UNIVERSITY OF PARDUBICE
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Design and Synthesis of Novel Potentially Cytotoxically Active Salicylamides

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References

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ABSTRACT

Presented doctoral thesis contains literary review describing biological activities and methods of salicylamide synthesis, describing peptide synthesis including reagents, additives and aspects of substrate racemization, and describing molecules able of proteasomal inhibition. Presented doctoral thesis describes the design and experimental verification of synthetical strategy to prepare salicylamides with great potential to have cytotoxic activities against tumour growth cells. Doctoral thesis presents several options for peptide synthesis including the functional group incorporation which is essential for desired proteasomal inhibition. The scope of peptide synthesis was mainly to solve the racemisation issue. Steglich amidation was found to be the crucial step and was optimalized. By the replacement of used reagents such as dicyclohexylcarbodiimide, *N*,*N*-dimethylaminopyridine and triethylamine was the racemisation issue solved. Salicylamides with peptide motive in the length up to three amino acids with methylester, Weinreb amide or aldehyde functional group as well as their activities against various strains of leukemia (K562, THP-1, CEM) or multiple myeloma (U266) are presented in the end of presented thesis.

Keywords

Aliphatic salicylamides, proteasome inhibition, peptide synthesis, amidation reagents, racemization.

ABSTRAKT

Předkládaná dizertační práce obsahuje literární rešerši zaměřenou na metody syntézy a popsané biologické aktivity salicylamidů, na možnosti syntézy peptidů včetně činidel, aditiv a aspektů racemizace substrátu, a na molekuly schopné inhibice proteasomu. Předkládaná dizertační práce dále popisuje návrh a experimentální ověření strategie pro syntézu saliyclamidů s potenciálem na výraznou cytotoxicitu vůči nádorovým onemocněním. Popisuje několik možných přístupů ke konstrukci peptidového řetězce včetně zavedení funkční skupiny důležité pro inhibici. V rámci výstavby peptidového řetězce byla intenzivně řešena problematika racemizace substrátu. Přípravou série modelových molekul byla jako kritický stupeň určena a optimalizována amidace dle Steglicha prováděná za přítomnosti dicyklohexylkarbodiimidu, *N*,*N*-dimethylaminopyridinu a triethylaminu. Úpravou reakčních podmínek byla racemizace potlačena. V závěru práce je uveden přehled připravených originálních salicylamidů s peptidovým motivem o délce až tří aminokyselin zakončených methylesterovou, Weinreb amidovou nebo aldehydovou skupinou včetně výsledků pozorovaných aktivit vůči kmenům leukemie (K562, THP-1, CEM) a nádorového bujení (U266).

Klíčová slova

Alifatické salicylamidy, inhibice proteasomu, syntéza peptidů, amidační činidla, racemizace.

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INTRODUCTION

Salicylamides is a group of compounds well known for their biological activities and is used for over 60 years in clinical humane and veterinary praxis for treating tapeworms, nematodes and trematodes. At the end of 90_{th} years of 20_{th} century the inhibition activity of histidine kinase in two-component regulatory system in bacteria by an undescribed mechanism was discovered. The discovery exhibited the potential salicylamides had. Afterwards some salicylamides showed activities in inhibiting various types of mycobacterium, acetylcholinesterases, Zika virus, bacterial and viral infections, rheumatoid arthritis, type 2 diabetes or various types of tumour growth factors. One of the new possible applications of one group of aliphatic salicylamides with peptide side chain is tumour treatment of multiple myeloma, breast cancer or colon cancer.

First part of presented thesis contains literary review describing biological activities and methods of salicylamide synthesis, describing peptide synthesis including reagents, additives and aspects of substrate racemization, and describing molecules able of proteasomal inhibition.

Second part of presented thesis describes the design and experimental verification of synthetical strategy to prepare salicylamides with great potential to have cytotoxic activities against tumour growth cells. Doctoral thesis presents several options for peptide synthesis including the functional group incorporation which is essential for desired proteasomal inhibition.

The scope of peptide synthesis was mainly to solve the racemisation issue. Steglich amidation was found to be the crucial step and was optimized. By the replacement of used reagents such as dicyclohexylcarbodiimide, *N*,*N*-dimethylaminopyridine and triethylamine was the racemisation issue solved.

Salicylamides with peptide motive in the length up to three amino acids with methylester, Weinreb amide or aldehyde functional group as well as their activities against various strains of leukemia (K562, THP-1, CEM) or multiple myeloma (U266) are presented in the end of presented thesis.

AIMS

It was deduced, that salicylamides have perspective cytotoxic properties. Presented thesis aims for a convenient expansion of salicylamide group and obtain new derivatives with significant cytotoxicity and new biological properties.

The main goals of this thesis are:

- Revision of the literature and the current state of salicylamide biological activities and synthesis;
- Revision of the literature and the current state of peptide synthesis with the focus on reagents, additives and their effect on racemisation, mechanization and automatization of the synthesis, racemization problematics and methods of the analysis;
- Revision of the literature and the current state of proteasomal inhibition and its use in tumour cell treatment;
- Design and experimental verification of several possible synthetic routes for various salicylamides preparation. Structural variability of target compounds lies in incorporation of di-/tripeptide side chain terminated with various functional groups like aldehyde, Weinreb amide or others. Testing of biological activities will be conducted by an external institution.
- Conduct SAR study.

1. RESULTS AND DISCUSSION

Presented thesis extends scientific efforts of Assoc. Prof. Aleš Imramovský, Ph.D. research group on the field of biologically active aliphatic salicylamides and completes the logical matrix of possible structural motive combinations. [1–3] In the original series of salicylamides the salicylic moiety bears only one amino acid, which is terminate by substituted aniline in the form of amide.

This thesis presents several ways of structural modifications which branches the basic group to new salicylamide derivatives. The first group of salicylamides keeps one amino acid and replaces anilide with aliphatic amide or allylamine moiety. The SAR study is conducted and the shift in biological activities compared to the original series is observed and commented. This basic topic is further expanded by a fellow doctoral student Pratibha Magar in her own thesis. Her course of research is mentioned here to illustrate the width of this topic.[4] For detailed structures see **Figure 1**.

