Synthesis and antimicrobial activity of amino acid and peptide derivatives of mycophenolic acid

Agnieszka Siebert [a], Magdalena Wysocka [b], Beata Krawczyk [b], Grzegorz Cholewi nski [a, *], Janusz Racho n [a]

[a] Department of Organic Chemistry, Gdansk University of Technology, ul. G. Narutowicza 11/12, 80-233 Gdansk, Poland
[b] Department of Molecular Biotechnology and Microbiology, Gdansk University of Technology, ul. G. Narutowicza 11/12, 80-233 Gdansk, Poland

a b s t r a c t

The series of 16 novel amino acid and peptide mycophenolic acid (MPA) derivatives was obtained as potential antibacterial agents. Coupling of MPA with respective amines was optimized with condensing reagents such as EDCI/DMAP and T3P/TEA. Amino acid analogs were received both as methyl esters and also with the free carboxylic group. The biological activity of the products was tested on five references bacterial strains: Klebsiella pneumoniae ATCC 700603 (ESBL), Escherichia coli ATCC 8739, Pseudomonas aeruginosa ATCC 27853, Staphylococcus aureus MRSA ATCC 43300, Staphylococcus aureus MSSA ATCC 25923. Peptide derivatives proved to be the most versatile ones, their MIC values relative to most strains was lower than MPA alone. It has been noted that the activity of amino acid derivatives depends on the configuration at the chiral center in the amino acid unit and methyl esters indicated better antimicrobial activity than analogs with free carboxylic group.

Keywords: Mycophenolic acid Amino acids Peptides Antimicrobial activity Tuftsin

1. Introduction

Mycophenolic acid **1** (MPA) possesses interesting scope of biological properties including antimicrobial, antifungal, antiviral, immunosuppressive and antitumor features. MPA **1** (Fig. 1) was discovered in 1896 by Italian physician Bartolomeo Gosio, who collected the fungus from damaged maize and called it *Penicillium glaucum* [1–3]. It was found that the fungus exhibited antimicrobial activity against *anthrax bacterium* [4]. Although MPA **1** occurred to be the first antibiotic isolated in pure and crystalline form, further studies were not continued for a long time [5].

In the 1970s, the biochemical causes of immunodeficiency were investigated in children, and inosine 5′-monophosphate dehydrogenase (IMPDH) was established as an enzyme responsible for the undesired immune response in autoimmune diseases. Studies towards a suitable inhibitor were started, followed by a successful clinical trial with the compound under the trade name Cell Cept **2** (Fig. 2), which was approved for use in kidney transplantation by the US Food and Drug Administration in 1995 [6].

MPA **1** is a competitive and reversible inhibitor of inosine-5′-monophosphate dehydrogenase (IMPDH), predominantly isoforms II, which is present in tumor cells and in activated lymphocytes [7,8].

MPA **1** is an active substance of immunosuppressive drugs for the prevention of acute and chronic rejection of allogenic organ transplants. So far, there are clinically applied two derivatives of MPA: mycophenolate mofetil **2** (MMF) known as CellCept produced by Roche and mycophenolate sodium **3** (MPS) (Fig. 2) under the trade name Myfortic manufactured by Novartis [9–11].

MPA **1** possesses significant antimicrobial properties e.g. against *Anthrax bacterium* [4], *Botrytis cinerea* [12]), antifungal like against *Rhizoctonia Solani* [13], antiviral for instance against Dengue virus [14], avian reoviruses (ARV) [15], coronaviruses (MERS_CoV) [16] and rotavirus [17]. Mycophenolic acid **1** exhibits also a wide spectrum of anticancer properties [18–20].

Recent studies in 2015 showed that the combination of rapamycin with MPA significantly improves clinical symptoms (erythema, edema, rash and rash) at atopic dermatitis, also reduced epidermal exfoliation, edema and cellulite [21].

On the other hand, multidrug resistance is also a significant problem in treatment of bacterial infections, and structural modifications of MPA could provide promising antimicrobial agents.

Taking into account such a large number of positive aspects of the MPA, we attempted to modify its structure towards
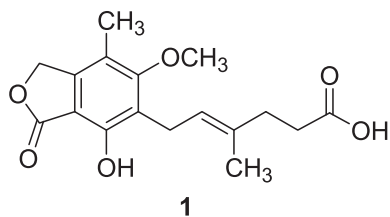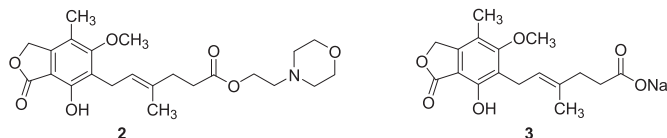
Fig. 1. Structure of MPA [1].

Fig. 2. Structures of MMF **2** and MPS **3** [9].

microbiological activity. We optimized synthesis of several new amino acid and peptide MPA derivatives by conjugation with various condensation reagents.

New synthesized compounds have been tested on five reference strains: *Klebsiella pneumoniae* ATCC 700603 (ESBL), *Escherichia* coli ATCC 8739, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* MRSA ATCC 43300, *Staphylococcus aureus* MSSA ATCC 25923.

2. Results and discussion

2.1. Chemistry

According to research in molecular modeling, polar group at the end of the side chain of MPA **1** interacts with Ser 276 of IMPDH and is one of the crucial factors for maintenance of its activity [7]. In literature were reported amide MPA analogs bearing glycine [22–24] alanine [22] valine, glutamic acid, leucine and phenylalanine [3] moieties as potential anticancer and immunosupressive agents. In order to obtain an amide bond between the MPA molecule and the amino acid, a very large amount of condensing agents was checked, for example: 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) with pyridine, *N,N′*-dicyclohexylcarbodiimide (DCC) with NMM *O*-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU) with *N*-methylomorpholine (NMM) or method of mixed anhydrides (isobutyl chloroformate). Unfortunately, none of the above methods occurred to be effective, since low conversion of substrates or difficulties with purification of target amides [3]. EDCI (1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide) was successfully used as a condensing reagent in the presence of DMAP without any racemization suppressant in the synthesis of optically active amino acid derivatives, where amino group of chiral amino acid is acylated [25–27]. This method proved to be suitable for the synthesis of most of compounds **11–17**. Amino acid MPA derivatives **11–17** were obtained by means of a condensation reagent 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide (EDCI) in the presence of 4-(dimethylamino)pyridine (DMAP) acting as a base in anhydrous *N,N*-dimethylformamide (DMF) (Scheme 1).

In case of derivatives of MPA with isoleucine **14** or malonate **17** moiety the best results were received using the T3P/Et₃N procedure, where triethylamine acts as a base and propanephosphonic acid anhydride (T3P) is a condensing reagent (Scheme 2).

The method using T3P in the presence of TEA provides amides in high yields with very low epimerization and is particularly well-suited for the coupling of racemization prone acids and amines, including relatively non-nucleophilic anilines, with easy reaction setup and product isolation [28,29]. Finally, synthesis of compounds **14** and **17** [30] was optimized using T3P/TEA method. The results of the synthesis of MPA analogs **11–17** are given in Table 1.

In order to investigate the influence of methyl ester on activity against chosen microorganisms, methyl esters were hydrolyzed with lithium hydroxide monohydrate to produce derivatives with free carboxylic group **18–23** (Scheme 3). The yields of the obtained analogs were presented in Table 2.

The next step was to obtain pentapeptide derivatives containing in their structure tuftsin and retro-tuftsin **24, 27, 28**. The protected tuftsin and retro-tuftsin derivatives, and pentapeptides, were synthesized by the mixed anhydride method with isobutyl chloroformate and *N*-methylmorpholine (NMM) in anhydrous DMF [31–37]. Tuftsin is known as an natural immunomodulator of a wide range of biological properties, occurring in human blood and other mammals, is able to stimulate certain white blood cells (monocytes, macrophages, and neutrophils). This tetrapeptide provides also antitumor, antimicrobial, anticoagulant and analgesic properties [38–41].

