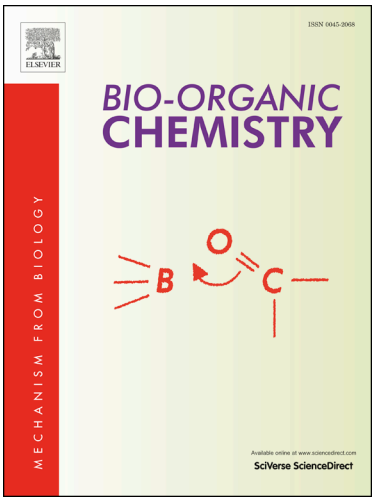


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Milos Jovanovic, Jelena Radivojevic, Kevin O'Connor, Stevan Blagojevic, Biljana Begovic, Vera Lukic, Jasmina Nikodinovic-Runic, Vladimir Savic

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# Rhamnolipid inspired lipopeptides effective in preventing adhesion and biofilm formation of *Candida albicans*

Milos Jovanovic[a], Jelena Radivojevic[b], Kevin O'Connor[c], Stevan Blagojevic[d], Biljana Begovic[d], Vera Lukic[e], Jasmina Nikodinovic-Runic[b], Vladimir Savic[a]

[a] University of Belgrade, Faculty of Pharmacy, Department of Organic Chemistry, Vojvode Stepe 450, Belgrade, Serbia

[b] University of Belgrade, Institute of Molecular Genetics and Genetic Engineering, Vojvode Stepe 444 a, Belgrade, Serbia

[c] UCD School of Biomolecular and Biomedical Science, University College Dublin, Belfield, Dublin 4, Ireland

[d] University of Belgrade, Institute of General and Physical Chemistry, Studentski trg 12/V, Belgrade, Serbia

[e] University of Belgrade, School of Medicine, Institute of Forensic Medicine, Deligradska 31 a, Belgrade, Serbia

e-mail adresses

Milos Jovanovic - mjovanovic@pharmacy.bg.ac.rs

Jelena Radivojevic - jelenaradivojevic@imgge.bg.ac.rs

Kevin O'Connor - Kevin.oconnor@ucd.ie

Stevan Blagojevic - stevan.blagojevic@gmail.com

Biljana Begovic - bbegovic63@gmail.com

Vera Lukic - vera_lukic2006@yahoo.com

Jasmina Nikodinovic-Runic - jasmina.nikodinovic@imgge.bg.ac.rs

Vladimir Savic - vladimir.savic@pharmacy.bg.ac.rs

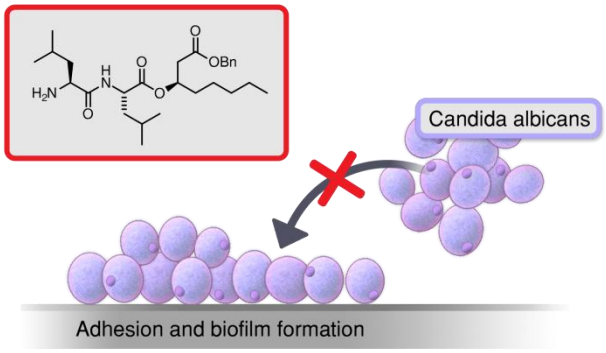
Corresponding Authors

Jasmina Nikodinovic-Runic - jasmina.nikodinovic@imgge.bg.ac.rs

Vladimir Savic- vladimir.savic@pharmacy.bg.ac.rs

Electronic supplementary information (ESI) available: Experimental procedures, structural data and copy of $^{1}H$ and $^{13}C$ NMR spectra of final products.

## Graphical abstract




## Abstract

Rhamnolipids are biodegradable low toxic biosurfactants which exert antimicrobial and anti-biofilm properties. They have attracted much attention recently due to potential applications in areas of bioremediation, therapeutics, cosmetics and agriculture, however, the full potential of these versatile molecules is yet to be explored. Based on the facts that many naturally occurring lipopeptides are potent antimicrobials, our study aimed to explore the potential of replacing rhamnose in rhamnolipids with amino acids thus creating lipopeptides that would mimic or enhance properties of the parent molecule. This would allow not only for more economical and greener production but also, due to the availability of structurally different amino acids, facile manipulation of physico-chemical and biological properties.

Our synthetic efforts produced a library of 43 lipopeptides revealing biologically more potent molecules. The structural changes significantly increased, in particular, anti-biofilm properties against *Candida albicans*, although surface activity of the parent molecule was almost completely abolished. Our findings show that the most active compounds are leucine derivatives of 3-hydroxy acids containing benzylic ester functionality. The SAR study demonstrated the further increase in activity with aliphatic chain elongation. The most promising lipopeptides **15**, **23** and **36** at 12.5 µg/mL concentration allowed only 14.3%, 5.1% and 11.2% of biofilm formation, respectively after 24 h. These compounds inhibit biofilm formation by preventing adhesion of *C. albicans* to abiotic and biotic surfaces.

**Keywords:** adhesion, biofilm, *Candida albicans*, lipopeptides, rhamnolipids.

## 1. Introduction

Rhamnolipids (Figure 1) are environmentally friendly, surface active molecules produced mostly by strains of *Pseudomonas aeruginosa* [1]. They are composed of one or two rhamnose molecules and up to three molecules of 3-hydroxy acids. On rare occasions rhamnose moiety is esterified or the terminal hydroxy acid is methylated [2]. In bacteria rhamnolipids fulfil a number of roles. They assist bacteria in assimilating and metabolising hydrophobic substrates, regulate biofilm formation and are involved in making channels and pores in the structure of mature biofilms thus allowing the inter-bacterial communication and the flow of nutrients [2-5]. As the biofilms mature, rhamnolipids facilitate the dispersion of bacteria from the biofilm [5,6]. Rhamnolipids also have antimicrobial properties against a large variety of Gram-positive and Gram-negative bacteria, primarily due to their ability to penetrate biological membranes [7]. Most of the abovementioned features are a direct result of the remarkable surfactant properties of these molecules. Rhamnolipids can reduce the surface tension of water to 25–30 mN/m, have relatively low critical micellar concentration (between 20 and 225 mg/L) and are effective over a wide range of pH and temperatures [8]. It should be also noted that they are biodegradable and hence potentially environmentally benign natural products considered to be a green alternative to synthetic surfactants [9]. These versatile molecules have a potential for application in pharmaceutical, food, cosmetic industry, bioremediation and in protection of animals and plants against microbes [10-13]. The inefficient production of rhamnolipids in pure form, however, significantly limits wider exploration of the chemical space defined by these molecules and their commercial application [14]. Although several chemical synthetic pathways have been developed the routes are not economical and require, due to nature of reacting components, many equivalents of protecting groups and stoichiometric amount of the glycoside bond forming agent [15-17]. Furthermore, rhamnose is a relatively expensive compound, almost twenty times more expensive than glucose. Synthesis of the other structural component of rhamnolipids, 3-hydroxyalkanoic acid fragment with longer chain, particularly in chiral form, has been reported in the literature but is challenging and suffers from several drawbacks [18]. To resolve these problems, it would be necessary to replace rhamnose with other carbohydrates or to completely redesign the rhamnolipid structure and create *de novo* compounds with preserved overall properties of these molecules. Our tactic to address this setback is based on the latter approach and the results of our research are the subject of this paper.

