UNIVERSITY OF PARDUBICE

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Advanced derivatives of biologically active salicylamides

Theses of the Doctoral Dissertation

Pardubice 2023

Study program: Organic technology

Study field: Chemistry and Chemical Technology

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Year of the defence: 2023

References

E. Pilařová. Advanced derivatives of biologically active salicylamides. Pardubice. 2023. 125 pages. Dissertation thesis (PhD.). University of Pardubice, Faculty of Chemical Technology, Institute of Organic Chemistry and Technology. Supervisor Prof. doc. Aleš Imramovský, Ph.D.

Abstract

The content of the thesis is a description of proteasome inhibitors including their function. The following chapters discuss the actual synthesis of peptides, which is carried out in two ways. The liquid phase method and the solid phase method. The solid-phase method is then discussed from a technological point of view. We have used this method for the preparation of some of our intermediates in collaboration with Apigenex Ltd. A separate chapter is also devoted to current trends in proteasomal inhibition and also potential proteasome inhibitors prepared by our research group, where the design of new compounds is then discussed.

Abstrakt

Obsahem disertační práce je popis inhibitorů proteasomu včetně jejich funkce. V dalších kapitolách se diskutuje vlastní syntéza peptidů, která se provádí dvěma způsoby. Metodou v kapalné fázi a metodou v pevné fázi. Metoda v pevné fázi je poté diskutována i z technologického hlediska. Tuto metodu jsme využili pro přípravu některých našich meziproduktů ve spolupráci s firmou Apigenex s.r.o. Samostatná kapitola je i věnována současným trendům v proteasomální inhibici a také potenciálním inhibitorům proteasomu připravenými naší výzkumnou skupinou, kde se poté diskutuje i návrh nových látek.

Key worlds

Proteasome, proteasome inhibitors, solid phase peptide synthesis, technology of organic compounds

Klíčová slova

Proteasom, inhibitory proteasomu, SPPS, technologie organických látek

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LIST OF ABBREVIATIONS

PI	Proteasome inhibitor
SPPS	Solid-phase peptide synthesis
SPS	Liquid-phase synthesis
L-Leu	*L*-Leucine
L-Trp	*L*-Tryptophane
L-Pro	*L*-Proline
L-Phe	*L*-Phenylalanine
L-NLeu	*L*-Norleucine
L-CHA	*L*-Cyclohexylalanine
UPS	Ubiquitin-proteasome system
MM	Multiple myeloma
RT	Room temperature
DMF	Dimethylformamide
TFA	Trifluor acetic acid
TIS	Triisopropylsilane
ACN	Acetonitrile
AMK	Aminoacid

SUMMARY

Salicylanilides are widely used substances that are derived from salicylic acid. They are effective against gram-positive strains including *Staphylococcus aureus* (golden staphylococcus) and *Enterococcus faecium*. These strains represent a clinical problem nowadays because they are resistant to some commonly used antibiotics. It is necessary that these substances contain an electron acceptor group on the aromatic part and a hydrophobic group on the anilide part so they can work. They are also used as anti-fungal and antihelminthic agents and also as proteasome inhibitors (PI).

This work focuses mainly on proteasome inhibitors, namely their description, distribution, production and technology. The preparation of new potential PI by our research group is based on salicylanilide intermediates, which can also be used for the preparation of potential antibacterial substances.

AIM OF THE THESIS

1. The aim of this thesis is to describe in the theoretical part what the proteasome is and what parts it consists of, including its function in cells. It also focuses on proteasomal inhibition. The next chapter is already devoted to the preparation of the peptide chain, describing two methods, namely solid-phase (SPPS) and liquid-phase (SPS), where subsequently more attention is paid to the solid-phase method. The principle of this method is described, giving representatives of carriers and linkers.
2. Next, the theoretical part deals with the actual technology of peptide preparation, namely the solution of scale-up (transferring the synthesis to a larger scale). From the technological point of view, the SPPS method is described in more detail, and it is explained how the intermediates used in the preparation of some substances were prepared, when these intermediates were prepared by the company Apigenex Ltd.
3. And the following is listed a summary of individual studies published by our research group in the past and how this dissertation builds on these studies is presented below. Lastly, the theoretical section focuses on salicylanilides as antibacterial agents.
4. The experimental part deals with tripeptide aldehydes and vinyl sulfones, which complements the study previously carried out by our working group.
5. There is also an extension to tetrapeptidic aldehydes and vinyl sulfones to investigate the effect of chain elongation on biological activity. In another study, tripeptidic and tetrapeptidic aldehydes and vinyl sulfones having only *L*-Leu and *L*-Trp in the chain are described. The compounds with *L*-Tryptophan were found to be potential inhibitors of the pathogen proteasome. [1]
6. Since the preparation of these compounds is based on monopeptide acids and this derivative is very interesting in terms of biological activities, we decided to prepare a series of trifluortoluidines based on these intermediates as well.
7. In the last study discussed in this work, compounds with acrylamide moiety are discuised. Similar compounds have been published [2] and are identified in the literature as potential inhibitors of protein kinases.