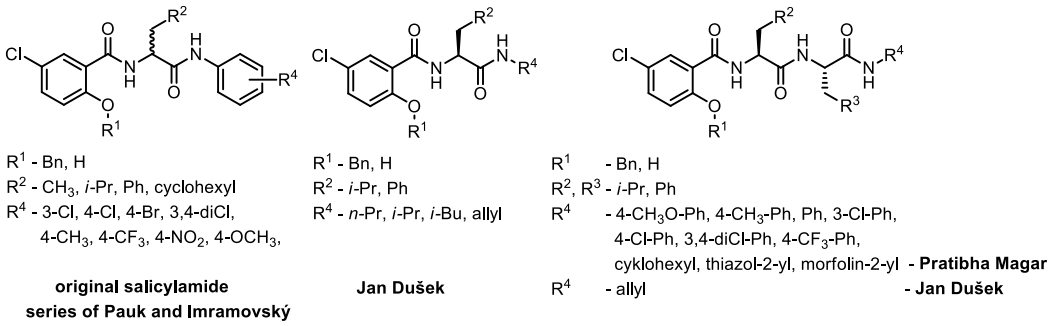


Figure 1: Salicylamides of the original series terminated with substituted anilides and novel series terminated with aliphatic, alicyclic, aromatic and heterocyclic amides

Second group of salicylamides extends the amino acid motive to di-/tripeptide and implements various electron-acceptor functional groups with the intension of achieving new biological activities like those of peptide proteasomal inhibitors. These compounds have proven themselves as valuable treatment for various types of tumour growth such as leukemia, multiple myeloma, breast or column cancer. The SAR study is conducted in detail and the achieved biological activities are compared to the original proteasomal inhibitors. For general structures see **Figure 2**.

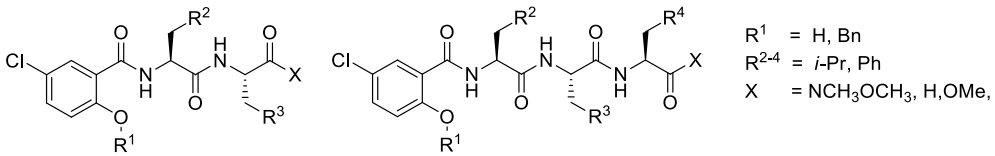
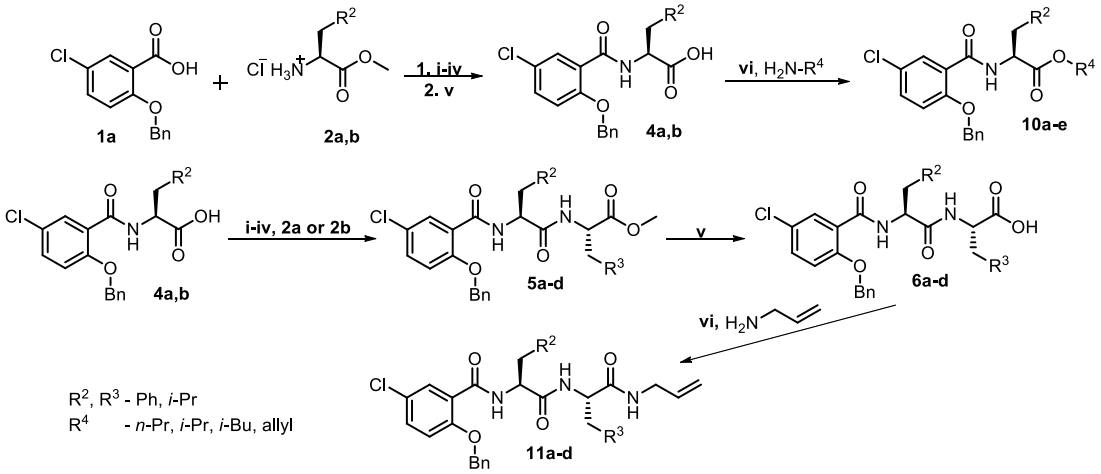


Figure 2: Salicylamides terminated with ester, Weinreb amide and aldehyde functional group

All biological activities and cytotoxicity surveys were conducted at Laboratory of Growth Regulators, Faculty of Science, Palacký University Olomouc and the Institute of Experimental Botany, Czech Academy of Sciences.

1.1 Synthesis and biological activities of salicyl-(di/tri)-amides terminated with aliphatic amides or allylamides

The synthesis of the basic line of salicylamides with one amino acid and terminated with an aliphatic amide (diamides) was conducted the same way the original line with one amino acid and terminated with anilide. The synthesis started with the O-Bn 5-chlorosalicylic acid (**1a**), which was connected to amino acid methyl ester (**2a,b**) in the way of Steglich amidation in the presence of carbodiimides. The amidation conditions were subject to complicated optimisation and will be discussed later. Next the carboxylic functional group was liberated in the presence of LiOH. Next the aliphatic amine or allylamine was connected to the deprotected intermediate (**4a,b**) under the same amidation conditions. Thus, the first line of compounds (**10a-e**) of the first group was synthesized. The second line of compounds (**11a-d**) was prepared by prolonging the amino acid motive to a dipeptide (triamide) by the same reactions as mentioned above. The final step was to terminate the dipeptide moiety with allylamine. For reaction see **Scheme 1**.



Scheme 1: Peptide chain synthesis and preparation if first group salicylamides **10a-e** and **11a-d**
i DCC (1,1 eq.), cat. DMAP, DCM, 25 °C, 20 h; **ii** EDCI·HCl (1,4 eq.), HOBt (1 eq.), TEA (1 eq.), DCM, 25 °C, 16 h; **iii** EDCI·HCl (1,4 eq.), HOBt (1 eq.), DIPEA(1 eq.), DCM, 25 °C, 16 h; **iv** EDCI·HCl (1 eq.), HOBt (1 eq.), K_2CO_3 (1 eq.), DCM, 25 °C, 2 h; **v** LiOH (10 eq.), 1,4 dioxane/H_2O (1/1), 50 °C, 1h; **vi** EDCI·HCl (1 eq.), HOBt (1 eq.), DCM, 25 °C, 2 h.