Despite of such advantageous properties of tuftsin, the peptide is not stable, its half-life in blood is 16 min. It is hydrolyzed by leucine aminopeptidase, carboxypeptidase B, proteinase and subtilisin to tripeptides such as H-Lys-Pro-Arg-OH and H-Thr-Lys-Pro-OH. The resulting compounds inhibit the activity of tuftsin [42], but tuftsin analogs with biologically active compounds such as MPA could improve its metabolic properties. Another way to stabilize the tuftin molecule is to attach another amino acid to the ε-amino group of lysine, thus forming a pentapeptide, which could be also implemented in designed conjugates.
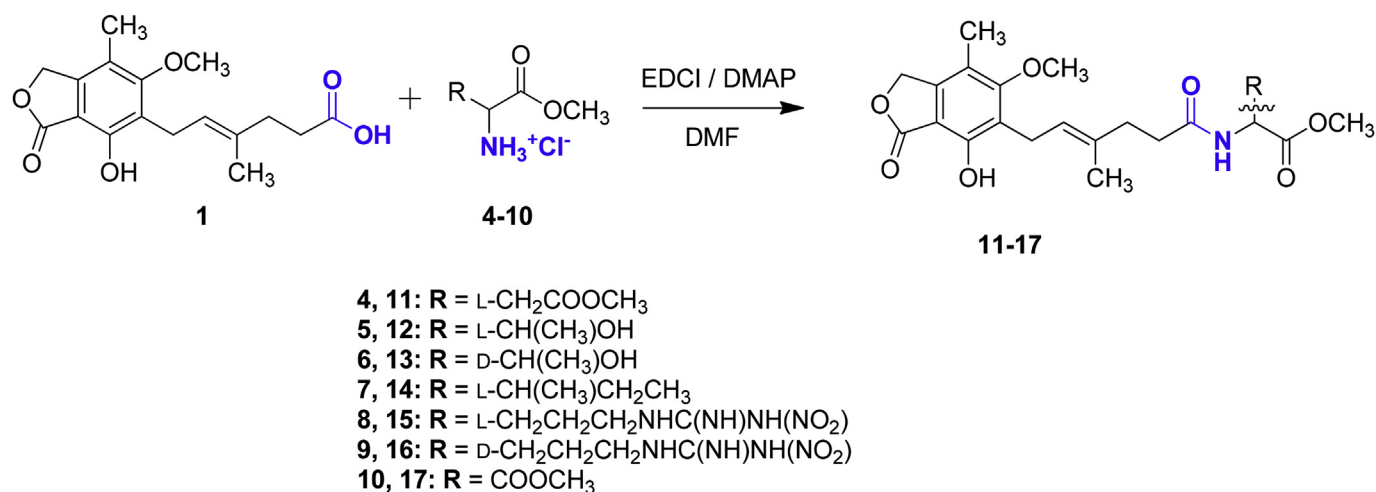
In the course of optimization of the consecutive stages towards final peptides we applied condensation reagents such as EDCI, EEDQ, or T3P. In the case of tuftsin analog **24** the most effective one occurred to be T3P procedure in the presence of TEA (Scheme 4), whereas compounds bearing retro-tuftsin **27, 28** were optimized with EDCI in the presence of DMAP (Scheme 5). Mycophenolic acid and peptides were coupled in anhydrous DMF at 0 °C to produce MPA derivatives **25, 29, 30** in reasonable yields. The results of these experiments are shown in Table 3. The Fmoc protective group was removed with 20–30% diethylamine solution towards target compounds **26, 31, 32**. Fmoc removal yields were within the range of 80–85%.

2.2. Microbiological results

The present study evaluated the effect of the obtained compounds **11–23, 26, 31, 32** on five reference strains: *Klebsiella pneumoniae* ATCC 700603 (ESBL), *Escherichia* coli ATCC 8739, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* MRSA ATCC 43300, *Staphylococcus aureus* MSSA ATCC 25923. Selected compounds differ in the structure of the skeleton to be able to see if any of its components are of particular importance for antibacterial activity.

The micro dilution method in the broth gave the possibility of determining the minimum concentration of microbial growth inhibitor (MIC) within a narrow and precise concentration range. By carrying out a 96-well microplate study, the analysis of results may consist of a visual assessment of the degree of turbidity in a given well or a spectrophotometer (OD600) [43,44]. When testing water-insoluble products (compounds with arginine), incompletely dispersible, may interfere with reading of absorbance, it is advisable to use colored growth indicators of microorganisms. The activity of sparingly soluble compounds may be erroneous.

In the presented work, a redox indicator - resazurin was used as

4, 11: R = L-CH$_2$COOCH$_3$
5, 12: R = L-CH(CH$_3$)OH
6, 13: R = D-CH(CH$_3$)OH
7, 14: R = L-CH(CH$_3$)CH$_2$CH$_3$
8, 15: R = L-CH$_2$CH$_2$CH$_2$NHC(NH)NH(NO$_2$)
9, 16: R = D-CH$_2$CH$_2$CH$_2$NHC(NH)NH(NO$_2$)
10, 17: R = COOCH$_3$

Scheme 1. Synthesis of amino acid derivatives of MPA **11—17** using the EDCI/DMAP method.
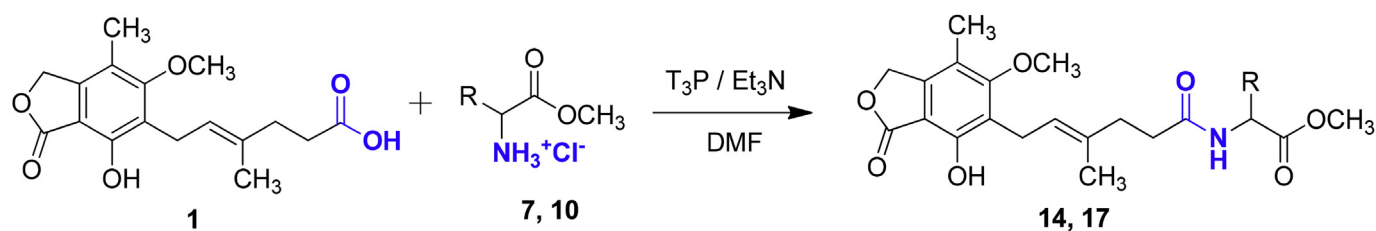
Scheme 2. Synthesis of amino acid derivatives of MPA **14**, **17** [30] using the T3P/TEA method.

Table 1
Yields of obtained compounds **11—17**.

Compound	Compound no	Yield [%]
MPA-Asp(OMe)-OMe	**11**	73
MPA-Thr-OMe	**12**	71
MPA-D-Thr-OMe	**13**	70
MPA-Ile-OMe	**14**	68
MPA-Arg(NO$_2$)-OMe	**15**	70.5
MPA-D-Arg(NO$_2$)-OMe	**16**	66
MPA-Mal-(OMe)$_2$	**17**	45
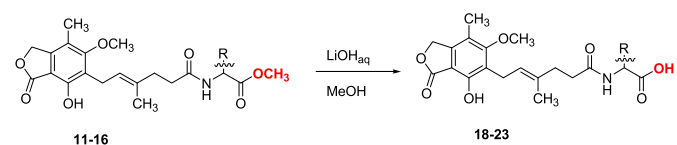
Scheme 3. Synthesis of amino acid analogues of MPA with free carboxylic group.

Table 2
Yields of carboxylic acid analogs **18—23**.

Compound	Compound no	Yield [%]
MPA-Asp-OH	**18**	87
MPA-Thr-OH	**19**	88
MPA-D-Thr-OH	**20**	86
MPA-Ile-OH	**21**	80
MPA-Arg(NO$_2$)-OH	**22**	87
MPA-D-Arg(NO$_2$)-OH	**23**	89

a measure of the presence of live microorganisms present in tested compounds. Resazurin is subjected to reduction of activity by active metabolic microorganisms by changing the color from blue (resazurin) to pink (resorufin) and the intensity of color correlates with the size of the population. It has been found in this and other works that the use of resazurin as an indicator of metabolic activity of bacteria increases the sensitivity of detection [45].

For standard antibiotics and MPA for *E. coli* it was possible to determine MIC90 (bold type in Table 4), for the remaining compounds for all tested bacteria MIC50 was determined.