TEORETICAL PART

1.1. Proteasom and its inhibition

Every eukaryotic cell contains a multicatalytic enzyme complex proteasome 26S located in the nucleus and cytoplasm. This proteasome is barrel-shaped and approximately 2.5 MDa in size. Over the years, it has been found to serve not only to degrade damaged proteins but also to be essential for the cell cycle. However, its primary function is protein degradation. Substrates of the proteasome include, for example, tumor suppressors, signaling molecules, cell cycle regulators, transcription factors, inhibitory molecules, and others. [3,4]

The 26S proteasome is composed of a barrel-shaped proteolytic core complex, referred to as the 20S proteasome, and this core is terminated at either one or both ends by 19S regulatory complexes (sometimes referred to as PA700) that recognize ubiquitin-tagged proteins. The 19S regulatory complexes are further tasked with unwinding and translocating the ubiquitin-tagged peptides into the nucleus, where they are degraded. Normal healthy cells make proteins and those that are damaged or unnecessary are broken down in the proteasome. [4,5]

Proteasome inhibitors or also inhibitors of the Ubiquitin-proteasome system (UPS) are generally substances capable of inhibiting the activity of the proteasome and thus increasing its protein content. Long-term stress caused by proteasome inhibition leads to cell cycle arrest and protein accumulation in the proteasome, resulting in a disruption of homeostasis and the cell is forced to induce apoptosis. This approach is mainly used for the treatment of multiple myeloma (MM). [6,7,8]

The largest group of PI are short peptides ending with a functional group, by means of which they bind to the active site in the proteasome and thus prevent it from functioning properly. The binding can be either reversible (bortezomib, ixazomib), when one covalent bond is formed, but after a certain period of time it is broken and the proteasome returns to its original function. In the case of irreversible binding (carfilzomib, oprozomib), two covalent bonds are formed, and the inhibition takes much longer due to the difficulty of re-releasing the proteasome. This only occurs when carfilzomib (or another irreversible IP) is expelled from the body and a new proteasome is formed. [7]

2.1. Synthesis of peptidic chain and technology

The peptide chain can be prepared in two ways in terms of synthesis. Either in solution (SPS) or in solid phase (SPPS), where the solid phase method is nowadays already used as the majority method in the preparation of small or medium-sized peptides. Hybrid methods are used for the preparation of large peptides and/or small proteins. [9,10]

The SPPS method is currently the most used method for peptide synthesis both in the laboratory and in industry due to its automation. [9,11]

The synthesis of peptides by this method proceeds from the C-terminus to the N-terminus, whereas the synthesis of ribosomal proteins proceeds from the N-terminus to the C-terminus. At the beginning, the amino acid is anchored to a polymeric solid support. This is in the form of small solid spheres on the order of millimeters in size, which contain small organic groups called 'linkers' on their surface. [9,12]

The classical reactor for peptide preparation by the SPPS method consists only of a cylinder equipped with a frit and a lid with a stirrer. The resin can also be mixed by bubbling nitrogen, but this requires more complex equipment.**Figure 1** shows an SPPS device for small-scale peptide synthesis. [13]

In the framework of this dissertation, such compounds were prepared using advanced intermediates. We were able to improve the production of these intermediates so much that the racemization of these compounds was as small as possible (the molecules contain more chiral sites) and thus also bring it to a technological scale in collaboration with Apigenex Ltd., a company involved in the preparation of peptides by the SPPS method (**figure 1**). This company prepared tripeptidic acids with a chain composed of *L*-Leu and *L*-Trp linked to 5-Cl-*O*-Bn salicylic acid (which was supplied) for us. These tripeptidic acids were subsequently used to prepare tetrapeptidic aldehydes and vinyl sulfones.