However, before successfully preparing salicylamides **11a-d** a major issue with racemisation was detected after adding second amino acid to the structure of **5a-d**. By gaining the second stereo centre the molecule became diastereomeric and thus in case of any racemisation visible in non-chiral environment such as ^{1}H NMR, where this issue was detected. The compounds were isolated with -20% ee and thorough investigation of the cause was done.

The amidation conditions and the use of DCC were suspected to cause the racemisation. Usage of DCC (**i**) was causing problems in the purification phase as well, where the corresponding urea by-product DCU was particularly difficult to remove even by repeated column chromatography. The carbodiimide was replaced for EDCI·HCl, which in its structure contains a tertiary nitrogen, which could be easily protonated and thus removing the urea by-product by a simple extraction. The next change was based on the

need of a tertiary base to be able to in situ liberate amino group of compound **2a-d**, which was in the form of a hydrochloride. TEA was tested first (**ii**) and the ee% were significantly improved from -20 to 60 ee%, however the racemisation was still present. TEA was suspected to react with the slightly acidic α-H in the amino acid so it was changed for DIPEA, which is sterically bulkier and might not affect the α-position so much (**iii**). The ee% was improved from 60 to 80 ee%. The next adjustment excluded TEA or DIPEA entirely from the reaction (**iv**). Instead the liberation of amino group took place separately in the presence of inorganic weak base such as K_2CO_3. The amino acid methylester was then subjected to the amidation protocol with EDCI·HCl and HOBt in equivalent amounts. Racemization was reduced and the product **5a-iv** was isolated with 92 ee%. The time needed for the reaction was reduced from 20 to 1 hour. The amidation conditions were optimized to provide robust method of amide synthesis (**iv**), which is used often in further parts of presented work. For compared amidation conditions and achieved ee % see **Table 1**. Compared ^{1}H NMR spectra of compounds **5a-i-iv** and the detail of successfully suppressed racemisation is presented on **Figure 3**.

Table 1: Owerview of reactionconditions

Product	Conditions	ee (%)
5a-i	DCC (1,1 eq.), cat. DMAP, DCM, 25 °C, 20 h	-20
5a-ii	EDCI·HCl (1,4 eq.), HOBt (1 eq.), TEA (1 eq.), DCM, 25 °C, 16 h	60
5a-iii	EDCI·HCl (1,4 eq.), HOBt (1 eq.), DIPEA(1 eq.), DCM, 25 °C, 16 h	80
5a-iv	EDCI·HCl (1 eq.), HOBt (1 eq.), K_2CO_3 (1,3 eq.), DCM, 25 °C, 1 h	92
9	EDCI·HCl (1,4 eq.), HOBt (1 eq.), DIPEA(1 eq.), DCM, 25 °C, 16 h	82

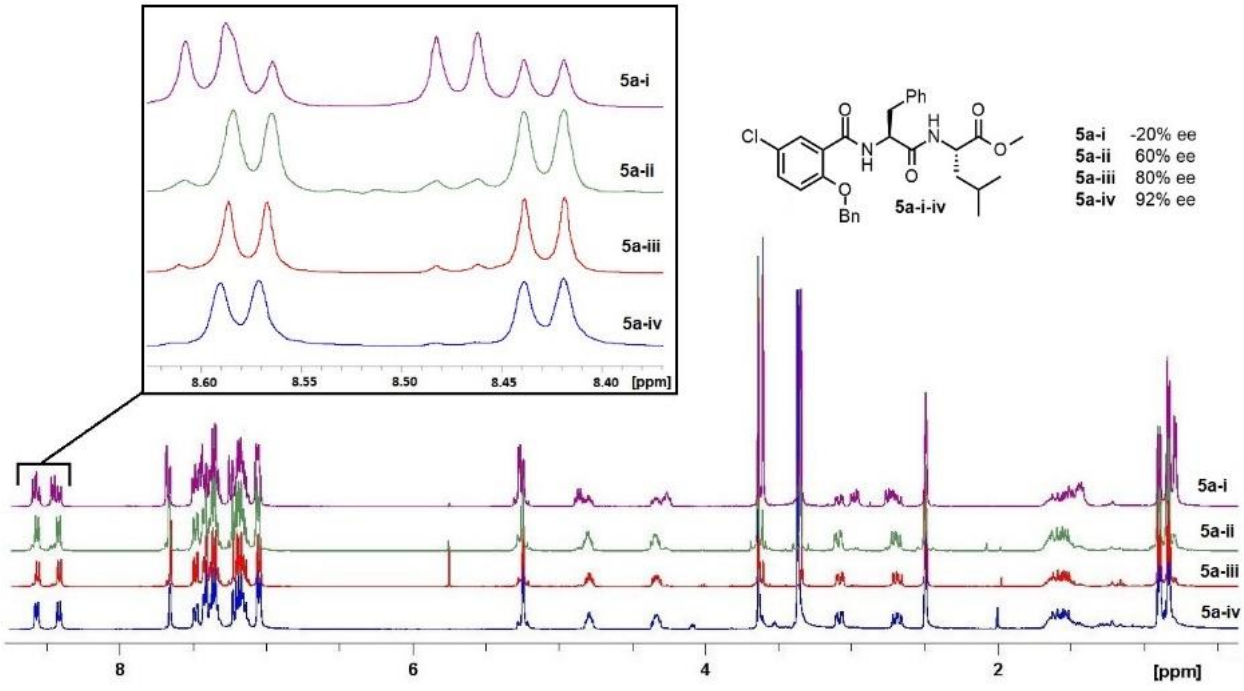
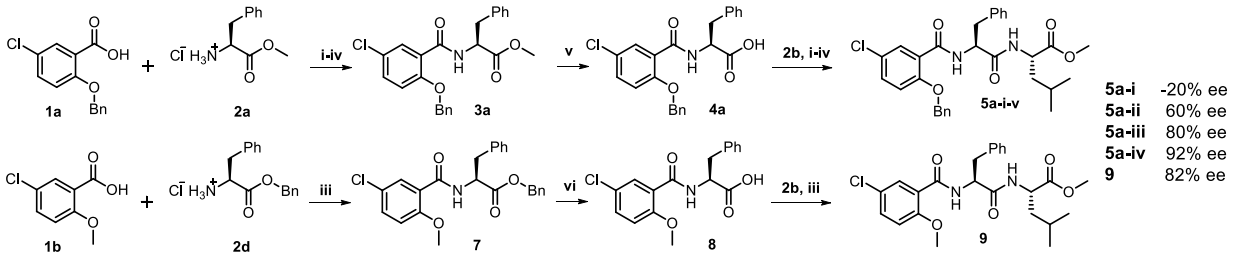