Inhibitory concentrations of tested compounds **11—23, 26, 31, 32** for selected microorganisms (μg/ml) is shown in Table 4. Figure S-1 (See electronic supplementary information) compares MIC values [μg/mL] of compounds and standard drug against different bacteria species.

The scope of the examined derivatives MPA was chosen individually. The methodological basis for further elaboration in the implementation and interpretation of minimal inhibitory concentrations is EUCAST, supplemented by the recommendations of the Scientific Societies, data from the publication and own experience [46].

Investigated compounds exerted a variety of effects depending on the species of bacteria. It was demonstrated that most of tested bacterial species (except for *Acinetobacter baumannii* for which MIC MPA was the same, and *P. aeruginosa*, where no activity was observed) were inhibited by compound **26** (MIC 32—128 μg/ml) as compared to MPA (128- ≥1500 μg/ml). In case of compound **17** the inhibition of *K. pneumoniae* was comparable to the control effect of ampicillin (128 μg/ml). The MIC50 for this compound is at MIC50 of MPA. The MIC50 for compound **15** is lower than MIC50 of MPA. For the remaining bacteria there is no clear activity (at the concentration limit). Compounds **11, 12, 13, 15, 26, 31, 32** exhibited the greatest activity against *S. aureus*, but the MSSA strain was more sensitive than MRSA. In case of analog **21**, for *S. aureus* MSSA, the
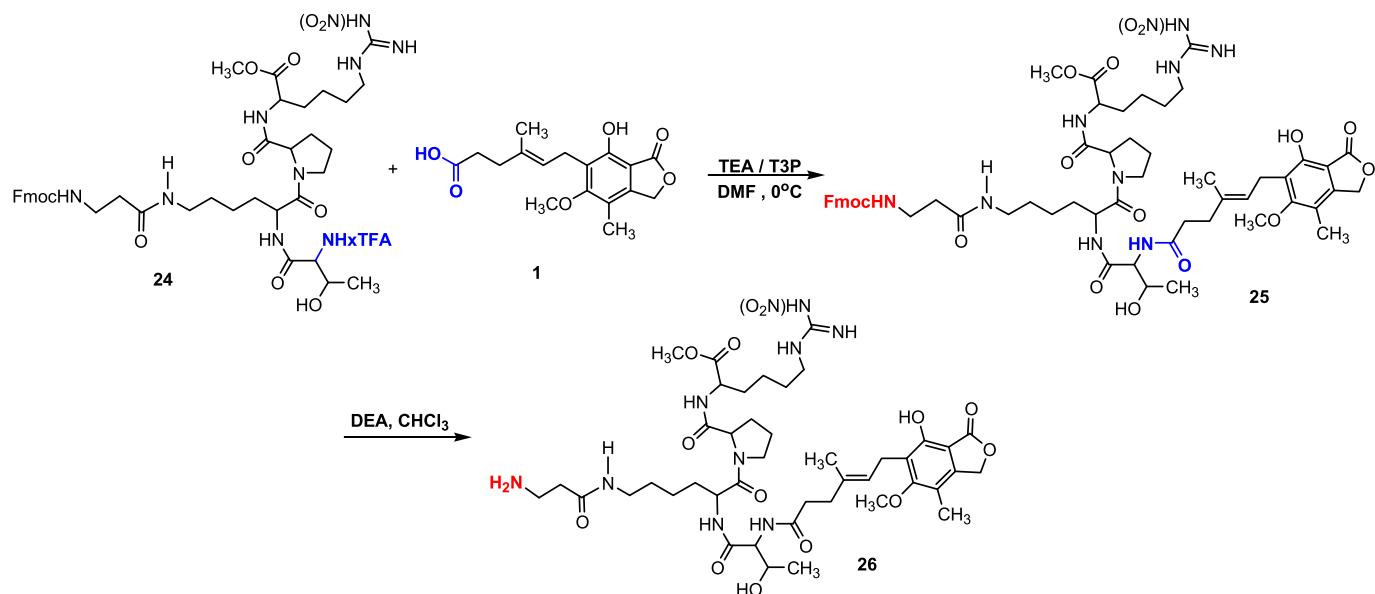
Scheme 4. Synthesis of pentapeptide analog of MPA **26** containing in its structure tuftsin.
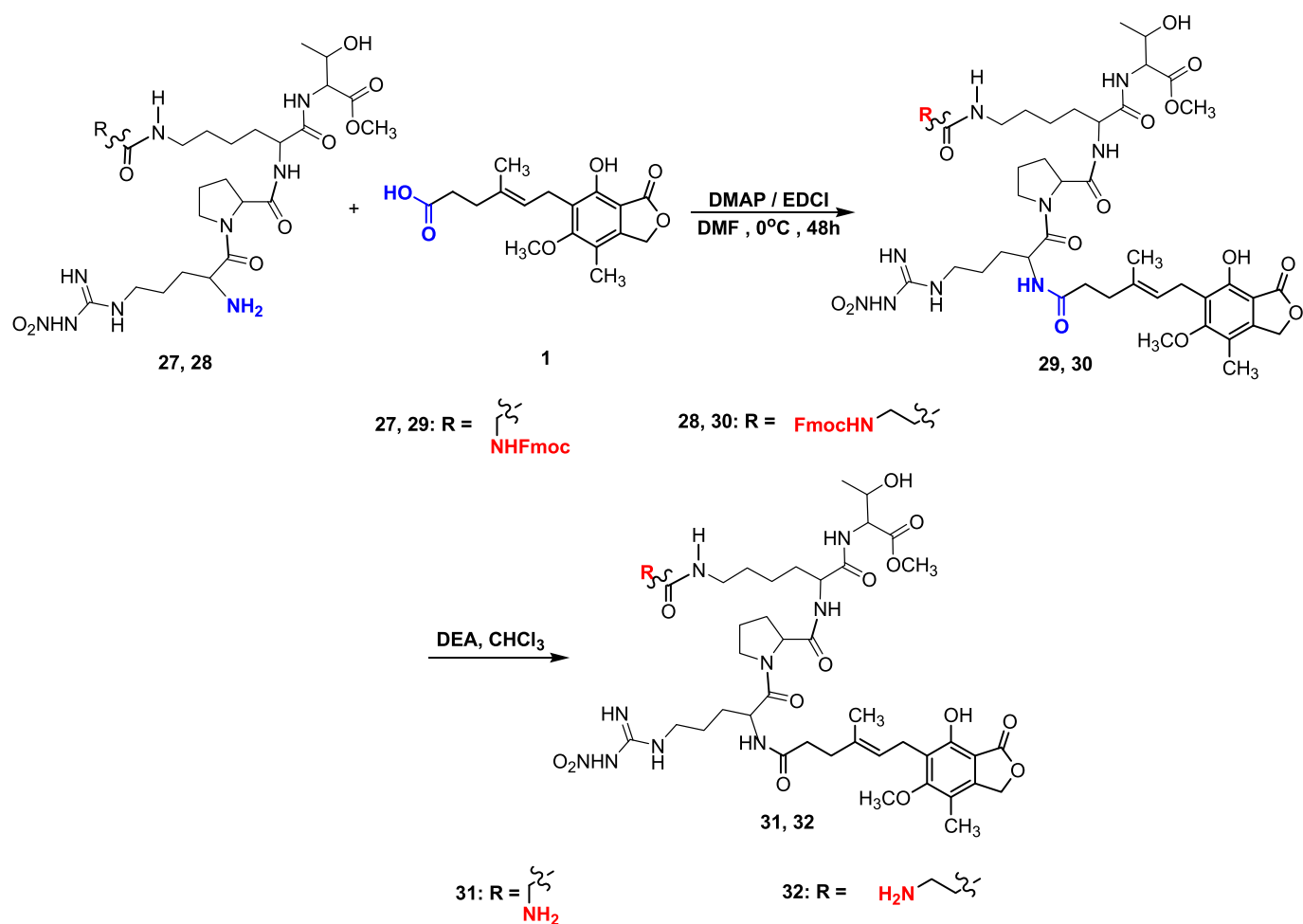
Scheme 5. Synthesis pentapeptides analogs of MPA **31, 32** containing in their structures retro-tuftsin.