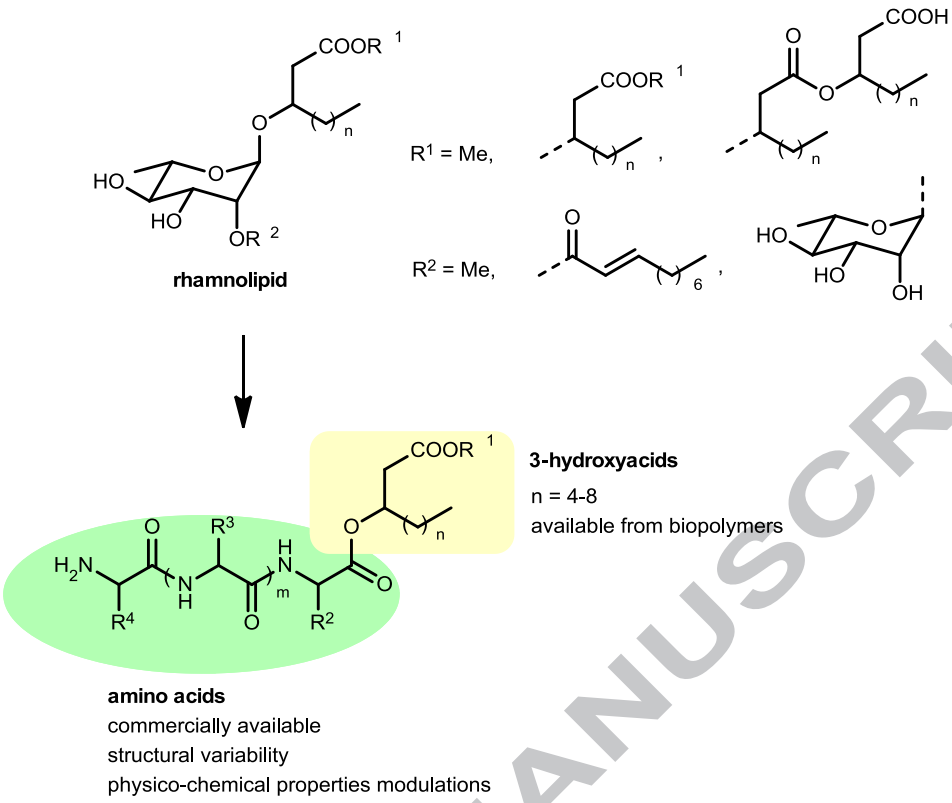



**Figure 1**. Rhamnolipids and structural variants proposed in this study (Please use colour for this figure)

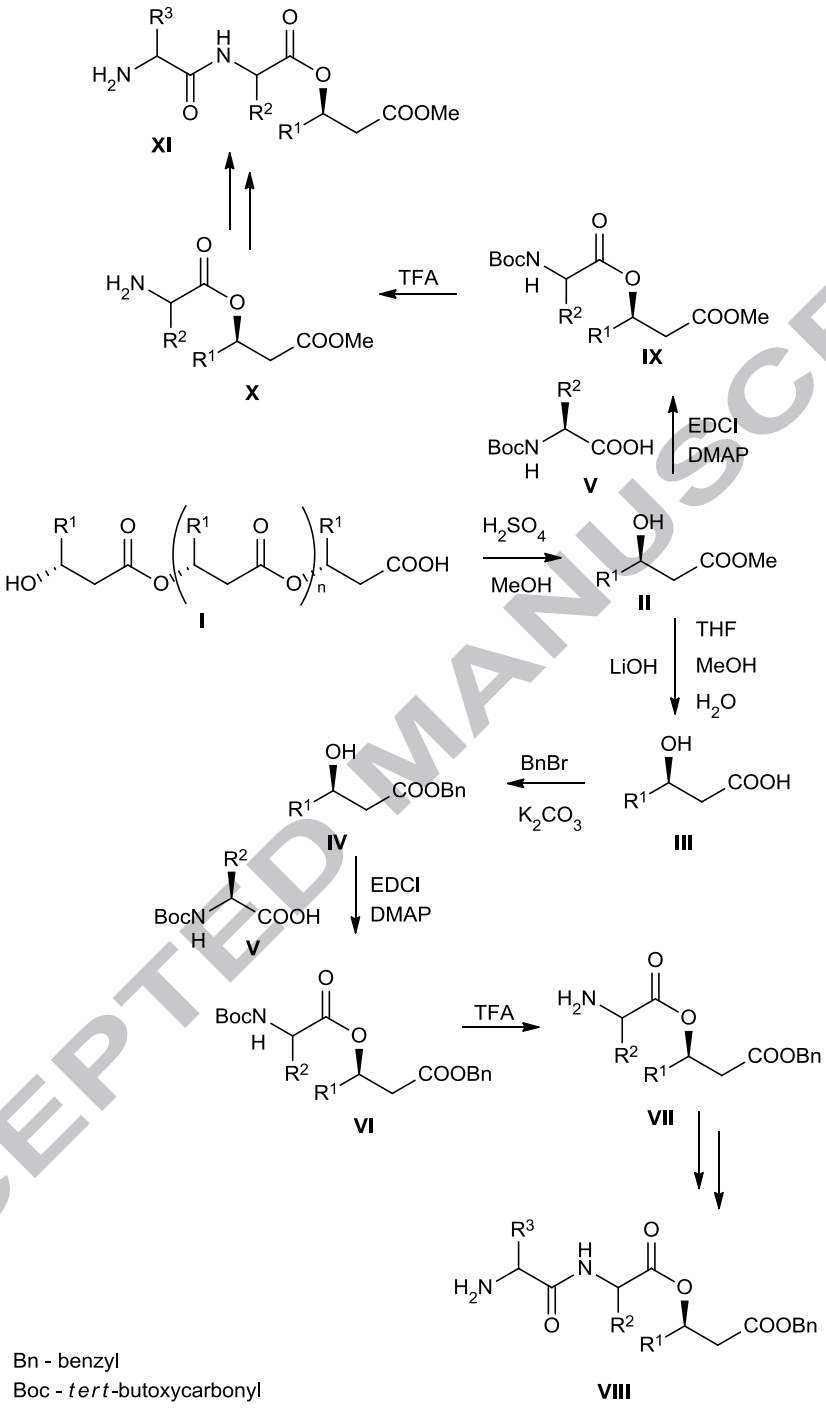
## 2. Results and Discussion

Inspired by rhamnolipids, we embarked on the development of synthetically accessible alternatives which would preserve or improve their biological profile or some of the key features. Having previously briefly explored the carboxylic end of the natural molecule, which revealed the amide derivatives with improved biological profile compared to the parent derivatives, we were particularly interested in replacing the sugar component of rhamnolipids with amino acids and peptides (Figure 1) [19]. This approach finds its support in observation that numerous peptides and lipopeptides have been shown to possess antimicrobial properties [20,21]. We postulated that the availability and structural diversity of amino acids could offer an opportunity to rationally design novel compounds and to enhance the biological activity compared to rhamnolipid derivatives. Our envisaged targets were planned to retain 3-hydroxy acid moiety present in rhamnolipids, which is also frequent structural motif found in numerous biologically active lipopeptides. In addition, 3-hydroxy acid building blocks are readily available from polyhydroxyalkanoate (PHA) biopolymers [22,23].

### *2.1. Variation of Amino Acids*

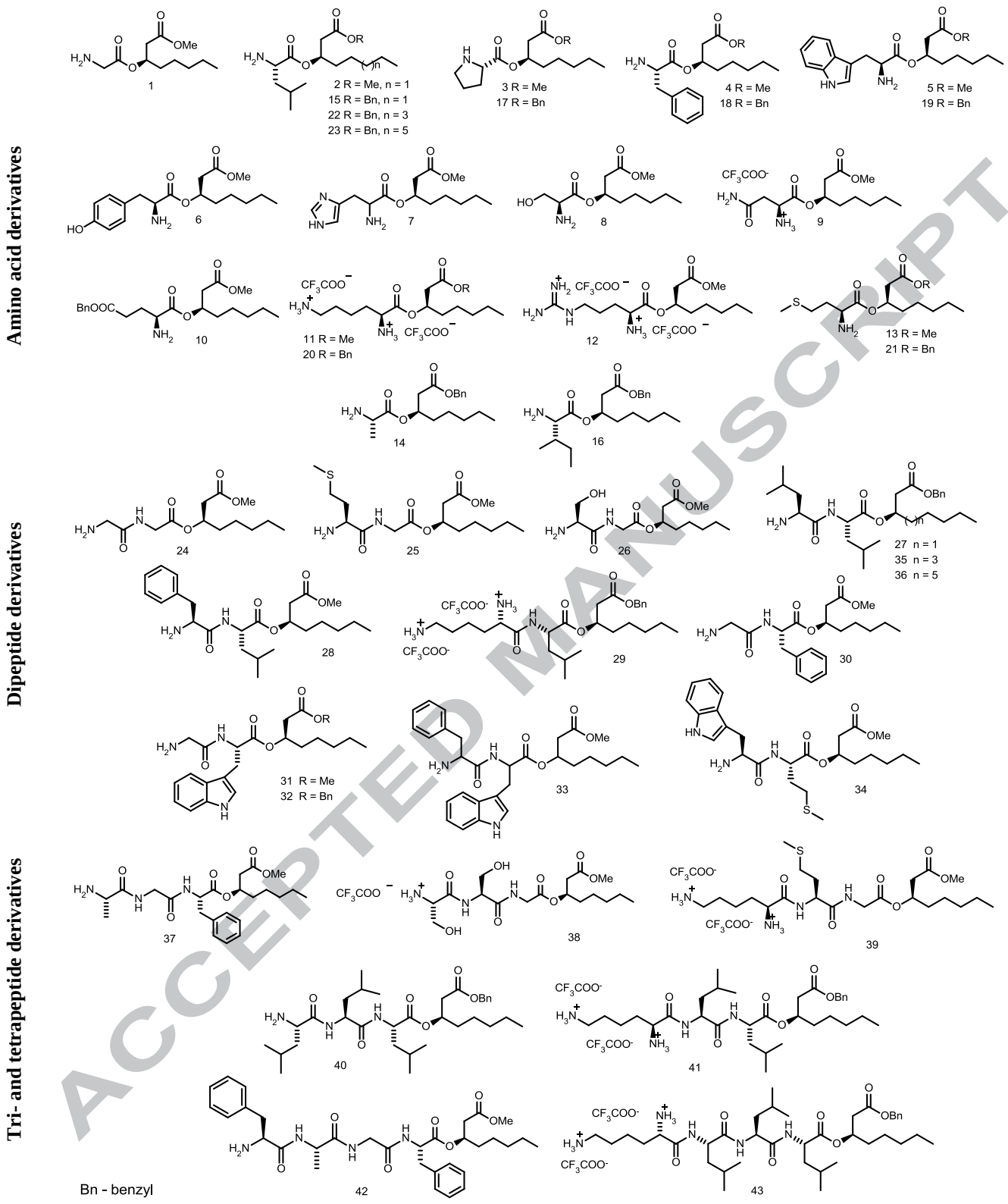
Synthesis of amino acid derivatives of rhamnolipids is outlined in Scheme 1. Prerequisite $C_8$-β-hydroxy acid ester **II**, as a starting point of the envisaged synthetic route, was prepared by methanolysis of bacterial polyhydroxylalkanoates (PHA) polymer **I** [22]. The use of PHA for monomer synthesis offers the opportunity to incorporate microbial production in the preparation of structural components, and, thus, leads towards greener