (12 Hz versus 31 Hz) and become sharp at 80 °C (3 Hz for H-3), in consequence of the higher speed of the tautomerism at higher temperatures.

In the spectra of compound **V**—taken in MeOH-d_4—the signals of the atoms concerned were as sharp as the respective signals of compound **VI** in DMSO-d_6 at 80 °C. As only one set of signals can be observed in both ^{1}H and ^{13}C NMR spectra, the structure of compound **V** is one of the possible four stereoisomers, but—due to conformational equilibria—the relative configuration of the stereogenic centers at C-2′ and C-3′ positions could not be established.

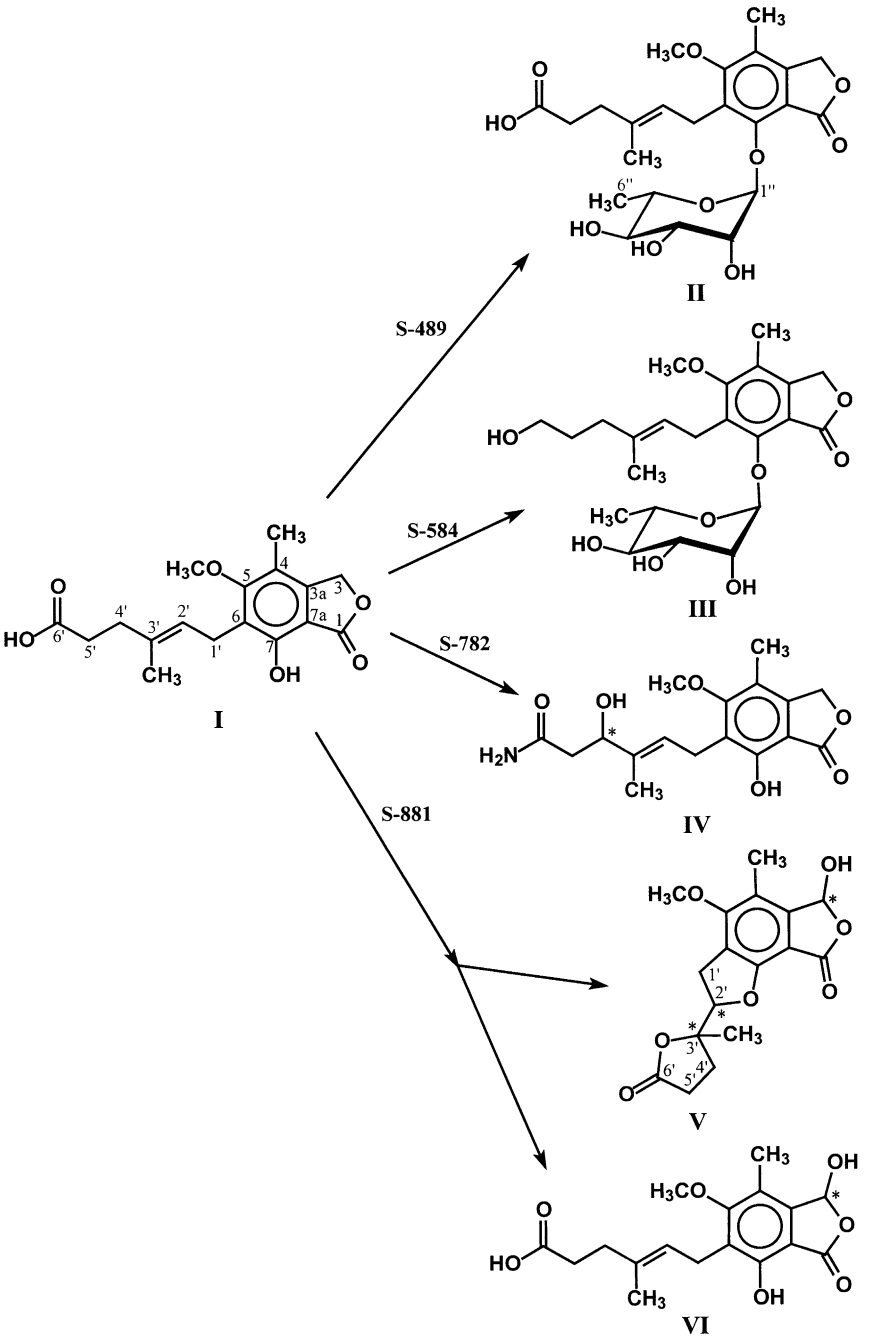

Fig. 1. Bioconversion of mycophenolic acid.

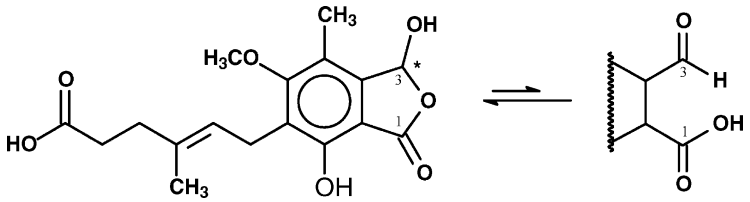

Fig. 2. Tautomerism of the hydroxylactone ring of compound **VI**.

The immunosuppressive activity of compounds **II**–**VI**, as a part of a study on several other mycophenolic acid derivatives, will be published elsewhere.

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