Figure 3: Comparison of ^{1}H NMR spectra of compounds **5a-i-iv** and the effect of suppressed racemization

Second possible cause of the racemization is the step of deprotecting the carboxylic group in the presence of strong inorganic base, which was suspected to be partially

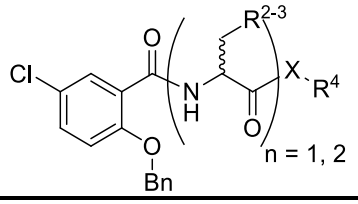
responsible. Parallel series of model compounds (**7-9**) was prepared with switched protective groups, see **Scheme 2**. The phenolic hydroxy group on salicylic acid was protected in the form of methyl ether (**1b**) and the carboxylic group of an amino acid was protected in the form of benzylester (**2d**). This protection was removed in the presence of H_2 and 10% Pd/C so the model molecule was not exhibited to the strong base. The amidation was conducted under the same conditions (**iii**) as the main compounds not to implement two adjustments at once. The levels of racemisation of compounds **5a-iii** and **9** were determined by ^{1}H NMR and were considered comparable. The result is, that the presence of strong inorganic base in large excess does not causes the racemisation and thus the amidation conditions are the main cause.



Scheme 2: Exchange of protective groups in the study of the cause of racemizaction
i DCC (1,1 eq.), cat. DMAP, DCM, 25 °C, 20 h; **ii** EDCI·HCl (1,4 eq.), HOBt (1 eq.), TEA (1 eq.), DCM, 25 °C, 16 h; **iii** EDCI·HCl (1,4 eq.), HOBt (1 eq.), DIPEA(1 eq.), DCM, 25 °C, 16 h; **iv**-EDCI·HCl (1 eq.), HOBt (1 eq.), K_2CO_3 (1 eq.), DCM, 25 °C, 2 h; **v** LiOH (10 eq.), 1,4-dioxane/H_2O (1/1), 50 °C, 1h; **vi** H_2, 10% Pd/C, EtAc, 25 °C, 20 h.

Prepared salicylamides with one amino acid terminated with aliphatic amide or allylamide (**10a-e**) and salicylamides with two amino acids terminated with allylamide (**11a-d**) were tested for their biological and antiproliferative activities against various leukemia cell lines (K562 – chronical myelogenic leukemia, THP-1 – acute monocytic leukemia, CEM – acute lymphoblastic leukemia). All salicylamides achieved mid micromolar range antiproliferative activities, which compared to activities of the original salicylamides with one amino acid terminated with anilide or compounds synthesized by Pratibha Magar is lower and not so perspective. The SAR outcome can be summarized into few points. Length of peptide moiety is not crucial to the overall activity. Aliphatic amides terminating the peptide moiety are less active than anilides, which achieve better antiproliferative activities in low micromolar range. The most important attribute of most potent derivatives is however the free phenolic hydroxy group, which is crucial for inhibition mechanism. For prepared salicylamides and achieved activities see **Table 2**.

Table 2: Antiproliferative activities of first series of salicylamides



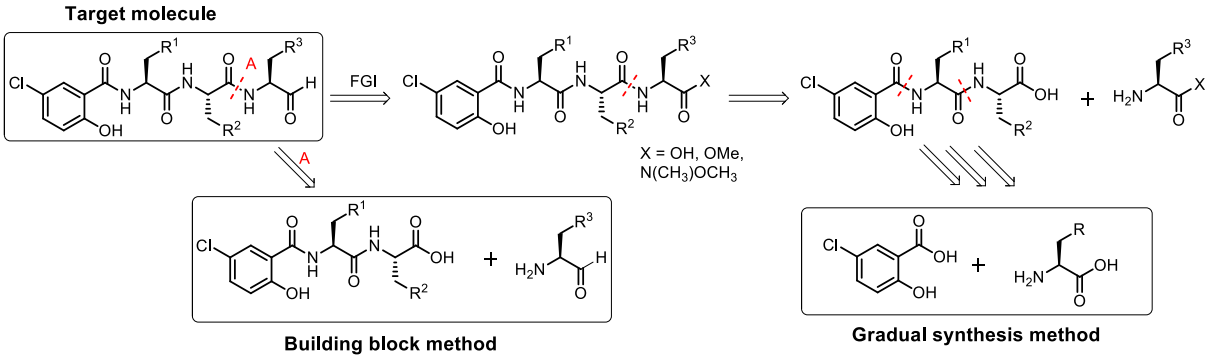
X - O: 3a,b, 4a,b, 5a-d, 6a-d
X - NH: 10a-e, 11a-d

Prod.	n	Amino acids	X	R^4	GI_{50} (μM)		
					K562	THP-1	CEM
3a	1	L-Phe	O	Me	70,6	39,1	53,0
3b	1	L-Leu	O	Me	68,1	32,4	43,0
4a	1	L-Phe	O	H	74,7	45,8	59,4
4b	1	L-Leu	O	H	85,7	38,0	53,0
5a	2	L-Phe-L-Leu	O	Me	55,0	**19,3**	44,2
5b	2	L-Leu-L-Phe	O	Me	69,1	**14,4**	45,5
5c	2	L-Leu-L-Leu	O	Me	59,5	32,8	49,9
5d	2	L-Phe-L-Phe	O	Me	76,4	24,2	59,9
6a	2	L-Phe-L-Leu	O	H	83,8	42,2	54,8
6b	2	L-Leu-L-Phe	O	H	80,7	36,8	56,8
6c	2	L-Leu-L-Leu	O	H	93,3	45,9	52,4
6d	2	L-Phe-L-Phe	O	H	65,3	45,4	56,5
10a	1	L-Phe	NH	allyl	81,4	40,5	55,4
10b	1	L-Phe	NH	*i*-Bu	81,3	44,0	41,8
10c	1	L-Phe	NH	*n*-Pr	89,4	42,4	57,3
10d	1	L-Phe	NH	*i*-Pr	52,7	**19,5**	37,1
10e	1	L-Leu	NH	allyl	65,9	32,6	37,9
11a	2	L-Phe-L-Leu	NH	allyl	76,3	27,4	55,8
11b	2	L-Leu-L-Phe	NH	allyl	49,5	37,3	62,1
11c	2	L-Leu-L-Leu	NH	allyl	63,3	31,3	39,8
11d	2	L-Phe-L-Phe	NH	allyl	73,0	30,9	49,9
Roscovitin					30,9	14,0	16,3