production process. Methyl ester **II** was also used for the synthesis of benzyl ester **IV** utilising routine synthetic procedures. The initial set of mono-amino acid derivatives was prepared using $C_8$-β-hydroxyesters **II** and **IV** employing EDCI as coupling reagent followed by TFA promoted deprotection to yield product **VII** or **X**. These

compounds were then utilised for the preparation of other di-, tri- and tetrapeptide derivatives. As coupling reagent for these processes HBTU was used under basic conditions and in the presence of hydroxybenzotriazole.




**Scheme 1**. Synthesis of amino acid derivatives of rhamnolipids

The initial set of synthesised amino acid derivatives (**1** – **23**) is outlined in Figure 2. Various amino acids used in the synthetic process were selected to cover all typical physicochemical features represented by these molecules.




**7** was isolated as 9:1 mixture of diastereomers at C-2 of the amino acid (partial isomerisation under reaction conditions)
**22** and **23** were isolated as 1:1 mixture of diastereomers at C-3 of the carboxylic ester (starting 3-hydroxy acid was used as racemate)
**33** was isolated as 2:1 mixture of diastereomers (partial isomerisation under reaction conditions)
**35** and **36** were isolated as 1:1 mixture of diastereomers at C-3 of the carboxylic ester (starting 3-hydroxy acid was used as racemate)

**Figure 2**. Chemical structures of the synthesised and tested compounds

In order to establish antimicrobial properties of the mono-amino acid derivatives the minimum inhibitory concentration (MIC) against planktonic *P. aeruginosa* and *C. albicans* was determined (Table 1). In general, weak activities were observed, without detectable effect on planktonic growth on both pathogens at concentrations as high as 500 μg/mL, with compound **15** (MIC of 50 μg/mL, Table 1) showing the highest anti-*Candida* potential.