inhibitory force (MIC50 = 8 μg/ml) is significant if compared to MPA (MIC50 ≈ 187.5 μg/ml), which causes very poor growth

inhibition. In case of *K. pneumoniae* for compounds **11, 12, 14** MIC50 is equal to 256 μg/ml (concentration limit) and is higher than for

Table 3
Yields of obtained conjugates of MPA and Fmoc-protected peptides **25, 29, 30.**

Compound	Compound no	Yield [%]
MPA-T-β-Ala	**25**	53
MPA-RT-Gly	**29**	58
MPA-RT- β-Ala	**30**	57

MPA (MIC50 = 128 μg/ml). These modifications do not affect the improvement of antibacterial activity for this bacterium. Conversely, compounds **15** and **26, 31, 32** indicated lower MIC, (MIC50 = 64, 16, 32, 32 μg/ml respectively) and show enhanced antimicrobial effects compared to MPA. By analyzing the results it can be concluded that MPA modifications such as **11, 12, 15, 26, 31, 32** are promising for *S. aureus*, while **15, 26, 31, 32** modifications are promising for *K. pneumoniae*. There was no bacteriostatic effect of the tested compounds on *Pseudomonas aeruginosa*. The remaining compounds **18**−**23** caused very poor growth inhibition (<10% control) or no inhibitory effect on the growth of bacteria. The bactericidal effect was not found for any MPA derivative. Compounds **17**−**23** exhibit poor activity against all groups of microorganisms, no susceptibility was observed.

2.3. Structure-activity analysis

Analyzing the results, it can be seen that the peptide derivatives of MPA **26, 31, 32** have better activity than the parent compound **1** itself. In case of strains *K. pneumoniae* ATCC 700603 (ESBL) and *S. aureus* MRSA ATCC 43300 their values MIC were lower (16−32 for *K. pneumoniae* ATCC 700603 (ESBL), 32−64 for *S. aureus* MRSA ATCC 43300) than for the antibiotics themselves: kanamycin and ampicillin. A slightly better microbiological activity for *S. aureus* MRSA strains revealed derivatives **26, 32** containing in his peptidic structure β-alanine moiety. There was no difference in effect between tuftsin skeleton and retro-tuftsin.

Microbiological activity of amino acid esters depends both on

substituent and configuration in amino acid moiety. It can be clearly seen, that in case of threonine **12, 13** and arginine **15, 16** D enantiomer is definitely less active against all strains. Although D-amino acids can enhance stability of the active substance due to its improved resistance against proteases [47], obtained results suggest that natural configuration of the tested compounds is privileged against investigated bacterial strains.

Deprotection of methyl esters **18**−**23** changed activity considerably. We observed a significant advantage of derivatives protected by methyl ester over those with a free carboxylic group. This is noticeable for all strains of bacteria, and could be explained by better cell membrane penetration due to lower polarity [19].

3. Summary

We have received 16 new MPA derivatives using EDCI and T3P condensation reagents. The obtained compounds were tested on five different bacterial cell lines. By analyzing the results it can be concluded that MPA modifications such as **11, 12, 15, 26, 31, 32** are promising for *S. aureus*, while **15, 26, 31, 32** modifications are promising for *K. pneumoniae*. It was noted that the compounds with the free carboxyl group **17**−**23** exhibit poor activity against all groups of microorganisms. Microbiological activity of amino acid esters depends both on substituent and configuration in amino acid moiety. It was found that derivatives of D configuration **13, 16** were much less active than those of L configuration **12, 15**.

These results confirm that structure modifications of MPA derivatives can be designed towards antimicrobial properties.

4. Experimental section

All reactions were performed in inert atmosphere with magnetic stirring. DMF was purified by distillation from benzene/water. The reactions were monitored by TLC on Merck F254 silica gel precoated plates. The following solvent systems (by volume) were used for TLC development: CH_2Cl_2:MeOH:CH_3COOH (15:1:0.1, v/v/

Table 4
Inhibitory concentrations of test compounds for selected microorganisms (μg/ml).

Compound	MIC range (ug/ml)	MIC [ug/ml]					
		Gram-positive bacteria		Gram-negative bacteria			
		S. aureus MSSA ATCC 25923	*S. aureus* MRSA ATCC 43300	*A. baumannii* ATCC 17978	*E. coli* ATCC 8739	*K. pneumoniae* ATCC 700603 (ESBL)	*P. aeruginosa* ATCC 27853
MPA	5.9−3000	>>187.5	750	128	**750**	128	>>1500.0
11	0.125−256	32	32	256	256	256	NI
12	0.125−256	8	32	256	256	256	NI
13	5.9−3000	NI	NI		>>1500.0	NI	>>1500.0
14	0.125−256	16	256	256	256	256	NI
15	0.125−256	32	64	128	256	64	NI
16	5.9−3000	NI	NI		>>375.0	NI	>>1500.0
17	0.125−256	256	256	256	256	128	NI
18	5.9−3000	NI	NI		>>750.0	NI	>>1500.0
19	5.9−3000	NI	NI		>>750.0	>>375.0	>>1500.0
20	5.9−3000	NI	NI		>>1500.0	NI	>>1500.0
21	5.9−3000	NI	>>3000	NI	>>375.0	>>187.5	>750.0
22	5.9−3000	NI	NI		>>750.0	NI	NI
23	5.9−3000	NI	NI		>>1500.0	NI	>>1500.0
26	0.125−256	32	32	128	128	32	NI
31	0.125−256	64	64	128	256	16	NI
32	0.125−256	32	32	128	256	32	NI
Kanamycin	2−1000	**7.8**	**125**	NI	**15.6**	NI	1500
Ampicillin	0.125−256	**0.125**	**128**	**2**	**8**	**128**	**256**

The value in bold type - MIC, is the lowest concentration causing complete inhibition of growth or decreasing the number of bacterial population by over 90%; another - MIC50.
Value with the ">" sign - concentration inhibiting growth by 10−50% relative to positive control.
Value with the sign ">>" - concentration causing very poor growth inhibition (less than 10% of control).
"NI" - no inhibitory effect at the compound concentrations used or stimulation of growth.

v) (A), CH_2Cl_2:MeOH (50:1, v/v) (B), $CHCl_3$:MeOH:NH_3 (9:1:0.2, v/v/v) (C), $CHCl_3$:MeOH:NH_3 (5:1:0.2, v/v/v) (D). ^{1}H NMR and ^{13}C NMR spectra were taken on the camera Varian Unity 500 Plus in $CDCl_3$, CD_3OD or DMSO. Mass spectra were performed at the Laboratory of Mass Spectrometry MALDI-TOF on the matrix DHB (BIFLEX III Bruker).

Conditions of chromatographic HPLC separation and detection of examined compounds (**11–24, 27, 28**): column - Poroshell EC-C18 (3.0 × 150 mm), 2.7 μm, Agilent Technologies; column temperature - 40 °C; injection volume - 2 μL; flow rate - 0.4 mL/min; eluents: (A) 0.1% HCOOH in water, (B) 0.1% HCOOH in ACN/MeOH (1:1, v/v); gradient program (Table 5):

4.1. General procedure for the preparation amino acid analogues of MPA 11−23

Amino acid methyl ester hydrochloride **4–10** (0.181 mmol), MPA **1** (0.166 mmol) and DMAP (0.270 mmol) were dissolved in 2 mL anhydrous DMF. Then the solution cooled to 0 °C in an ice bath, then added (0.271 mmol) of EDCI (in case of **7, 10** 0.271 mmol of T3P in 50% DMF was used [30]). Stirring was continued under nitrogen at 0 °C for 2 h and then at room temperature. The progress of the reaction was controlled by TLC. After completion of the synthesis, the solvent was evaporated under reduced pressure using a vacuum pump. The product was purified by preparative thin layer chromatography. Structures of synthesized derivatives **11–17** were established by spectroscopic methods (^{1}H NMR, ^{13}C NMR) and MS, HPLC-MS.