1.2 Synthesis and biological activities of tripeptide salicylamides as potential proteasomal inhibitors

One possibility of tumour cell treatment is to inhibit the proteasome, a protein complex responsible for degradation of damaged proteins.[5] By its inhibition the inner cellular space is filled with metabolic by-products and unneeded proteins, which disrupt homeostasis and leads to cellular death. Tumour cells in general, but especially multiple myeloma, breast or colon cancer are susceptible to such inhibition, because they are not able to remove such inhibition. Several of very promising inhibitors are in advanced stages of clinical trials, compounds such as bortezomib (2003), carfilzomib (2012) or ixazomib (2015) are already FDA approved for multiple myeloma treatment. The inhibitors can be distinguished by several criteria – the non/covalency and the ir/reversibility of the bond formed between the inhibitor and the proteasome, the

selectivity of inhibitor to β1/β2/β5 subunits of 20S proteasome core unit, the typical chemical structure or functional group of inhibitor such as non/cyclic peptides, *β*-lactones, epoxyketones, ketoaldehydes, vinylsulphones, aldehydes, boronates or syrbactines.[6] Following part of presented work aims to design and implement such changes in the structure of salicylamides so they can be easily and quickly synthesized in good yields, tested and deemed effective in proteasomal inhibition of multiple myeloma cells. The target molecule was designed as noncyclic tripeptide salicylamide terminated with aldehyde or epoxyketone functional group. Two synthetic paths have been designed based on retrosynthetic analysis (see **Scheme 3**) and experimentally evaluated.



Scheme 3: Retrosynthetic analysis of target molecule of potential proteasome inhibitor

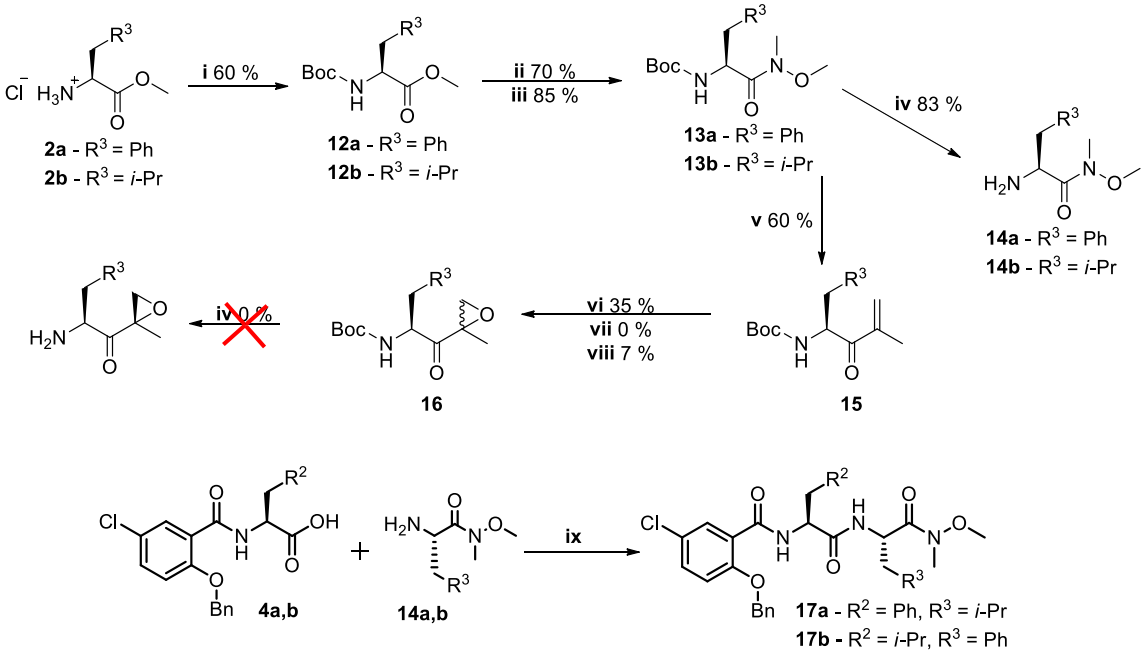
1.2.1 Building block method

First synthetic strategy is called the Building block method and its main point lies in the separate synthesis of the final functional group on the last *N*-protected amino acid. Such building block is subsequently connected via general amidation protocol to the rest of salicylamide substrate. Method was tested for synthesis of salicylamide tripeptides terminated with epoxyketone functional group.

Synthesis starts by protecting the amino group of **2a,b** into the form of *tert*-butyloxycarbonyl (**12a,b**) by general protocols. Next the Weinreb amide functional group is introduced (**13a,b**). Various methods and reagents are tested to obtain the reaction setting which gives the product in good purity and yield. Weinreb amide functional group is irreplaceable in modern organic synthesis due to its versatile reactivity and chemical properties such as being an excellent leaving group (nucleofuge) for nucleophilic substitution. Intermediate **13a,b** can be deprotected in the presence of TFA to obtain a building block **14a,b** suitable for inserting the Weinreb amide group to larger salicylamides (**17a,b**). Weinreb amide group in intermediate **13a,b** is replaced by isopropenyl motive (**15**), which is subjected to mild oxidation to obtain oxirane functional group (**16).** Several epoxidation protocols were tested, but none was acceptable. The reaction time, partial yield and difficult purifications lowered the already low total yield of the whole reaction procedure to overall unsuitable levels. Last step of the synthesis was not conducted successfully, although it is described in the literature.[7,8] Because of the low amounts of starting compound **16a** any further optimization of this last step was highly complicated. Building block strategy to synthesize the target molecules was considered unsuitable due to low overall yield and

several synthetically complicated steps. For reaction sequence see **Scheme 4**. The efforts to implement epoxyketone functional group to the final compound were abandoned due to poor reactivity of reagents even to these simple substrates.