**Table 1**. Minimal Inhibitory concentration of tested compounds against *C. albicans* and *P. aeruginosa*

| Derivatives | *C. albicans* MIC (μg/mL) | *P. aeruginosa* MIC (μg/mL) |
|---|---|---|
| amino acid | | |
| **5** | 150 | >500 |
| **14** | 150 | >500 |
| **15** | 50 | >500 |
| **16** | 200 | >500 |
| **17** | 200 | >500 |
| **22** | 200 | >500 |
| **23** | 100 | >500 |
| dipeptide | | |
| **27** | 60 | >500 |
| **28** | 150 | >500 |
| **29** | 100 | 100 |
| **31** | 200 | 500 |
| **32** | 50 | 500 |
| **35** | 200 | >500 |
| **36** | 100 | >500 |
| tripeptide | | |
| **41** | 80 | 100 |
| tetrapeptide | | |
| **43** | 50 | 100 |

We further explored the potential of synthesised mono-amino acid derivatives to inhibit biofilm formation and to disrupt already formed biofilms. Biofilm, both bacterial and fungal, is known as one of the most common causes of chronic infectious diseases [24]. It represents a surface-associated community of microorganisms embedded in a polymeric matrix [25]. These communities are often comprised of various bacterial or fungal species and sometimes even a synergistic combination of both [26]. Amongst fungal species *C. albicans* is the most commonly associated with the biofilm formation. The essential step in the pathogenesis of *C. albicans* is its adhesion to the host cell surface, mediated by cell walls adhesins [27]. Within biofilm organisms grow resistant to antimicrobial therapy rendering most antifungal drugs ineffective. The toxicity of many currently used antifungal drugs further aggravates the issue and emphasizes the need for a new class of molecules that could prevent biofilm formation [28]. Amongst the newly discovered potential antifungal molecules, antimicrobial peptides (AMPs), and their analogues displayed promising results [29-31]. Furthermore, many secondary metabolites including rhamnolipids and sophorolipids, as well as some lipopeptides are known to possess antifungal properties [32-35].

Tests to explore the inhibition of biofilm formation were performed under standard conditions at four different concentrations for both *C. albicans* and *P. aeruginosa*. Tested compounds showed little to no activity against *P. aeruginosa* biofilms and were significantly more potent against *C. albicans* biofilm formation ability. Noteworthy is the fact that these compounds had no ability to disrupt either *Pseudomonas* or *Candida* pre-formed biofilms (results not shown). The results are presented as a percentage of *C. albicans* biofilm formed after 24h of growth in the presence of the tested compound. For the comparison we also performed the same experiments with mono- and di-rhamnolipids (Figure 3A). Amongst the compounds possessing C-8 carboxylic side chain the most promising candidate was **15**. While at low concentration (6.25 μg/mL; 91%) compound **15** performed comparable to mono- and di-rhamnolipids (93%, for both), at slightly higher concentration (12.5 μg/mL) it demonstrated significantly better properties allowing the formation of only 14% of biofilm, in comparison to untreated control. Further increase in concentration (50 μg/mL) reduced biofilm formation to 3.5%, which was expected, as that was also the MIC concentration against *C. albicans* planktonic cells for this compound (Table 1). Very noticeable was the

detrimental effect of the ester group of this compound on its properties. Replacing the benzyl ester in **15** with methyl ester moiety (compound **2**), resulted in very weak activity. One of the reasons for this striking effect might be the outcome of changes in physicochemical properties in particular logP (calculated values: **2** logP 2.95, **15** logP 4.68)[36]. Given that cell surface hydrophobicity and hydrophobic interactions of mannoproteins play a pivotal role in *C. albicans* adhesion on epithelia and plastic surfaces it is likely that more lipophilic compound would interact more competitively with *C. albicans* cell wall proteins thus inhibiting their interaction with the host or plastic surface [37,38]. The other reason for reduction in the activity of methyl ester could be its faster degradation by the fungal esterases, as it is more likely that the sterically less demanding ester functionality is more prone to hydrolysis by these enzymes [39].

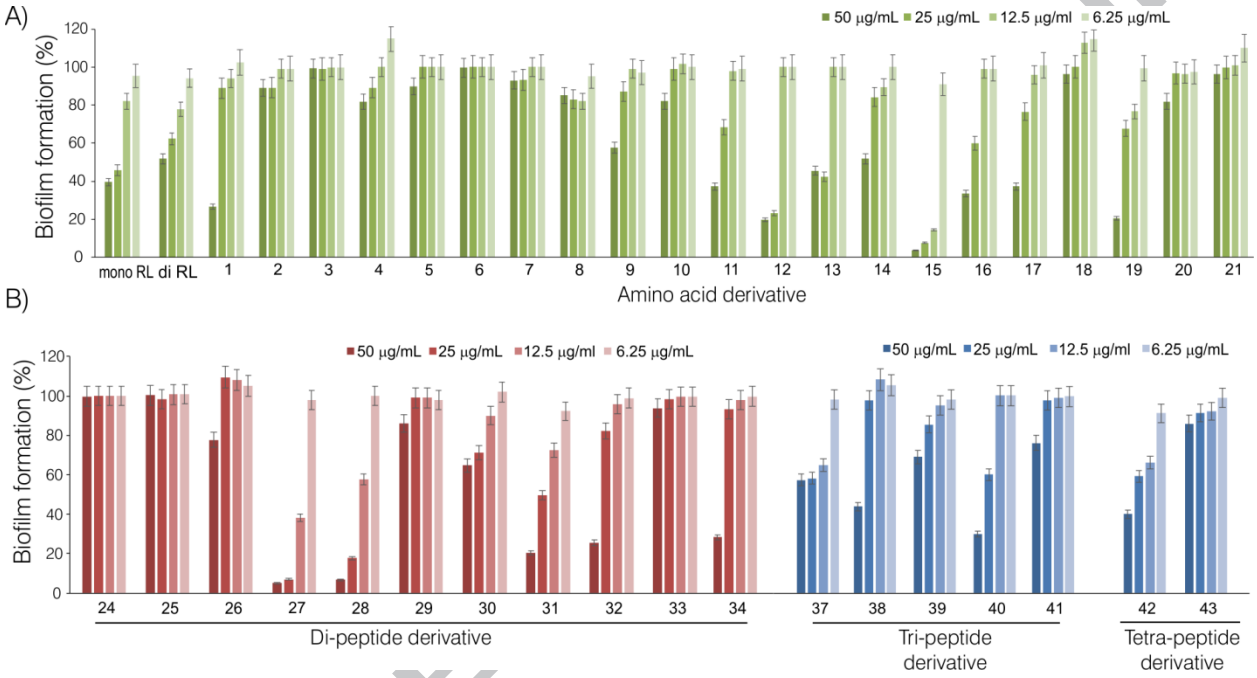



**Figure 3.** Inhibition of Biofilm Formation in *Candida albicans* in the presence of amino acid (A) and peptide derivatives (B). (Please use colour for this figure)