Figure 1: Synthesis of peptides by SPPS method

3.1. Overview of proteasome inhibitors within the research group

Proteasome 20S contains active sites, called pockets, where the inhibitor binds. It is therefore possible to deduce certain structural features that have been used to design compounds in the past and to apply this knowledge to new compounds. Active sites (pockets) (**figure 2**) are denoted by the letter 'S' and numbered from right to left of the functional group, whereas amino acid residues are denoted by the letter 'P', where the numbering is the same. [14]

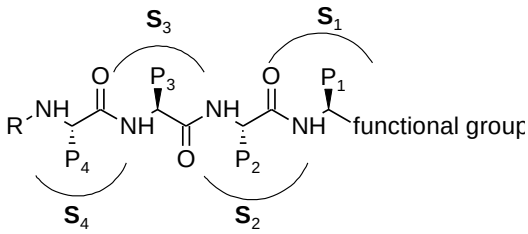
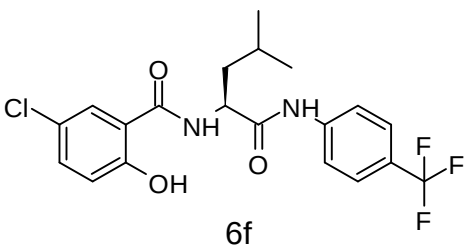


Figure 2: General structure with active sites

One approach to creating new molecules is to modify a known molecule. This can be modified in three ways, where only one approach can be chosen or their combination.

- By changing the functional group/brand new electrophilic functional group
- Changes in the peptide chain
- Changes at the N-terminus of the molecule

We have been working on proteasome inhibitors in our research group for many years. The basic substance that started it all is salicylamides. These were created by a shift as an unexpected product of salicylanilide prodrugs. Our research group later started to look at this switch and described it in terms of its generality, mechanism and biological activities, discovering the cytotoxic effect of the substances on tumour cell lines and affecting the proliferation of non-tumour cells. This crossover has been described using several different protected amino acids. This discovery entailed the design of additional molecules with the aim of further improving the existing biological activities, by altering the structure of the molecules under investigation. [15]

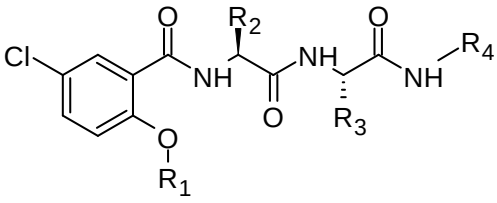


6f

This study was later supplemented with other compounds with different substituents, where the effect of each substituent on biological activity was

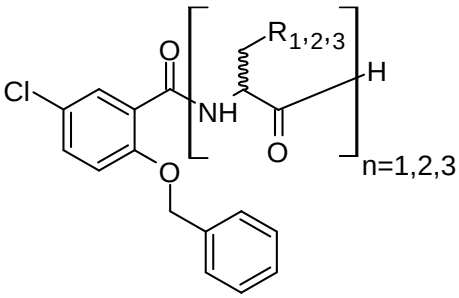
investigated. In the studied compounds, the substituents on both aromatic parts were varied and different amino acids were also used. The most active substance with a 5-Cl substituent was compound **6f** having a 4-trifluoromethyl group at the R_2 position. Substances with this group were later found to exhibit antibacterial activity. [15]

Since the substance with this substitution achieved better biological activities than the standards used, more substances were designed and synthesized. For these substances, the 5-Cl subtituent on the core was retained, based on the previous study, and the AMKs



used were changed, with the formation of already dipeptide substances and also the use of different substituents on the NH group. The biological activities of both the benzylated and the corresponding debenzylated compounds were measured. From the measured values it is clear that compounds with free OH group have higher biological activities than the benzylated ones.[16]