Hydrolysis of the esters **11–16** was carried out by removing the methyl ester from the carboxylic group. Methyl ester **11–16** (0.18 mmol) was dissolved in 1.8 ml of methanol. Then, was added 0.013 g (0.54 mmol) of lithium hydroxide monohydrate dissolved in 1.18 ml of water. The mixture was stirred on a magnetic stirrer for 24 h at room temperature. The solution was acidified with 2 N HCl and extracted with ethyl acetate (EtOAc). The extract was dried with $MgSO_4$, evaporated and purified by preparative thin layer chromatography with respective solvent system A-D to produce compounds **18–23**.

4.1.1. Dimethyl N-mycophenoyl-L-asparaginate 11
MPA-Asp(OMe)-OMe (11): ^{1}H NMR (DMSO-d_6) δ ppm: 1.71 (s, 3H), 2.06 (s, 3H), 2.099 (m, 2H), 2.14 (m, 2H), 2.60 (m, 1H, β-D3), 2.68 (dd, $J = 16.3$ Hz, $J = 6.3$ Hz, 1H, β-D3), 3.27 (d, $J = 6.8$ Hz, 2H, d), 3.58 (s, 3H, -COOCH$_3$), 3.59 (s, 3H, -COOCH$_3$), 3.68 (s, 3H), 4.54 (dd, $J = 14.3$ Hz, $J = 6.9$ Hz, 1H, α-D2), 5.09 (t, $J = 6.5$ Hz, 1H), 5.22 (s, 2H), 8.32 (d, $J = 12.8$ Hz, 1H, D-NH);
^{13}C NMR (DMSO-d_6) δ ppm: 11.48, 16.42, 22.85, 34.26, 35.24, 36.05 (β-D3), 48.81 (α-D2), 52.10 (COOCH$_3$), 52.55 (COOCH$_3$), 61.05, 69.030, 107.37, 116.17, 122.90, 123.06, 134.28, 146.21, 153.44, 162.99, 170.65, 170.86, 171.67, 172.22;
MS m/z calculated for $C_{23}H_{29}NO_9$ 463.18; found 462.18 [M+H]$^+$.
Rf = 0.5 (B).
89.15% pure by HPLC.

Table 5
Gradient program for HPLC.

Time [min]	% A	% B
0	90	10
20	0	100
30	0	100

Post time - 10 min; UV−Vis detection; wavelengths UV: 254 nm; Vis: 580 nm; peak width > 0.1 min (2s); ESI MS detection.

4.1.2. N-mycophenoyl-L-threonine methyl ester 12
MPA-ThrOMe (12): ^{1}H NMR (CDCl$_3$-d$_1$) δ ppm: 1.18 (d, $J = 6.4$ Hz, 3H, γ-T4), 1.83 (s, 3H), 2.16 (s, 3H), 2.33 (m, 2H), 2.40 (m, 2H), 3.4 (d, $J = 6.8$ Hz, 2H), 3.75 (s, 3H), 3.77 (s, 3H, -COOCH$_3$), 4.31 (m, 1H, β-T3), 4.59 (dd, $J = 8.8$ Hz, $J = 2.4$ Hz, 1H, α-T2), 5.21 (s, 2H), 5.27 (t, $J = 6.6$ Hz, 1H), 6.34 (d, $J = 8.7$ Hz, 1H, T-NH);
^{13}C NMR (CDCl$_3$-d$_1$) δ ppm: 11.56, 16.25, 19.84 (γ-T4), 22.68, 34.90, 34.98, 52.57 (COOCH$_3$), 57.07 (β-T3), 61.05, 68.04 (α-T2), 70.02, 106.45, 116.73, 122.19, 122.95, 134.39, 144.13, 153.64, 163.59, 171.61, 173.48;
MS m/z calculated for $C_{22}H_{29}NO_8$ 435.19; found 436.20 [M+H]$^+$.
Rf = 0.5 (A).
99.77% pure by HPLC.

4.1.3. N-mycophenoyl-D-threonine methyl ester 13
MPA-D-ThrOMe (13): ^{1}H NMR (DMSO-d_6) δ ppm: 0.98 (d, $J = 6.4$ Hz, 3H, γ-T4), 1.72 (s, 3H), 2.05 (s, 3H), 2.12 (m, 2H), 2.19 (m, 2H), 3.26 (d, $J = 6.8$ Hz, 2H), 3.59 (s, 3H), 3.67 (s, 3H, -COOCH$_3$), 4.02 (m, 1H, β-T3), 4.21 (dd, $J = 8.5$ Hz, $J = 3.5$ Hz, 1H, α-T2), 5.1 (t, $J = 6.5$ Hz, 1H), 5.21 (s, 2H), 7.94 (d, $J = 8.5$ Hz, 1H, T-NH);
^{13}C NMR (CDCl$_3$) δ ppm: 11.56, 16.25, 19.84 (γ-T4), 22.68, 34.90, 34.98, 52.57 (COOCH$_3$), 57.07 (β-T3), 61.05, 68.04 (α-T2), 70.02, 106.45, 116.73, 122.19, 122.95, 134.39, 144.13, 153.64, 163.59, 171.61, 173.48;
MS m/z calculated for $C_{22}H_{29}NO_8$ 435.19; found 436.11 [M+H]$^+$.
Rf = 0.5 (A).
99.72% pure by HPLC.

4.1.4. N-mycophenoyl-L-isoleucine methyl ester 14
MPA-Ile-OMe (14): ^{1}H NMR (DMSO-d_6) δ ppm: 0.76 (m, 6H, δ-I5, ε-I6), 1.08 (m, 1H, γ-I4), 1.30 (m, 1H, γ-I4), 1.66 (m, 1H, β-I3), 1.718 (s, 3H), 2,07 (s, 3H), 2.1 (m, 2H), 2.19 (m, 2H), 3.27 (d, $J = 7.4$ Hz, 2H), 3.583 (s, 3H), 3.67 (s, 3H, COOCH$_3$), 4.13 (dd, $J = 7.8$ Hz, $J = 6.8$ Hz, 1H, α-I2), 5.06 (t, $J = 6.6$ Hz, 1H), 5.231 (s, 2H), 8.06 (d, $J = 8.1$ Hz, 1H, I-NH);
^{13}C NMR (DMSO-d_6) δ ppm: 11.51, 11.51 (δ-I5), 15.84 (ε-I6), 16.50, 22.85, 25.21 (γ-I4), 34.11 (β-I3), 35.36, 36.71, 51.96 (COOCH$_3$), 56.60 (α-I2), 61.06, 69.06, 107.40, 116.39, 122.90, 122.92, 134.42, 146.21, 153.17, 163.02, 170.60, 172.50, 172.67;
MS m/z calculated for $C_{24}H_{33}NO_7$ 447.23; found 448.24 [M+H]$^+$.
Rf = 0.46 (B).
98.37% pure by HPLC.

4.1.5. N-mycophenoyl-L-arginine methyl ester 15
MPA-Arg(NO$_2$)OMe (15): ^{1}H NMR (CDCl$_3$) δ ppm: 1.58 (m, 3H, γ-R4, β-R3), 1.77 (s, 3H), 1.84 (m, 1H, β-R3a), 2.10 (s, 3H), 2.22 (m, 2H), 2.34 (m, 2H), 3.23 (m, 2H, δ-R5), 3.32 (d, $J = 6.5$ Hz, 2H), 3.70 (s, 3H), 3.72 (s, 3H, -COOCH$_3$), 4.56 (m, 1H, α-R2), 5.16 (s, 2H), 5.21 (t, $J = 6.6$ Hz, 1H), 8.64 (m, 1H, R-NH);
^{13}C NMR (CDCl$_3$) δ ppm: 11.5, 16.12, 22.60, 24.12 (β-R3), 30.60 (γ-R4), 34.70, 34.99, 40.35 (δ-R5), 50.68 (α-R2), 52.68 (COOCH$_3$), 60.99, 70.12, 106.29, 116.96, 121.95, 122.90, 134.24, 144.26, 153.45, 159.27, 163.60, 172.66, 172.92;
MS m/z calculated for $C_{24}H_{33}N_5O_9$ 535.55; found 535.23 M$^+$.
Rf = 0.4 (A).
100% pure by HPLC.