Based on the observed results, we explored the potential to influence the properties of these compounds further by introducing additional amino acids. Therefore, a small set of dipeptide derivatives was synthesised as outlined in Figure 2. To establish antifungal potential MICs were determined but as in case of mono amino acid derivatives only moderate activity was observed against *C. albicans* and almost no or very weak activity against *P. aeruginosa* (Table 1). Anti-biofilm studies of peptide derivatives produced some interesting results (Figure 3B). Amongst the most active C-8 carboxylic derivatives were compounds **27** and **28**, both possessing non-polar amino acids as structural components. They showed better activity than mono- and di-rhamnolipids, in particular at 25 µg/mL (**27** - 7% biofilm formation, **28** - 18% biofilm formation) and at 50 µg/mL concentrations (**27** - 5% biofilm formation, **28** - 7% biofilm formation) while **27** was significantly more active even at 12.5 µg/mL (**27** - 18% biofilm formation).

Finally, we made additional tri- and tetrapeptide derivatives as outlined in Figure 2. While some improvements were observed MIC values confirmed a general trend of weak activity against planktonic *C. albicans* and *P. aeruginosa* (Table 1). Interestingly, contrary to the previously discussed derivatives, compounds possessing non-polar internal and polar terminal amino acids (**41**, **43**) showed better potency against planktonic organisms than compounds comprised only of nonpolar amino acids. Increasing the peptide length in general had a negative effect on the anti-biofilm potency of the compounds. Only on rare occasions and at the highest concentration did the

synthesised tri- and tetrapeptide derivatives perform better than mono- and di-rhamnolipids (i.e leucine derivative **40**).

Overall the majority of the most active compounds were benzyl esters with a noticeable predominance of non-polar amino acid residues such as leucine, phenylalanine and methionine. In all the active compounds except **12**, the presence of leucine residue and phenyl group whether in the form of benzyl ester or as a phenyl group in phenylalanine was conspicuous. On the other hand, only a slight change in the structure of **15** (leucine derivative) resulted in almost complete loss of activity (**16** isoleucine derivative). Some of the abovementioned structural features are also reported in the recent work of Pierce et al. [40]. Screening the library of 20 000 small molecules the group suggested that the most potent molecules for inhibition of biofilm formation were derivatives of diazaspiro-decane which contained *para*-isopropyl benzyl side chain on the piperidine nitrogen. These results imply that the overall physicochemical properties are not the only factor influencing the activity of these compounds. Specific structural motifs may play an important role as well.

*2.2. Variations of Chain Length of Carboxylic Acids*

A further extension of the initial structure activity relationship (SAR) of the rhamnolipid inspired amino acid derivatives focused on the carboxylic moiety. As discussed above, the initial set of compounds was based on C-8 carboxylic derivative **II** prepared from the depolymerisation of PHA polymer to produce a single enantiomer. To explore the influence of this structural fragment on the biological profile of the peptide derivatives a small series of C-10 and C-12 congeners was synthesised and explored. The C-10 and C-12 carboxylic derivatives were prepared as previously described and were used as racemic mixtures [41]. Replacing the C-8 fragment of the most active compounds **15** and **27** with C-10 or C-12 units afforded derivatives **22**/**23** and **35**/**36** respectively. Extending the carboxylic chain of both mono- amino acid and dipeptide derivatives did not have a beneficial effect on the activity against planktonic *C. albicans or P. aeruginosa* (Table 2). On the other hand, anti-biofilm properties improved further for mono-amino acid derivatives **22** and **23** compared to the parent C-8 analogue **15** at all studied concentrations (Table 2). The same trend was observed for dipeptide derivatives **35** and **36** (Table 2). It is also worth noting that all four compounds, **22**/**23** and **35**/**36**, showed better anti-biofilm properties than both mono- and di-rhamnolipids at all studied concentrations. All C-10 and C-12 compounds, **22** (logP 5.57), **23** (logP 6.46), **35** (logP 6.3) and **36** (logP 7.2), have higher calculated logP than mono-rhamnolipid (logP 3.8) and di-rhamnolipid (logP 3.9) used for comparison. This supported our previous observation suggesting that anti-biofilm potential is likely to be influenced, at least in part, by the lipophilicity.

**Table 2**. Effects of the selected amino acid derivatives on biofilm formation of *C. albicans*, their cytotoxic effect given as $IC_{50}$ value on healthy human fibroblasts (MRC5 cell line) and physicochemical parameters.

| Compound | C-length | Biofilm formation Concentration (μg/mL) | | | | MIC[a] (μg/mL) | $BIC_{50}$[b] (μg/mL) | $IC_{50}$[c] (μg/mL) | LogP |
|---|---|---|---|---|---|---|---|---|---|
| | | 6.25 | 12.5 | 25 | 50 | | | | |
| **15** | C8 | 91.1 | 14.3 | 7.5 | 3.4 | 50 | 8 | >100 | 4.68 |
| **22** | C10 | 56.2 | 10.8 | 4.7 | 3.1 | 200 | 6 | 20 | 5.57 |
| **23** | C12 | 40.3 | 5.1 | 4.2 | 2.1 | 100 | 4.5 | 15 | 6.46 |
| **27** | C8 | 98.2 | 38.4 | 7.1 | 4.9 | 60 | 10 | >100 | 5.40 |
| **35** | C10 | 63.2 | 39.8 | 6.1 | 5.6 | 200 | 6.5 | 12 | 6.29 |
| **36** | C12 | 42.7 | 11.2 | 8.8 | 7.2 | 100 | 4.5 | 8 | 7.18 |

[a]Minimum inhibitory concentration

[b]Concentration causing 50% biofilm inhibition. Results represent mean of two independant experiments done in hexaplicate, with standard deviation between 1-5%.

[c]Concentration causing 50% cell killing under treatment of 48 h. Results represent mean of two independant experiments done in quadriplicate, with standard deviation between 1-5%

Selected derivatives were found to be effective against *C. albicans* biofilm formation at concentrations much lower than the ones needed to affect fungal growth (Table 2). This emphasises the importance of the anti-biofilm potential of these compounds as they do not directly target microbial survival but rather act on properties that

influence microbial population as a whole, and as such are unlikely to cause the development of drug resistance in *C. albicans* [42].