The aim of the research on these compounds was to achieve an increase of biological activities by a change in the structure, where two changes were considered, namely in the



anilide part of the molecule, or a change in the middle part of the molecule, namely the use of various both natural and atypical aminoacids. This led to the design and synthesis of new compounds in which the middle part was altered (different aminoacids) and an aldehyde functional group was introduced based on the literature and similarity to clinically used PI. Subsequently, the middle part of the molecule was extended to dipeptide and then tripeptide. From the measured values it is clear that tripeptidic aldehydes have the highest biological activities. Based on this, part of the experimental work is aimed at extending this series of tripeptidic aldehydes using additional aminoacids. [17]

Since we have so far only investigated substances with an aldehyde group and this group is somewhat unstable, the introduction of some other group is suggested to solve the stability problem. Such a group could be, for example, the vinyl sulfonic group. [18,19]

RESULTS AND DISCUSSION

The prepared materials were designed based on a literature search and previous research by our Imramovsky research group. The aim was to supplement some existing series with additional substances and to use other atypical amino acids. The first series contained aldehydes and vinyl sulfones with a tipeptide chain consisting of *L*-Leu, *L*-Phe, *L*-CHA, *L*-NLeu, *L*-Pro and *L*-Trp. The substances containing tryptophan showed the best proteasomal inhibition. As a result, we prepared another series of aldehydes and vinyl sulfones having tryptophan located at the S1, S2, or S3 position. Other derivatives contained more than one Trp, where these substances were prepared based not only on our previous research but also on the literature. [1]

Table 1: Biological activities of tripeptidic aldehydes

				MCF-7 cell lines			MCF-7	U2OS-PI-GFP
				Caspase(β1)	Tryspine (β2)	Chymotrypsine (β5)	Chymotrypsine	
				20 µM			IC_{50} (µM)	c (µM)[a]
Comp.	**R_1**	**R_2**	**R_3**	**% of ctrl± SD**	**% of ctrl± SD**	**% of ctrl± SD**	**data±SD**	**data**
55a	*i*-bu	*i*-bu	*i*-bu	45.2±1.0	91.1±1.0	10.0±0.1	0.37±0.03	1,6
55b	benzyl	*i*-bu	*i*-bu	75.4±3.6	≥100	9.4±0.1	1.75±0.01	4
55c	*i*-bu	butyl	*i*-bu	40.0±0.7	96.1±1.6	7.3±0.0	0.27±0.02	1,6
55d	*i*-bu	cyclohexylmethyl	*i*-bu	48.3±1.1	87.6±2.4	8.7±0.1	2.34±0.00	3,2
55e	*i*-bu	Pro	*i*-bu	≥100	≥100	90.6±4.3	>20	>5
55f	*i*-bu	1*H*-indol-3-yl	*i*-bu	26.2±1.4	87.6±1.5	7.0±0.2	0.61±0.02	1,6
55g	*i*-bu	*i*-bu	benzyl	≥100	≥100	30.8±1.3	6.22±0.60	>5
55h	*i*-bu	*i*-bu	butyl	86.5±1.3	≥100	16.2±0.3	3.27±0.07	>5
55i	*i*-bu	*i*-bu	cyclohexylemethyl	82.7±1.1	≥100	35.8±1.7	7.58±0.08	>5
55j	*i*-bu	*i*-bu	Pro	95.4±3.8	96.6±6.8	≥100	>20	>5
55k	*i*-bu	*i*-bu	1*H*-indol-3-yl	63.5±0.1	94.4 ±1.4	11.1±0.9	2.22±0.06	>5
55l	1*H*-indol-3-yl	*i*-bu	*i*-bu	-	-	-	-	-
55m	1*H*-indol-3-yl	*i*-bu	1*H*-indol-3-yl	93,7	99,8	96,8	>20	-
55n	1*H*-indol-3-yl	1*H*-indol-3-yl	1*H*-indol-3-yl	94,2	102,1	47,9	17,86	-
bortezomib 1000 nM				27.5±6.5	85.9±2.0	7.3±1.2	0.008±0.00	Not tested