4.1.6. N-mycophenoyl-D-arginine methyl ester 16
MPA-D-Arg(NO$_2$)OMe (16): ^{1}H NMR (DMSO-d_6) δ ppm: 1.47 (m, 3H, γ-R4, β-R3) 1.65 (m, 1H, β-R3), 1.72 (s, 3H), 2,06 (s, 3H), 2.11 (m, 2H), 2.14 (m, 2H), 3.10 (m, 2H, δ-R5), 3.27 (d, $J = 6.7$ Hz, 2H), 3.59 (s, 3H), 3.67 (s, 3H, -COOCH$_3$), 4.16 (q, $J = 13.2$ Hz, 1H, α-R2), 5.10 (t, $J = 6.6$ Hz, 1H), 5.22 (s, 2H, b), 8.18 (d, $J = 7.5$ Hz, 1H, R-NH);
^{13}CNMR (CDCl$_3$) δ ppm: 11.5, 16.12, 22.60, 24.12 (β-3R), 30.60 (γ-R4), 34.70, 34.99, 40.35 (δ-R5), 50.68 (α-R2), 52.68 (COOCH$_3$), 60.99,

70.12, 106.29, 116.96, 121.95, 122.90, 134.24, 144.26, 153.45, 159.27, 163.60, 172.66, 172.92;

MS: m/z calculated for $C_{24}H_{33}N_5O_9$ 535.55; found 535.45 M$^+$.

Rf = 0.46 (A).

100% pure by HPLC.

4.1.7. Dimethyl N-mycophenoylomalonate 17

NMR and MS data are presented in our recent publication [30].

Rf = 0.6 (B).

99.5% pure by HPLC.

4.1.8. N-mycophenoyl-L-aspartic acid 18

MPA-Asp-OH (18): ^{1}H NMR (DMSO-d_6) δ ppm: 1.71 (s, 3H), 2.06 (s, 3H), 2.12 (m, 4H), 2.49 (m, 1H, β-D3), 2.63 (dd, J = 15.7 Hz, J = 6.3 Hz, 1H, β-D3), 3.27 (d, J = 6.6 Hz, 2H), 3.67 (s, 3H), 4.35 (dd, J = 14.1 Hz, J = 7.0 Hz, 1H, α-D2), 5.07 (t, J = 6.5 Hz, 1H), 5.21 (s, 2H), 7.94 (d, J = 7.7 Hz, 1H, D-NH);

^{13}C NMR (DMSO-d_6) δ ppm: 11.48, 16.44, 22.97, 34.43, 35.39, 36.86 (β-D3), 50.01 (α-D2), 61.03, 66.97, 107.47, 115.72, 122.97, 133.96, 146.27, 161.06, 170.88, 171.49, 171.89, 173.36;

MS m/z calculated for $C_{23}H_{29}NO_9$ 435.47; found 436.16 [M+H]$^+$.

Rf = 0 in A-D solvent systems.

99.49% pure by HPLC.

4.1.9. N-mycophenoyl-L-threonine 19

MPA-ThrOH (19): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.97 (d, J = 6.3 Hz, 3H, γ-T4), 1.74 (s, 3H), 2.08 (s, 3H), 2.16 (m, 2H), 2.25 (m, 2H), 3.29 (d, J = 6.7 Hz, 2H), 3.69 (s, 3H), 3.99 (m, 1H, β-T3), 4.08 (dd, J = 8.2 Hz, J = 3.5 Hz, 1H, α-T2), 5.12 (t, J = 6.4 Hz, 1H), 5.23 (s, 2H), 7.66 (d, J = 8.2 Hz, 1H, T-NH), 7.95 (s, 1H, COOH);

^{13}C NMR (400 MHz, CDCl$_3$) δ ppm: 11.49, 16.45, 20.39 (γ-T4), 21.60, 34.49, 35.50, 55.36 (β-T3), 61.06, 66.32 (α-T2), 69.04, 106.45, 107.41, 116.03, 122.97, 123.21, 134.34, 146.21, 162.71, 163.08, 170.81, 172.34, 172.52;

MS m/z calculated for $C_{21}H_{27}NO_8$ 421.44; found 422.57 [M+H]$^+$.

Rf = 0.39 (D).

98.65% pure by HPLC.

4.1.10. N-mycophenoyl-D-threonine 20

MPA-D-ThrOH (20): ^{1}H NMR (DMSO-d_6) δ ppm: 0.93 (d, J = 8.6 Hz, 3H, γ-T4), 1.72 (s, 3H), 2.06 (s, 3H), 2.11 (m, 2H), 2.17 (m, 2H), 3.26 (d, J = 6.7 Hz, 2H), 3.67 (s, 3H), 3.94 (m, 1H, β-T3), 4.03 (m, 1H, α-T2), 5.09 (t, J = 6.5 Hz, 1H), 5.22 (s, 2H), 7.56 (d, J = 8 Hz, 1H, T-NH);

^{13}C NMR (CDCl$_3$) δ ppm: 11.49, 16.45, 20.39 (γ-T4), 21.60, 34.49, 35.50, 55.36 (β-T3), 61.06, 66.32 (α-T2), 69.04, 106.45, 107.41, 116.03, 122.97, 123.21, 134.34, 146.21, 162.71, 163.08, 170.81, 172.34, 172.52;

MS m/z calculated for $C_{21}H_{27}NO_8$ 421.44; found 422.37 [M+H]$^+$.

Rf = 0.46 (D).

99.02% pure by HPLC.

4.1.11. N-mycophenoyl-L-isoleucine 21

MPA-Ile-OH (21): ^{1}H NMR (DMSO-d_6) δ ppm: 0.77 (d, J = 7.3 Hz, 3H, δ-I5), 0.79 (t, J = 7.5 Hz, 3H, ε-I6) 1.071 (m, 2H, γ-I4), 1.66 (m, 1H, β-I3), 1.72 (s, 3H), 2.05 (s, 3H), 2.05 (m, 2H), 2.09 (m, 2H), 3.26 (d, J = 6.7 Hz, 2H), 3.66 (s, 3H), 4.05 (m, 1H, α-I2), 5.07 (t, J = 6.5 Hz, 1H), 5.21 (s, 2H), 7.81 (d, J = 8.5 Hz, 1H, I-NH);

^{13}C NMR (DMSO-d_6) δ ppm: 11.49, 11.77 (δ-I5), 16.04 (ε-I6), 16.50, 22.92, 25.15 (γ-I4), 34.29 (β-I3), 35.52, 36.84, 57.07 (α-I2), 61.03, 69.0, 107.41, 115.79, 122.97, 123.28, 134.20, 146.22, 154.06, 163.07, 170.86, 172.22, 174.12;

MS: m/z calculated for $C_{23}H_{31}NO_7$ 433.49; found 434.38 [M+H]$^+$.

Rf = 0.674 (D).

100% pure by HPLC.

4.1.12. N-mycophenoyl-L-arginine 22

MPA-Arg(NO$_2$)OH (22): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.53 (m, 3H, γ-R4, β-R3) 1.68 (m, 1H, β-R3), 1.73 (s, 3H), 2.07 (s, 3H), 2.14 (m, 2H), 2.18 (m, 2H), 3.13 (m, 2H, δ-R5), 3.28 (d, J = 6.7 Hz, 2H), 3.68 (s, 3H), 4.06 (m, 1H, α-R2), 5.11 (t, J = 6.3 Hz, 1H), 5.23 (s, 2H), 7.86 (d, J = 7.3 Hz, 1H, R-NH), 8.52 (s, 1H, COOH);

^{13}C NMR (400 MHz, DMSO-d_6) δ ppm: 11.48, 16.46, 21.65, 22.96 (β-R3), 29.23 (γ-R4), 34.53, 35.51, 40.57 (δ-R5), 52.71 (α-R2), 61.03, 69.00, 107.41, 122.97, 123.42, 134.17, 146.22, 159.74, 163.09, 170.91, 172.08, 172.59;

MS: m/z calculated for $C_{23}H_{31}N_5O_9$ 521.52; found 522.47 [M+H]$^+$.

Rf = 0.22 (D).

100% pure by HPLC.