Building block method can be used to synthesize dipeptide Weinreb amides (**17a,b**), which were prepared by the bellow mentioned Gradual synthesis method (see **1.2.2**) as well. The compounds are equal in the terms of quality and purity. The drawbacks of Building block method were already partially mentioned. The method takes longer to prepare target compounds. Poor reactivity in some stages and further loss of product in many purification steps makes the Building block method a synthetically possible but practically unsuitable way.

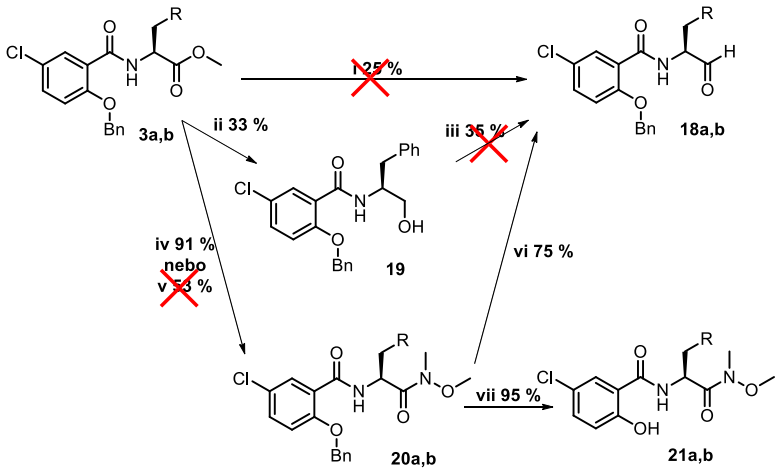


Scheme 4: Synthesis of building block for epoxyketone and Weinreb amide functional group insertion **i** for **12a**: $(Boc)_2O$, TEA, THF, 20 h, 0 °C to 25 °C; for **12b**: $(Boc)_2O$, EtOH, guanidine HCl, 35-40°C, **ii** *i*-PrMgCl, *O*,*N*-diethylhydroxylamine·HCl, dry THF, N_2, -40-0°C – 3 h; **iii** LiHMDS, *O*,*N*-diethylhydroxylamine·HCl, dry THF, N_2, -20 °C – 20 minute, -10 °C – 2 h; **iv** TFA, DCM, 25 °C - 45 minute; **v** *i*-propenylMgBr, THF, 0°C – 6 h, N_2; **vi** *m*-CPBA, DCM, 25 °C – 4 days; **vii** tetrahydrothiopyrane-4-on, oxone = 2 $KHSO_5 \cdot KHSO_4 \cdot K_2SO_4$, $NaHCO_3$, CH_3CN, H_2O, Na_2EDTA, 25 °C – 24 h; **viii** oxone, $NaHCO_3$, 1,1,1-trifluoroacetone, H_2O, CH_3CN, 0-25 °C – 5 h; **ix** EDCI·HCl, HOBt, CH_2Cl_2, 2 h, 25 °C.

1.2.2 Gradual synthesis method

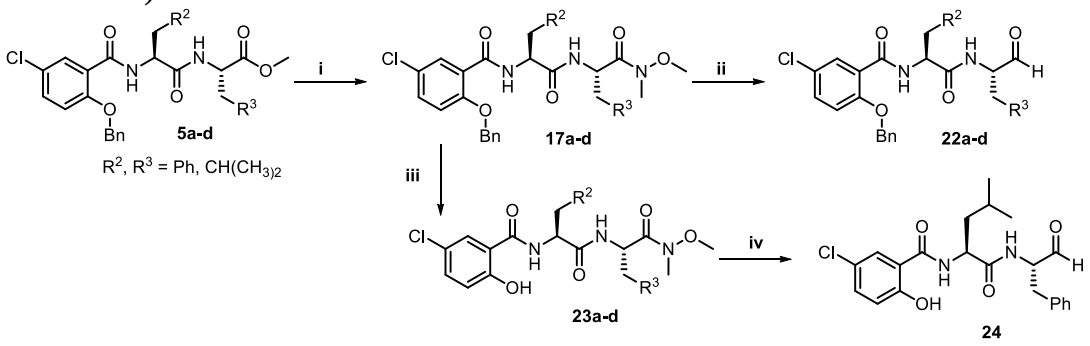
For Gradual synthesis method the general amidation methods optimized in chapter **1.1** are used as well as the peptide chain construction strategy. The peptide chain is built starting from *O*-Bn-5-chlorosalicylic acid (**1a**) step by step by repeating the amidation (EDCI·HCl (1 eq.), HOBt (1 eq.), K_2CO_3 (1,3 eq.), DCM, 25 °C, 1 h) and carboxylic group deprotection (LiOH (10 eq.), 1,4 dioxane/H_2O (1/1), 50 °C, 1h). L-Leucine and L-phenylalanine were used exclusively as amino acids. After the peptide chain is built to desired length the functional group is introduced to the *C*-terminus of the peptide. For aldehyde functional group implementation see **Scheme 5**. The direct reduction of

methylester **3a,b** with DIBAL in dry THF gave aldehyde **18a,b** in poor yield with difficult purification. Next method to obtain the aldehyde **18a** was based on total reduction of methylester **3a** by $NaBH_4$ to alcohol **19** followed by mild oxidation by Dess-Martin periodnane. This two-stage synthesis was also low yielding and problematic to purify. The final method was based on introducing the Weinreb amide group **20a,b**. There are several reaction protocols for this task, the method with LiHMDS was experimentally verified as the most useful considering the reaction time, the yield given and the complexity of purification. Weinreb amide functional group is an excellent nucleofuge and thus is it suitable for $LiAlH_4$ nucleophilic substitution to give aldehyde **18a,b** in the best achieved yield. The deprotection of phenolic hydroxy group with H_2 on 10% Pd/C was tested successfully as well.