Table 2: Biological activities of tripeptidic vinyl sulfones

				MCF-7 cell lines			MCF-7	U2OS-PI-GFP
				Caspase (β1)	Tryspine (β2)	Chymotrypsine (β5)	Chymotrypsine	
				20 µM			IC_{50} (µM)	c (µM)[a]
Comp.	**R_1**	**R_2**	**R_3**	**% of ctrl± SD**	**% of ctrl± SD**	**% of ctrl± SD**	**data±SD**	**data**
53a	*i*-bu	*i*-bu	*i*-bu	76.7±1.3	≥100	11.2±0.1	2.27±0.10	2,5
53b	benzyl	*i*-bu	*i*-bu	77.5±0.9	≥100	21.0±0.5	3.38±0.03	5
53c	*i*-bu	butyl	*i*-bu	63.1±1.0	89.1±0.7	8.7±0.2	3.26±0.07	3,2
53d	*i*-bu	cyclohexylmethyl	*i*-bu	75.8±2.2	93.8±4.3	44.2±1.3	5.58±0.57	>5
53e	*i*-bu	Pro	*i*-bu	≥100	≥100	93.0±1.2	>20	>5
53f	*i*-bu	1*H*-indol-3-yl	*i*-bu	73.9±0.3	95.7±1.9	19.0±0.3	5.31±0.30	5
53g	*i*-bu	*i*-bu	benzyl	52.5±0.8	89.5±0.4	5.9±0.3	1.71±0.01	2,5
53h	*i*-bu	*i*-bu	butyl	70.2±3.4	≥100	11.2±0.5	3.97±0.14	4
53i	*i*-bu	*i*-bu	cyclohexylemethyl	74.9±2.3	96.2± 0.4	69.6±2.7	>20	>5
53j	*i*-bu	*i*-bu	Pro	≥100	≥100	94.8±0.1	>20	>5
53k	*i*-bu	*i*-bu	1*H*-indol-3-yl	54.8±0.2	88.1± 1.6	7.9±0.2	1.77±0.33	3,2
53l	1*H*-indol-3-yl	*i*-bu	*i*-bu	-	-	-	-	-
53m	1*H*-indol-3-yl	*i*-bu	1*H*-indol-3-yl	-	-	-	-	-
53n	1*H*-indol-3-yl	1*H*-indol-3-yl	1*H*-indol-3-yl	96,4	93,2	95,8	>20	-
bortezomib 1000 nM				27.5±6.5	85.9±2.0	7.3±1.2	0.008±0.00	Not tested

The results of the measured inhibitory concentrations (IC) of aldehydes and vinyl sulfones (**table 1** and **2**) clearly show that aldehydes generally have higher biological activity. However, the IC of the standard, where bortezomib was chosen as the standard in this case, are much lower. Bortezomib, however, has many side effects, including the relatively rapid development of resistance to the agent. Therefore, pressure is being put on the synthesis of new potential proteasome inhibitors lacking these side effects, even though they may not reach the inhibitory concentrations of bortezomib just mentioned. In the case of aldehydes, a few representatives (**figure 3**) with high biological activities have been synthesised. In particular, these are compounds **55a**, **55c** and **55f**, where compound **55a** contains only *L*-leucines in its chain, compounds **55c** and **55f** are aldehydes with *L*-leu in the R_1 and R_3 positions, with *L*-norleucine in the R_2 position in the case of **55c** and *L*-tryptophan in the case of **55f**. Thus, it can be said that substances having an *L*-leucine in the R_3 position are more active than substances with another AMK in this position. [20]

The corresponding vinyl sulfones were prepared for the aldehydes (**table 2**). This functional group was chosen because of its higher stability compared to aldehydes. Of all the representatives prepared, the derivative with three *L*-leucines (**53a**) is again one of the most active, but the trend is reversed here in contrast to the aldehydes. The most active compounds have an *L*-leucine in the R_2 position and another amino acid in the R_3 position. These are substance **53g** with *L*-phenylalanine and substance **53k** with *L*-tryptophan. [20]

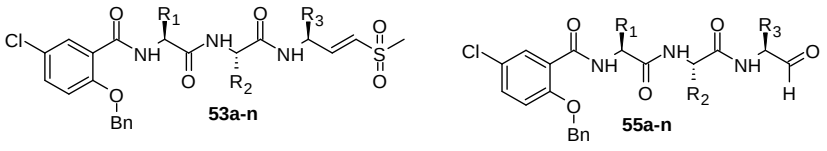


Figure 3: General structure of tripeptidic aldehydes and vinyl sulfones

So far, only the influence of functional groups and amino acids used in tripeptides has been investigated. The next study was on tetrapeptide compounds containing tryptophan in combination with leucine (**figure 4**), like the previous compounds. Here we compared the effect of chain elongation on biological activity. Based on the literature

[1], the proposed molecules contain *L*-Trp or *L*-Leu in R4, *L*-Leu in R_3, and a combination of *L*-Leu and *L*-Trp in R_2 and R_1. The starting tripeptidic acids were supplied by Apigenex Ltd. A tetrapeptide aldehyde and a vinyl sulfone containing 4 *L*-Leu were also prepared for comparison of activities.