4.1.13. N-mycophenoyl-D-arginine 23

MPA-D-Arg(NO$_2$)OH (23): ^{1}H NMR (DMSO-d_6) δ ppm: 1.48 (m, 3H, γ-R4, β-R3) 1.62 (m, 1H, β-R3), 1.72 (s, 3H), 2.04 (s, 3H), 2.10 (m, 2H), 2.15 (m, 2H), 3.12 (m, 2H, δ-R5), 3.25 (d, J = 6.3 Hz, 2H), 3.66 (s, 3H), 3.96 (q, J = 13 Hz, 1H, α-R2), 5.06 (t, J = 6.4 Hz, 1H), 5.20 (s, 2H), 8.20 (d, J = 8.17 Hz, 1H, R-NH);

^{13}C NMR (DMSO-d_6) δ ppm: 11.48, 16.46, 21.65, 22.96 (β-R3), 29.23 (γ-R4), 34.53, 35.51, 40.57 (δ-R5), 52.71 (α-R2), 61.03, 69.00, 107.41, 122.97, 123.42, 134.17, 146.22, 159.74, 163.09, 170.91, 172.08, 172.59;

MS: m/z calculated for $C_{23}H_{31}N_5O_9$ 521.52; found 522.39 [M+H]$^+$.

Rf = 0.195 (D).

99.06% pure by HPLC.

4.2. General procedure for the preparation tuftsin/retro-tuftsin derivatives 24, 27 and 28

The procedure for the synthesis of compounds **24, 27 and 28** by the mixed anhydride method has been published previously [31–37]. The Boc-protecting groups was removed by treatment with TFA.

4.3. General procedure for the preparation MPA-Thr-Lys(FmocβAla)-Pro-Arg(NO$_2$)-ThrOCH$_3$ 25

Peptide (0.055 mmol), MPA (0.05 mmol) and TEA (0.026 mmol) were dissolved in 1 ml of anhydrous DMF. The solution was then cooled to 0 °C in an ice bath, followed by the addition of (0.103 mmol) of T3P in 50% DMF. The reaction mixture was stirred in a spherical flask filled with inert gas (nitrogen) at 0 °C for 2 h and then at room temperature for 48 h. The progress of the reaction was controlled by TLC plates. After completion of the synthesis, the solvent was distilled off under reduced pressure using a vacuum pump. The product was purified using preparative thin-layer chromatography.

4.3.1. MPA-Thr-Lys(FmocβAla)-Pro-Arg(NO$_2$)-ThrOCH$_3$ 25

MS m/z calculated for $C_{58}H_{76}N_{10}O_{16}$ 1155.25; found 1155.54 M$^+$.

Rf = 0.5 (C).

4.4. General procedure for the preparation MPA-Thr-Lys(βAla)-Pro-Arg(NO$_2$)-ThrOCH$_3$ 26

Compound **25** (0.0286 mmol) was treated with 1 ml of chloroform followed by the addition of 0.2−0.3 ml of diethylamine (DEA). The deprotection reaction was carried out at room temperature for 12−24 h and controlled by TLC. Then, solvent was distilled off under reduced pressure using a vacuum pump. The product was purified using preparative thin-layer chromatography.

4.4.1. MPA-Thr-Lys(βAla)-Pro-Arg(NO₂)-ThrOCH₃ 26

MPA-T-βAla (26): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 0.97 (d, $J = 6.3$ Hz, 3H, γ-T4), 1.33 (m, 2H, γ-K4), 1.38 (m, 3H, δ-K, β-K3), 1.47 (m, 2H, γ-R4) 1.55 (m, 1H, β-R3), 1.63 (m, 1H, β-K3), 1.71 (m, 1H, β-R3), 1.74 (s, 3H), 1.78 (m, 1H, β-P3), 1.88 (m, 2H, γ-P4, γ-P4), 2.04 (m, 1H, β-P3), 2.08 (s, 3H), 2.16 (m, 2H), 2.27 (m, 2H), 2.46 (m, 2H, α-βA2), 2.89 (q, $J = 7.3$ Hz, 2H, β-βA3), 3.02 (m, 2H, ε-K6), 3.15 (m, 2H, δ-R5), 3.30 (d, $J = 7.0$ Hz, 2H), 3.5 (m, 1H, δ-P5), 3.65 (s, 3H, COOCH₃), 3.65 (m, 1H, δ-P5), 3.70 (s, 3H), 3.91 (m, 1H, β-T3), 4.18 (m, 2H, α-T2, α-R2), 4.35 (m, 1H, α-P2), 4.47 (m, 1H, α-K2), 5.13 (t, $J = 6.4$ Hz, 1H), 5.25 (s, 2H), 7.79 (m, 2H, α-KNH, α-TNH), 78.10 (m, 1H, ε-KNH), 8.33 (d, $J = 7.2$ Hz, 1H, α-RNH);

^{13}C NMR (400 MHz,CD₃OD-d_4) δ ppm: 10.07, 14.95, 18.82 (γ-T4), 22.18 (γ-K4), 22.24, 24.81 (γ-P4), 28.21 (γ-R4), 28.45 (β-R3), 29.03 (δ-K5), 30.46 (β-P3), 31.39 (β-K3), 34.33, 35.16, 35.88 (α-βA2), 38.54 (β-βA3), 40.18 (ε-K6), 42.15 (δ-R5), 46.92 (δ-P5), 48.48 (α-R2, α-K2), 51.49 (OCH₃), 58.63 (α-T2), 60.08 (α-P2), 60.22, 67.10 (β-T3), 69.47, 106.33, 116.53, 122.24, 123.01, 133.82, 145.28, 153.28, 163.44, 170.77 (T1), 171.13 (K1),171.25 (βA1), 172.15, 172.41 (R1), 173.04 (P1), 174.67;

MS m/z calculated for C₄₂H₆₄N₁₀O₁₄ 933.02; found 933.47 M⁺.
Rf = 0.37 (D).
94.8% pure by HPLC.

4.5. General procedure for the preparation peptide analogue of MPA and retro-tuftsin 29, 30

Peptide **27, 28** (0.0289 mmol), MPA (0.0212 mmol) and DMAP (0.0289 mmol) were dissolved in 1 ml of anhydrous DMF. The solution was then cooled to 0 °C in an ice bath, then of EDCI was added. The whole was stirred with magnetic stirrer in a spherical flask under inert gas (nitrogen) at 0 °C for 2 h and then at room temperature for 48 h. The progress of the reaction was controlled by TLC plates. After completion of the synthesis, the solvent was distilled off under reduced pressure using a vacuum pump. The product was purified using preparative thin-layer chromatography.

4.5.1. MPA-Arg(NO₂)-Pro-Lys(FmocGly)-ThrOCH₃ 29

MS m/z calculated for C₅₆H₇₂N₁₀O₁₆ 1141.23; found 1141.65 M⁺.
Rf = 0.474 (C).

4.5.2. MPA-Arg(NO₂)-Pro-Lys(FmocβAla)-ThrOCH₃ 30

MS m/z calculated for C₅₈H₇₆N₁₀O₁₆ 1155.25; found 1155.58 M⁺.
Rf = 0.2 (D).

4.6. Synthesis of peptide analogue of MPA and retro-tuftsin 31, 32

Compounds **29, 30** (0.017 mmol) was treated with 1 mL of chloroform followed by the addition of 0.2−0.3 mL of diethylamine (DEA). The deprotection reaction was controlled by TLC plates. The solvent was distilled off under reduced pressure using a vacuum pump. The product was purified using preparative thin-layer chromatography.