*2.3. Other biological and physicochemical properties*

To further evaluate the potential for their medical development, *in-vitro* cytotoxicity of the most potent derivatives against healthy human lung fibroblast cell line (MRC5) has been determined (Table 2). While compounds **15** and **27** showed no cytotoxic effects at the concentrations tested, their C-10 and C-12 analogues **22**/**23** and **35**/**36** killed almost 100% of the cells at concentrations 2-3 fold greater than their biofilm inhibition $BIC_{50}$ values (Table 2). This represents a potentially limiting factor in further exploration and development of C-10 and C-12 derivatives for biomedical applications. On the other hand, the low cytotoxicity and potent *Candida* anti-biofilm activity of **15** and **27** prompted further exploration. Using GFP and RFP reporter strains of *C. albicans* SC5314 we observed that both derivatives successfully prevented biofilm formation (Figure 4A) as well as adhesion onto A549 cells monolayer (Figure 4B). At these concentrations, biofilms under treatment appeared to contain predominantly the round yeast form of *Candida* in comparison to controls where an obvious network of elongated mycelia was observed (Figure 4A). Surprisingly, **15** and **27** did not prevent hyphal formation nor reduced hyphal length during the cell adhesion process (Figure 4B), which was also observed *in-vitro* on Spider medium (Supporting Figure 1S). Taken together, these results indicate that rhamnolipid derivatives act by effectively preventing adhesion of *C. albicans* to abiotic and biotic surfaces.

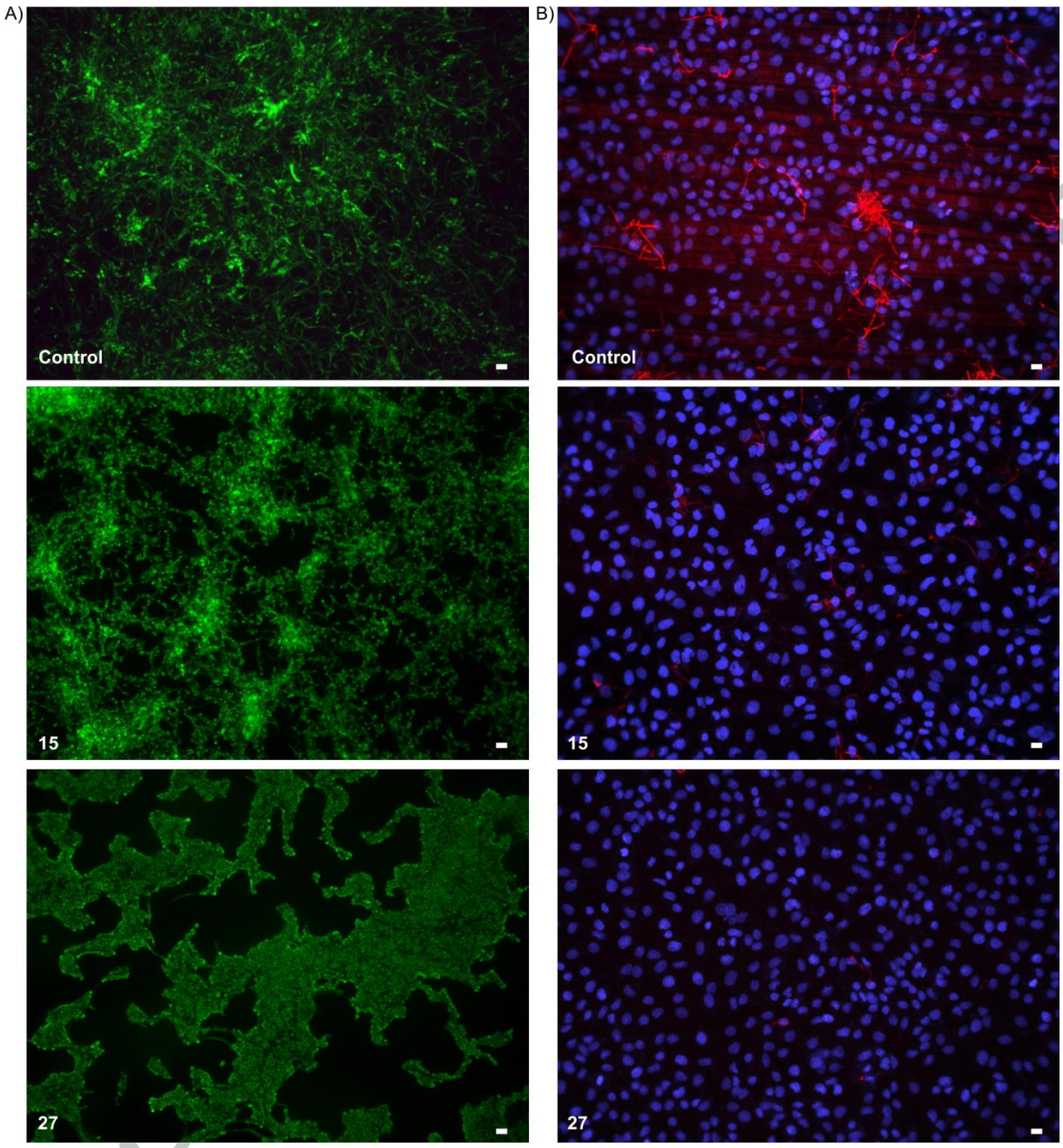



**Figure 4**. *C. albicans* SC5314 (GFP) biofilm formation in the presence of **15** and **27** at 12.5 µg/mL (A). Adhesion of *C. albicans* SC5314 (RFP) on A549 cell monolayer (stained with DAPI) in the presence of **15** and **27** at 25 µg/mL (B). Control represents 0.1% DMSO as vehicle solvent control and scale bars represent 10 µm. (Please use colour for this figure)

Apart from antimicrobial properties, main features of rhamnolipids also include, surface-active properties and biodegradability. We selected several representative molecules (amino acid derivative **11**, dipeptide derivative **26**, tripeptide derivative **40**, and tetrapeptide derivative **42**) in order to explore the influence of replacement of the sugar component with amino acids on the abovementioned features. Results presented in table 3 and supporting table 1S suggest that regardless of polarity or number of amino acids in peptide chain the compounds behave in a simillar manner. Overall the replacement of rhamnose almost completely negated the surface active properties as the critical micellar concentrations (CMC) were significantly greater than those of rhamnolipids (Table 3). Surface

tension is existent but at this concentration (0.1%) it is rather low. On the other hand the biodegradable properties of the parent molecule were retained as the set of studied compounds was almost completely biodegraded over the course of 25 days.