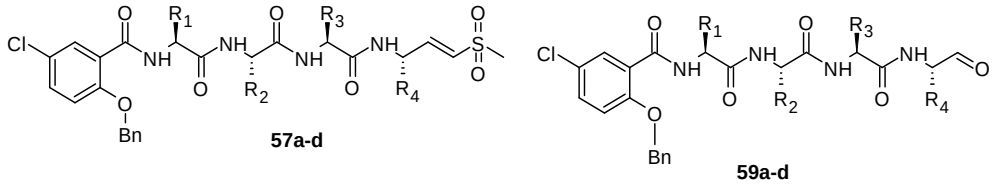


Figure 4: General structures of tetrapeptidic aldehydes and vinyl sulfones

Comp.	IC_{50} (μM)-MCF-7			% residual-MCF-7			GI_{50} (μM)	
	β1	β2	β5	β1	β2	β5	K562	MCF-7
57a	>20	>20	**0,86**	**73,7**	**58,5**	**3,9**	**1,67**	**3,17**
57b	>20	>20	>20	81,1	95,8	108,3	12,5	12,5
57c	>20	>20	>20	82,4	94,8	97,8	12,5	12,5
57d	>20	>20	>20	-	-	-	-	-
59a	>20	>20	**1,87**	**63,2**	**73,7**	**5,7**	**5,4**	**4,88**
59b	>20	>20	6,51	92,1	111,5	21,3	12,5	12,5
59c	>20	>20	3,68	77,4	74,0	12,4	7,86	12,5
59d	>20	>20	3,1	32,8	59,0	5,4	-	-

Table 3: Inhibition concentration of tetrapeptidic aldehydes and vinyl sulfones

	R_1	R_2	R_3	R_4
a	*i*-bu	*i*-bu	*i*-bu	1*H*-indol-3-yl
b	1*H*-indol-3-yl	*i*-bu	*i*-bu	1*H*-indol-3-yl
c	*i*-bu	1*H*-indol-3-yl	*i*-bu	1*H*-indol-3-yl
d	*i*-bu	*i*-bu	*i*-bu	*i*-bu

Table 4: Substituents of 57a-d and 59a-d

The results in **table 3** (and **table 4**) clearly show that the substance with the amino acid sequence Leu-Leu-Leu-Trp, either aldehyde or vinyl sulfone, achieves the highest biological activity. Tripeptidic and tetrapeptidic aldehydes and vinyl sulfones having a

peptide chain composed of only *L*-Leucine was also prepared but they don´t achieve so high biological activities as compounds **57a** and **59a**.

Monopeptide acids are a very interesting intermediate. We have therefore used them in other reactions, for example for the preparation of p-trifluorotholudine derivatives (**figure 5**), which according to the literature should have good antibacterial properties. Again, a group of compounds containing different amino acids was created. Both benzylated and debenzylated products were subjected to biological testing.