4.6.1. MPA-Arg(NO₂)-Pro-Lys(Gly)-ThrOCH₃ 31

MPA-RT-Gly (31): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.06 (d, $J = 6.6$ Hz, 3H, γ-T4), 1.29 (m, 2H, γ-K4), 1.38 (m, 3H, δ-K5, β-R3), 1.46 (m, 3H, β-K3, γ-R4), 1.65 (m, 2H, β-K3, β-R3), 1.73 (s, 3H), 1.81 (m, 2H, γ-P4, β-P3), 1.88 (m, 1H, γ-P4), 1.98 (m, 1H, β-P3), 2.06 (s, 3H), 2.13 (m, 2H), 2.19 (m, 2H), 3.07 (m, 2H, ε-K6), 3.11 (m, 2H, δ-R5), 3.26 (d, $J = 6.7$ Hz, 2H), 3.36 (m, 2H, α-G2), 3.51 (m, 1H, δ-P5), 3.60 (m, 1H, δ-P5), 3.63 (s, 3H, COOCH₃), 3.68 (s, 3H), 4.10 (m, 1H, β-T3), 4.26 (m, 1H, α-T2), 4.28 (m, 1H, α-K2), 4.34 (m, 2H, α-P2), 4.45 (m, 1H, α-R2), 5.12 (t, $J = 6.5$ Hz, 1H), 5.21 (s, 2H), 7.82 (d, $J = 8.4$ Hz, 1H, α-TNH), 7.97 (m, 3H, α-RNH, α-KNH, ε-KNH);

^{13}C NMR (400 MHz,CD₃OD-d_4) δ ppm: 10.04, 14.99, 18.97 (γ-T4), 22.59, 24.65 (γ-K4), 28.43 (γ-P4), 29.22 (γ-R4), 31.05 (β-R3), 33.94 (δ-K5), 35.05 (β-P3), 38.65 (β-K3), 40.53, 42.17, 42.78 (ε-K6), 48.47 (δ-R5), 50.77 (δ-P5), 51,47 (α-R2), 53.28 (OCH₃), 56.93 (α-K2), 57.81 (α-T2), 60.11 (α-P2), 60.15, 66.99 (β-T3), 69.33, 106.38, 122.41, 123.16, 133.57, 145.29, 159.56, 163.41, 171, 171.13 (T1), 172.56 (P1), 172.97 (K1), 173.25 (R1), 174.23 (G1);

MS m/z calculated for C₄₁H₆₂N₁₀O₁₄ 918.99; found 919.45 M⁺.
Rf = 0.454 (C).
86% pure by HPLC.

4.6.2. MPA-Arg(NO₂)-Pro-Lys(βAla)-ThrOCH₃ 32

MPA-RT-βAla (32): ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm: 1.04 (d, $J = 7.0$ Hz, 3H, γ-T4), 1.27 (m, 2H, γ-K4), 1.38 (m, 3H, δ-K5, β-R3), 1.548 (m, 3H, β-K3, γ-R4), 1.65 (m, 2H, β-K3, β-R3), 1.73 (s, 3H), 1.82 (m, 2H, γ-P4, β-P3), 1.89 (m, 1H, γ-P4), 2.00 (m, 1H, β-P3), 2.07 (s, 3H), 2.10 (m, 2H), 2.16 (m, 2H), 2.44 (t, $J = 6.8$ Hz, 2H, α-βA2), 2.95 (t, $J = 5.3$ Hz, 2H, β-βA3), 3.03 (m, 2H, ε-K6), 3.13 (m, 2H, δ-R5), 3.27 (d, $J = 6.8$ Hz, 2H), 3.52 (m, 1H, δ-P5b), 3.615 (m, 1H, δ-P5a), 3.623 (s, 3H, COOCH₃), 3.686 (s, 3H, c), 4.102 (m, 1H, β-T3), 4.25 (m, 1H, α-T2), 4.28 (m, 1H, α-K2), 4.34 (m, 2H, α-P2), 4.44 (m, 1H, α-R2), 5.11 (t, $J = 6.9$ Hz, 1H), 5.22 (s, 2H), 7.85 (d, $J = 8.4$ Hz, 1H, α-TNH), 8.04 (m, 3H, α-RNH, α-KNH, ε-KNH);

^{13}C NMR (400 MHz,CD₃OD-d_4) δ ppm: 10.06, 14.98, 18.98 (γ-T4), 22.31, 24.66 (γ-K4), 28.30 (γ-P4), 29.22 (γ-R4), 31.00 (β-R3), 31.96 (β-βA3), 33.95 (δ-K5), 35.06 (β-P3), 36.06 (α-A2), 38.66 (β-K3), 40.56, 42.151, 46.868 (ε-K6), 48.33 (δ-R5), 50.85 (δ-P5), 51.80 (α-R2), 53.29 (OCH₃), 56.93 (α-K2), 57.81 (α-T2), 60.18, 67.01 (β-T3), 69.37, 106.37, 116.03, 122.37, 123.12, 133.63, 145.31, 159.56, 163.42, 170.96, 170.99 (T1), 172.51 (P1), 172.98, 173.26 (K1), 174.24 (R1, βA1);

MS m/z calculated for C₄₂H₆₄N₁₀O₁₄ 933.02; found 933.47 M⁺.
Rf = 0.3 (D).
100% pure by HPLC.

4.7. Microbiological activity evaluation

4.7.1. Bacterial strains

Susceptibility testing was performed for five reference strains: *Klebsiella pneumoniae* ATCC 700603 (ESBL), *Escherichia* coli ATCC 8739, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* MRSA ATCC 43300, *Staphylococcus aureus* MSSA ATCC 25923. All bacteria were routinely passaged and cultured in MHB liquid medium and MHA solid medium.

4.7.2. Preparing the compounds for testing

At the beginning of the experiment the effect of solvents DMSO ((at concentrations ranging from 0.01% to 12.5%) on bacterial cells viability was investigated by measurement OD₆₀₀ and by plating on a solid medium. The bacterial growth after 18−20 h incubation was compared to control strain without DMSO. No impact of DMSO on bacterial cells was determined at concentration below 6.0%. The research was done in triplicate. The stock solution for conjugates of mycophenolic acid was prepared in 6% DMSO. Serial dilutions were made with water and the growth medium to a final concentration of 3000 µg/l. The solutions were frozen and thawed only once and then discarded.

4.7.3. Determination of minimal-inhibitory concentrations (MICs)

The screening sensitive bacteria to MPA derivatives was performed with MICs tests. MICs were determined by the micro-dilution method in Mueller Hinton Broth II (MHB II) on 96-well polystyrene microtiterplates according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations [48,49].

The cell bacterial cultures from the logarithmic growth phase

were diluted in a medium at a ratio 1: 1000) and them suspension was diluted 50-fold in saline to received McFarland 0.5 scale.

On 96-well titration plates 100 μl of MHB II medium was applied. Then, 100 μl of the compound solution was added to each of the first wells in each column and *serial double dilutions were made*. In case of compounds **11, 12, 14, 15, 17** and **26, 31, 32** were in range from 256.0 μg/ml to 0.125 μg/ml in case of compound **21** were in the range from 3000.0 μg/ml to 5.9 μg/ml. Two conventional antibiotics: ampicillin (*Sigma*) or kanamycin (*Sigma*) was used as a control. For ampicillin the range of concentration was from 256.0 to 0.125 μg/ml, for kanamycin from 1000.0 to 2.0 μg/ml. Finally, 100 μl of suitable bacterial suspension was inoculated to each well to achieve 10^4 cells/ml. Inoculated plates were incubated at 35 ± 1 °C for 18−20 h and MICs were recorded. Evaluation was performed spectrophotometrically (*PlateReader AF2200 UV/VIS and Fluorescence Microplate Reader from Eppendorf*) OD$_{600}$ and visually.

MICs determined by spectrophotometric techniques were defined as the smallest concentration of the compound above which inhibition of bacterial cell growth was expressed as MIC50 and MIC90 (50% and 90%, respectively, of bacterial growth arrest).

For confirmation results the resazurin −based assay was also used. Resazurin is an oxidation-reduction indicator used for the evaluation of cell growth [45,49] - pink or uncolored indicates growth and blue inhibition of growth.

Resinazurin (10 μl of 0.015% aqueous solution) was added to each well. Samples were incubated at 37 °C for 2 h (in the dark) and measurements of absorbance and visual evaluation of color according to control were made.

The lowest concentration at which color change occurred is taken as the MIC value. MBC value was not possible to determine in each case.

4.7.4. Statistical analysis of results

MIC values were determined in 3 replicates in 3 independent experiments. Data were collected and statistically tested (ANOVA parametric test and Kruskal-Willis nonparametric test). Statistical work has been done using RStudio and using the Microsoft Excel 2010 in all calculations, assuming p < 0.05 as statistically significant.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejmech.2017.11.094.

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