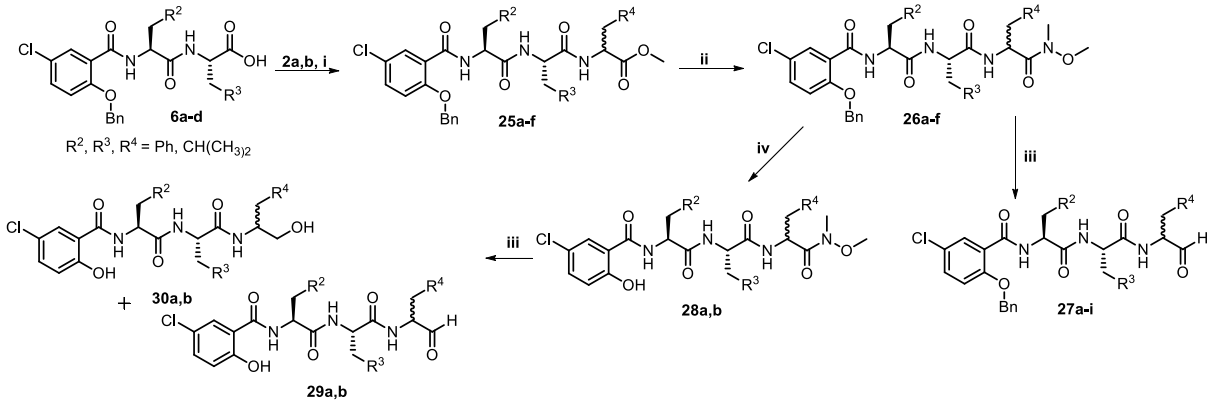
Scheme 5: Methods of aldehyde functional group synthesis
i DIBAL (1,2M in THF), dry THF, 3 h, -78 °C, N_2; **ii** $NaBH_4$, THF/H_2O – 3/1, 4 h; **iii** Dess-Martin periodnane, $NaHCO_3$, CH_2Cl_2, 1 h - 0°C, 1h - 25 °C; **iv** LiHMDS, *O,N*-dimethylhydroxylamine, dry THF, 1 h - -20 °C, then 4 h - -10 °C; **v** *i*-PrMgCl, *O,N*-dimethylhydroxylamine, dry THF, 1 h - -40 °C, then 3 h - 0 °C; **vi** $LiAlH_4$ (3 eq.), dry THF, 3 h - -20 °C, then 1 h - 25 °C; **vii** H_2, 10% Pd/C, EtAc, 20 h, 25 °C.

The reaction conditions and reagents for creating aldehyde functional group were tested on shorter derivatives (**3a,b**). The optimized reaction protocols were then applied to more advanced dipeptide salicylamides (see **Scheme 6**) and tripeptide salicylamides (see **Scheme 7**).



Scheme 6: Synthesis of dipeptide Weinreb amides and aldehydes
i LiHMDS, *O,N*-dimethylhydroxylamine, dry THF, 1 h - -20 °C, then 4 h - -10 °C; **ii** $LiAlH_4$ (3 eq.), dry THF, 0,5 h - -8 °C; **iii** H_2, 10% Pd/C, EtAc, 20 h, 25 °C; iv $LiAlH_4$ (3 eq.), dry THF, 1,5 h - -8 °C.

Totally 22 new original dipeptide salicylamides were synthesized. Out of them 4 bear carboxylic, 5 methylester, 8 Weinreb amide and 5 aldehyde functional group.



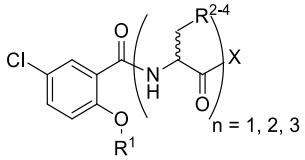
Scheme 7: Synthesis of tripeptide Weinreb amides and aldehydes
i EDCI·HCl, HOBt, K_2CO_3, CH_2Cl_2, 2 h, 25 °C; **ii** LiHMDS, *O,N*-dimethylhydroxylamin, suché THF, 1 h při -20 °C, pak 2 h při -10 °C, 16 h – 25 °C; **iii** $LiAlH_4$ (2 ekv), suché THF, 6 h při -8 °C; **iv** H_2, 10% Pd/C, EtAc, 3 h, 25 °C; v $LiAlH_4$ (6 ekv), suché THF, 3 h při -8 °C;

Totally 27 new original dipeptide salicylamides were synthesized. Out of them 6 methylester, 8 Weinreb amide, 11 aldehyde and 2 alcohol functional group. The detailed summary of prepared compounds can be seen in **Table 3**. Aldehydes are expected to achieve some perspective activities, but Weinreb amides were never tested for such purpose.

1.2.3 Biological activities of novel salicylamides

Novel series of di-/tripeptide salicylamide aldehydes (**22a-d**, **24**, **27a-i**, **29a,b**) and Weinreb amides (**17a-d**, **23a-d**, **26a-f**, **28a,b**) as well as other compounds prepared within this topic were tested for antiproliferative activities against various tumour cell lines (leukemia K562 and CEM and multiple myeloma U266). The results can be seen in **Table 3**. Derivatives bearing the benzyl protective group seem to achieve better activities than their deprotected analogues. The reason might be the presence of unprotected and highly polar phenolic hydroxyl group, which may influence the process of entering of the salicylamide inhibitor inside the proteasome. The tripeptide Weinreb amide derivatives achieve better mid-micromolar activities (**28a**,**b**, GI_{50} in range of 16,3-32,8 μM) compared to derivatives with mono- or dipeptide chain (**21a,b**, **23a-d**, GI_{50} in range of 48,2-100 μM). The aldehydes with tripeptide moiety achieve the best activities overall in the mid-nanomolar range (**27a-i**, GI_{50} in range of 0,15 1,45 μM). According to antiproliferative activities multiple myeloma cell line U266 seems to be highly susceptible to **27a-i** exposure. The results show the tested compounds achieve IC_{50} in mid-nanomolar values in range 57,0-197 nM, which is higher than IC_{50} achieved by bortezomib (4 nM). De/benzylated derivatives bearing phenylalanine next to the terminal aldehyde group achieve higher IC_{50} values than derivatives bearing leucine at the same position. The most perspective derivatives were chosen and further tested for U266 proteasomal inhibition. All biological activities and cytotoxicity surveys were conducted at Laboratory of Growth Regulators, Faculty of Science, Palacký University Olomouc and the Institute of Experimental Botany, Czech Academy of Sciences.