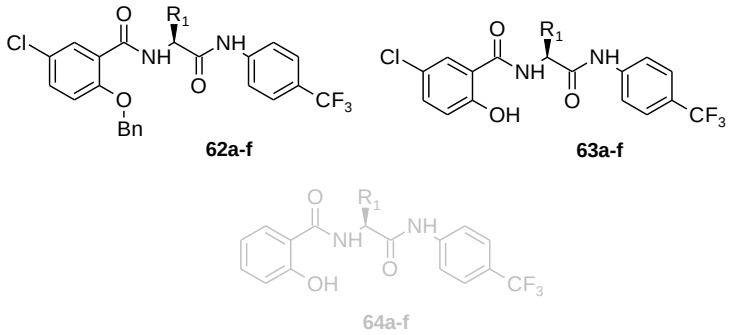


Figure 5: General structure of 4-CF_3-toluidin compounds

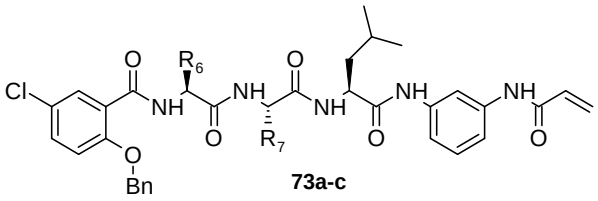
Comp.	MIC [μM]										IC_{50} [μM] THP-1@10% FBS 24h
	SA	MRSA1	MRSA2	MRSA3	EF	VRE1	VRE2	VRE3	MT	MS	
63a	-	-	-	-	-	-	-	-	-	-	>10
63b	2.41 **4.82**	4.82 **4.82**	4.82 **4.82**	4.82 **9.64**	38.6 **38.6**	38.6 **38.6**	38.6 **38.6**	38.6 **38.6**	77.1 **–**	77.1 **–**	>10
63c	-	-	-	-	-	-	-	-	-	-	7.3±1.2
63d	0.583 **1.17**	2.33 **2.33**	2.33 **4.66**	4.66 **4.66**	-	-	-	-	-	-	4.5±1.2
63e	1.07 **1.07**	1.07 **1.07**	1.07 **2.13**	2.13 **2.13**	546 -	546 -	546 -	546 -	-	-	1.4±1.1
63f	2.24 **2.24**	8.95 **8.95**	4.48 **8.95**	8.95 **8.95**	17.9 **35.8**	35.8 **35.8**	17.9 **35.8**	35.8 **35.8**	35.8 **–**	35.8 **–**	>10
L*-Leu	1.17 **1.17**	1.17 **1.17**	1.17 **1.17**	0.070 **0.070**	4.66 **9.33**	4.66 **18.7**	9.33 **18.7**	4.66 37.3	18.7 –	18.7 –	1.9±1.1
L*-Phe	1.08 **2.16**	2.16 **2.16**	1.08 **2.16**	0.270 **0.270**	277 -	277 -	277 -	277 -	-	-	3.3±1.0
APM	5.72 >5.72	45.8 >45.8	45.8 >45.8	45.8 >45.8	2.81 2.81	11.5 11.5	11.5 11.5	11.5 11.5	–	–	–
INH	–	–	–	–	–	–	–	–	36.6 –	117 –	–

Table 5: Biological activities of 4-CF_3-toluidine compounds

Table 5 shows the measured inhibition concentrations against the pathogens (SA = *Staphylococcus aureus* ATCC 29213; MRSA1-3 = clinical isolates of methicillin-

resistant *S. aureus* SA 3202, SA 630 and 63718; EF = *Enterococcus faecalis* ATCC 29213, and vancomycin-resistant *Enterococci* VRE1-3 = VRE 342B, VRE 368, VRE 725B, MT = *Mycobacterium tuberculosis* H37Ra/ATCC 25177; MS = *M. smegmatis* ATCC 700084; FSB = fetal bovine serum; hyphen "-" = no activity). These are values for debenzylated agents only, as benzylated agents were found not to inhibit these pathogens. In addition, the table is expanded to include two more derivatives containing *L*-Leu and *L*-Phe, where these substances have been prepared previously*. Focusing on individual substances, compounds **63f** with *L*-Leu have activity comparable to or higher than that of the standards (AMP-ampicillin, INH-isoniazid), substance **63c** has higher activity only in some strains, and substances **63f** with *L*-Phe show an antistaphylococcal effect. [21]

Lastly, we mention here the preparation of compounds terminated with an acrylamide group (**figure 6**), which could serve as a potential new functional group of PI. A problem in the syntheses of these compounds occured in the deprotection of the Boc group containing mentioned acryl group. We were not able to isolate some such compounds from the reaction mixture because of very poor solubility in many commonly used organic reagents. Finally, we were able to prepare only two dipeptide acrylamides and one tripeptide, but these substances did not show any biological activity.



73a-c

	R6	R7	výtezek
73a	-	isopropyl	43 %
73b	-	phenylmethyl	8 %, 20 %
73c	isopropyl	isopropyl	8,6 %

Figure 6: General structure of akrylamides and its substituents

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List of Students' Published Works

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