**Table 3**. Critical micelle concentration, surface tension at 23.0 °C, 36.5°C and foam index of selected compounds in comparison with commercially available rhamnolipid mixture Rha-90.

| Compound | CMC (mg/L) 23˚C | γ (mN/m)23˚C 0.1% | γ (mN/m) 36.5˚C 0.1% | Foam index 0/3 min |
|---|---|---|---|---|
| Rha-90 | 175 | 45.5 | 43.2 | 3/3 |
| 11 | 390 | 67.6 | 67.5 | 1/0 |
| 26 | 420 | 68.5 | 68.0 | 0/0 |
| 40 | 400 | 68.0 | 68.0 | 1/0 |
| 42 | 315 | 68.2 | 68.0 | 2/0 |

These results imply that contrary to rhamnolipids and other known anti-biofilm biosurfactants such as pseudofactin II, which act by reducing the hydrophobicity of cell surface, the molecules hereby presented may act by a different mechanism [43-45]. The molecules likely do not target nonspecific adhesion factors such as hydrophobicity, as they displayed anti-biofilm properties at concentrations much lower than their CMC. This is also illustrated by the strong dependency of anti-biofilm properties on structural characteristics of the molecules (e.g. **15** and **16**), thus suggesting that the more subtle interactions are at work. In this regard the mechanism is most likely related to the interaction of the molecules with more specific targets such as cell wall proteins, namely adhesins or mannoproteins [37,38,46].

## 3. Materials & Methods

*3.1 Chemistry*

Polyhydroxyalkanoate polymer **I** was purchased from Bioplastech Ltd. (Dublin, Ireland)and hydrolysed as described by de Roo et al. [22], rhamnolipid mixture R90 was purchased from AGAE Technologies and purified as described by Lotfabad et al. [47]. All amino acids and reagents were purchased from Acros Organics (Morris Plains, NJ, United States), unless otherwise stated. The NMR spectra were recorded on a Bruker Ascend 400 (400 MHz) or on a Bruker Avance III (500 MHz) spectrometer. Chemical shifts are given in parts per million (δ) downfield from tetramethylsilane as the internal standard. Deuterochloroform was used as a solvent, unless otherwise stated. Mass spectral data were recorded using an Agilent Technologies 6520 Q-TOF spectrometer coupled with an Agilent 1200 HPLC system or an Agilent Technologies 5975C MS system coupled with an Agilent Technologies 6890 N GC system. Optical rotations were measured on an AUTOPOL IV Automatic Polarimeter Rudolph Research Analytical. $[\alpha]_D$ values are given in deg mL $g^{-1}$ $dm^{-1}$. IR spectra were recorded on an IR Thermo Scientific NICOLET iS10 (4950) spectrometer. Flash chromatography employed silica gel 60 (230–400 mesh) while thin layer chromatography was carried out using alumina plates with a 0.25 mm silica layer (Kieselgel 60 F254, Merck). Compounds were visualized by staining with potassium permanganate solution or Dragendorff's reagent. The solvents were purified by distillation before use. Procedures for the preparation of lipopeptides and the corresponding structural data for the synthesized compounds can be found in the Supporting information.

*3.2. Microbial Strains and Growth Conditions.*

*Pseudomonas aeruginosa* PAO1 NCTC 10332, *Candida albicans* ATCC 1023 and *C. albicans* SC 5314 (ATCC MYA-2876; green fluorescence protein (GFP) and red fluorescence protein (RFP) reporter strains obtained from Prof Bernhard Hube, Department of Microbial Pathogenicity Mechanisms, Leibniz Institute for Natural Product Research and Infection Biology Hans Knoell Institute, Jena, Germany) were used in this study. *P. aeruginosa* was grown in Luria Bertani (LB; 10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.2) while *C. albicans* strains were grown in Sabouraud broth (SAB; 10 g/L peptone, 40 g/L dextrose, pH 5.6) on a rotary shaker at 180 rpm at 37°C.

*3.3. Antimicrobial Susceptibility Tests for Planktonic Cells*

The minimum inhibitory concentration (MIC) of test compounds were determined according to standard broth microdilution assays recommended by the National Committee for Clinical Laboratory Standards (M07-A8 and M27-A3, for bacteria and yeast respectively) [48,49]. Stock solutions of compounds were prepared in DMSO (50 mg/mL). The highest concentration used was 500 µg/mL. The inocula were $10^8$ and $10^5$ colony forming units (cfu)/mL for bacteria and fungi, respectively. MIC values were read after 24 h incubation at 37°C as the lowest concentration to exhibit an absence of growth.

*3.4. Anti-Biofilm Assays*

Biofilm quantification assays were performed in 96-well microtiter plate format using polystyrene flat bottom and round bottom plates for *P. aeruginosa* and *C. albicans*, respectively and a crystal violet (CV) staining of adherent cells. Biofilms were formed in the absence or presence (concentration range 100-1.5 µg/mL) of compounds for 24 h at 37°C for biofilm formation assay. The biofilm disruption ability was tested on developed biofilms in 96-well flat bottom and round bottom plates for *P. aeruginosa* and *C. albicans*, respectively as described previously [50,51]. Briefly, harvested from the overnight grown *P. aeruginosa* and *C. albicans* cells were washed twice in sterile PBS and collected by centrifugation, resuspended in RPMI-1640 with 2% glucose and adjusted to cell density of $1 \times 10^6$ cells/mL using Neubauer haemocytometer. Biofilms were formed using 100 µL of the prepared cell suspensions into wells of a microtiter plate and incubated for 24 h at 37°C. The established biofilms were rinsed with PBS and treated with two-fold serial dilutions of test compounds (concentration range 200-3.1 µg/mL) for 24 h at 37°C. After incubation, biofilms were washed twice with sterile PBS and adherent cells were stained with 0.1% (v/v) CV, the absorbance at 590 nm was read on Tecan Infinite 200 Pro multiplate reader (Tecan Group Ltd., Männedorf, Switzerland). Biofilm formation and disruption assays were performed in six replicates and repeated three times. Biofilm inhibition values were plotted against the log of concentration and a sigmoidal dose response curve was calculated by non-linear regression analysis using Graphpad Prism software version 5.0 for Windows (Graphpad Software, CA, USA). Biofilm inhibition concentrations for selected compounds are expressed as the concentration of the compound inhibiting biofilm by 50% ($BIC_{50}$). Biofilms of *C. albicans* SC5314 (GFP reporter strain) formed in the presence of selected compounds were also examined using fluorescence microscope (Olympus BX51, Applied Imaging Corp., San Jose, USA) at 20 × magnification.

*3.5. C. albicans Yeast to Hyphe Transition Assay*

Morphological changes of *C. albicans* in the presence and absence of selected compounds in subinhibitory concentrations ($MIC_{80;}$ 80% of MIC value determined for the planktonic growth) was observed upon *C. albicans* growth on Spider medium as previously described [52].

*3.6. In-vitro cytotoxicity on Human Fibroblast Cell Line*

Cytotoxicity (anti-proliferative activity) of rhamnolipids and synthetized amino- and peptide- analogues was determined by standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay [53]. MRC5 cells (human lung fibroblast, obtained from the American Type Culture Collection) were plated in a 96-well flat-bottom plate at a concentration of $1 \times 10^4$ cells per well, grown in humidified atmosphere of 95% air and 5%

$CO_2$ at 37 °C, and maintained as monolayer cultures in RPMI-1640 medium supplemented with 100 µg/mL streptomycin, 100 U $mL^{-1}$ penicillin, and 10% (v/v) fetal bovine serum (FBS) (all from Sigma, Munich, Germany). Assay was carried out after 48 h of cell incubation in the media containing test compounds at concentrations ranging from 0.1 to 250 µg/mL, MTT reduction assay was carried out and cell proliferation was determined from the absorbance at 540 nm on Tecan Infinite 200 Pro multiplate reader (Tecan Group, Männedorf, Switzerland). The percentage viability values were plotted against the log of concentration and a sigmoidal dose response curve was calculated by non-linear regression analysis using Graphpad Prism software version 5.0 for Windows (Graphpad Software, CA, USA). Cytotoxicity is expressed as the concentration of the compound inhibiting growth by 50% ($IC_{50}$).

*3.7. Cell Adhesion Assay*

The ability of *C. albicans* SC5314 (RFP reporter strain) to adhere to A549 cells (human epithelial adenocarcinoma, obtained from the American Type Culture Collection) were tested in the presence of selected compounds as previously described by Wachtler B, et al. [54]. *C. albicans* cells were added to monolayer of A549 cells and co-incubated for 1 h in serum-free RPMI-1640 medium (Gibco) in the presence and absence of selected test compounds (at 25 µg/mL) 37°C and 5% $CO_2$. Adherent *C. albicans* cells were visualized with a fluorescence microscope (Olympus BX51, Applied Imaging Corp., San Jose, USA) at 20 × magnification.

*3.8. Statistical Analysis*

Results are expressed as means ± standard deviation (SD). All results were analysed by ANOVA. A P value < 0.05 was considered significant.

*3.9. Conductivity and Surface Tension Measurements*

Conductivity measurements were applied to determine the CMC in various combinations of surfactants. Those measurements were carried out at 23.0 °C with digital conductivity meter SensION 5 (Hach, USA) with the accuracy ± 0.5 %, as well as with the 51975 conductivity probe that uses the 4–ring method. The CMC values at each surfactant formulation composition were determined by using the conventional method (Williams' method) as well as the method proposed by Carpena at al. [55]. Surface tensions of surfactant mixtures were measured by a stalagmometer (Traube stalagmometer Neubert Glass BN–0330–10–208), while surface tension was determined by drop counting method. Surface tension measurements were performed at 23.0 °C.

*3.10. Testing of Biodegradability (Closed Bottle Test)*

The Closed Bottle-Test for ”ultimate biodegradability” determination of dishwashing liquid surfactant mixtures in an aqueous medium primarily was established according to the standard method [SRPS] [56]. The preadapted microorganism from the river Sava, Belgrade, Serbia was used. The aqueous medium for preparing the cultures had a neutral pH (7.2) and contained minerals and micronutrients to support bacterial activity (2.75 g $L^{-1}$ $NH_4Cl$, 1.0 g $L^{-1}$ $K_2HPO_4$, 0.252 g $L^{-1}$ $MgSO_4$, 0.32 g $L^{-1}$ KCl and 0.0018 g $L^{-1}$ $FeSO_4$). Tested formulations (1 –5) were added to the microorganism media to give a concentration of about 100 mg $L^{-1}$. Each sample was inoculated with 1 mL of 15 g $L^{-1}$ suspension of aerobic microorganism. The samples in dark bottles were placed in a temperature controlled incubator (Velp Scientifica FOC 120E, Italy), and the incubator temperature was maintained at 25 °C. BOD was measured every five days during the analyzed time of 28 days ($BOD_5$), by using Sensor system 6 (Velp Scientifica, Italy).

*3.11. Foam Volume Test*

SRPS ISO 696: 2000 Surface active agents - Measurement of foaming power - Modified Ross-Miles method, Institute for Standardization of Serbia. Evaluation of foam ability of surfactant mixtures was done by foam volume measuring after free flows of 0.4 % solution. The reservoir was placed in such a way that the outlet tube was positioned in the centre of the basin and the distance between its lower edge and bottom was 1 m.

## 4. Conclusion

Our synthetic efforts created a library of lipopeptides inspired by structures of naturally occurring rhamnolipids and antimicrobial peptides. Biological and physicochemical assessment of these compounds established their potent antibiofilm properties, in particular against *C. albicans*, as their most prominent feature. Overall, lipopeptides containing benzylic esters and lipophilic amino acid residues (leucine, phenylalanine and methionine) displayed the highest activity on the inhibition of biofilm formation of *C. albicans*. The length of aliphatic chain improves the potency of compounds but also increases their *in vitro* cytotoxicity. The optimal number of amino acids in the side chain was found to be one or two, as the further elongation of the peptide chain decreased their effect on biofilm formation. Taken together these observations suggest that for anti-biofilm potential of these compounds, the combination of specific structural motifs and lipophilicity may play more important role than their surface activity. Evidence of successful inhibition of *C. albicans* biofilm formation by prevention of the adhesion to abiotic and biotic surfaces has been provided for synthesized compounds, however, the exact mode of action still remains to be elucidated. The compounds may have specific targets such as adhesins or mannoproteins on *Candida* cell surface. The most potent amongst the synthesized compounds were able to significantly inhibit biofilm formation whilst not affecting the fungal growth thus minimizing the chances of resistance development. The strategy of targeting biofilm formation as a specific phenomenon may offer alternative therapeutic opportunities. Our lipopeptide library, in that regard, represents a synthetically accessible starting point for the development of new strategies against *C. albicans* infections and could represent the basis for further mechanistic and compatibility studies that may yield compounds which completely prevent biofilm formation of *C. albicans*.

## Conflict of interest

The authors declare no conflict of interest.

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# Rhamnolipid inspired lipopeptides effective in preventing adhesion and biofilm formation of *Candida albicans*

Milos Jovanovic[a], Jelena Radivojevic[b], Kevin O'Connor[c], Stevan Blagojevic[d], Biljana Begovic[d], Vera Lukic[e], Jasmina Nikodinovic-Runic[b], Vladimir Savic[a]

## Highlights

Substituting rhamnose in rhamnolipids with amino acids yielded 43 lipopeptides.

Their most prominent feature was anti-biofilm activity against *C. albicans*.

Lipopeptides inhibit biofilm formation of *C. albicans* by preventing its adhesion.

Compounds containing leucine residue and benzyl ester proved to be the most effective.

Compounds effectively prevent adhesion of *C. albicans* at sub MIC levels.