Table 3: Antiproliferative and antiproteasomal actvities of novel salicylamides

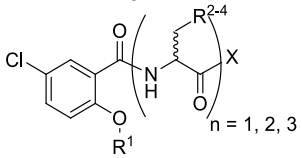


Prod.	n	R^1	Amino acids	X	GI_{50} (μM)[a]			Proteasomal inhibition IC_{50} (μM) [a]
					K562	CEM	U266	
18a	1	Bn	L-Phe	H	33,3	17,7	63,8	>1
18b	1	Bn	L-Leu	H	23,6	24,3	55,8	>1
20a	1	Bn	L-Phe	NCH_3OCH_3	50,9	65,2	92,3	NT
20b	1	Bn	L-Leu	NCH_3OCH_3	42,7	45,2	54,1	NT
21a	1	H	L-Phe	NCH_3OCH_3	99,1	55,5	72,4	NT
21b	1	H	L-Leu	NCH_3OCH_3	>100	58,6	>100	NT
17a	2	Bn	L-Phe-D-Leu	NCH_3OCH_3	25,8	32,2	67,4	NT
17b	2	Bn	L-Leu-L-Phe	NCH_3OCH_3	22,5	19,5	81,9	NT
17c	2	Bn	L-Leu-L-Leu	NCH_3OCH_3	33,2	22,2	35,2	NT
17d	2	Bn	L-Phe-L-Phe	NCH_3OCH_3	29,6	28,6	72,1	NT
22a	2	Bn	L-Phe-DL-Leu	H	17,3	15,1	17,7	>1
22b	2	Bn	L-Leu-L-Phe	H	16,3	11,0	14,8	>1
22c	2	Bn	L-Leu-DL-Leu	H	21,0	16,3	37,0	>1
22d	2	Bn	L-Phe-L-Phe	H	27,1	15,5	21,7	>1
23a	2	H	L-Phe-L-Leu	NCH_3OCH_3	90,9	64,1	71,6	NT
23b	2	H	L-Leu-L-Phe	NCH_3OCH_3	93,6	48,2	79,2	NT
23c	2	H	L-Leu-L-Leu	NCH_3OCH_3	98,1	63,0	69,0	NT
23d	2	H	L-Phe-DL-Phe	NCH_3OCH_3	79,5	54,2	68,7	NT
24	2	H	L-Leu-L-Phe	H	57,9	27,7	64,9	>1
25a	3	Bn	L-Leu-L-Leu-D-Leu	OMe	>100	51,0	82,0	NT
25b	3	Bn	L-Leu-L-Leu-L-Leu	OMe	87,5	48,4	84,9	NT
25c	3	Bn	L-Leu-L-Leu-L-Phe	OMe	70,3	31,4	72,2	NT
25d	3	Bn	L-Leu-L-Phe-L-Leu	OMe	58,5	48,5	83,7	NT
25e	3	Bn	L-Leu-L-Phe-D-Leu	OMe	100	68,2	88,5	NT
25f	3	Bn	L-Leu-L-Phe-L-Phe	OMe	72,0	48,0	82,1	NT
26a	3	Bn	L-Leu-L-Leu-D-Leu	NCH_3OCH_3	34,0	11,5	23,7	NT
26b	3	Bn	L-Leu-L-Leu-L-Leu	NCH_3OCH_3	55,6	30,6	55,2	NT
26c	3	Bn	L-Leu-L-Leu-L-Phe	NCH_3OCH_3	44,2	15,1	47,2	NT
26d	3	Bn	L-Leu-L-Phe-L-Leu	NCH_3OCH_3	16,4	19,0	48,6	NT
26e	3	Bn	L-Leu-L-Phe-D-Leu	NCH_3OCH_3	28,9	17,6	22,6	NT
26f	3	Bn	L-Leu-L-Phe-L-Phe	NCH_3OCH_3	34,7	35,7	56,3	NT
27a	3	Bn	L-Leu-L-Leu-DL-Leu	H	**0,61**	**0,49**	**0,2**	**0,099**
27b	3	Bn	L-Leu-L-Leu-L-Leu	H	**0,76**	**0,41**	**0,20**	**0,057**
27c	3	Bn	L-Leu-L-Leu-D-Leu	H	**0,98**	**0,58**	**0,38**	**0,182**
27d	3	Bn	L-Leu-L-Leu-DL-Phe	H	**1,05**	**0,79**	**0,54**	**0,197**
27e	3	Bn	L-Leu-L-Phe-L-Leu	H	**1,04**	**0,43**	**0,17**	**0,067**
27f	3	Bn	L-Leu-L-Phe-D-Leu	H	**0,73**	**0,40**	**0,21**	**0,075**
27g	3	Bn	L-Leu-L-Phe-DL-Leu	H	**0,57**	**0,28**	**0,15**	**0,090**
27h	3	Bn	L-Leu-L-Phe-L-Phe	H	**1,45**	**0,44**	**0,40**	**0,110**
27i	3	Bn	L-Leu-L-Phe-DL-Phe	H	**1,28**	**0,40**	**0,46**	**0,168**
28a	3	H	L-Leu-L-Phe-L-Leu	NCH_3OCH_3	30,9	17,2	32,8	NT
28b	3	H	L-Leu-L-Phe-L-Phe	NCH_3OCH_3	22,2	16,3	24,1	NT
29a	3	H	L-Leu-L-Phe-L-Leu	H	**2,10**	**0,78**	**0,67**	**2,37**
29b	3	H	L-Leu-L-Phe-L-Phe	H	**6,90**	**2,30**	**2,23**	**0,740**
30a	3	H	L-Leu-L-Phe-L-Leu	CH_2OH	22,4	8,45	11,8	NT
30b	3	H	L-Leu-L-Phe-L-Phe	CH_2OH	27,3	10,8	18,9	NT
Bortezomib					0,007	0,001	0,001	0,004

[a] Value is calculated from at least two measurments. NT – not tested.

Although at least minimal coinhibition of all proteolytic subunits of the 20S proteasome core is necessary for the desired apoptosis induction, major inhibition of β5 subunit is still considered as essential.[9] Bortezomib and carfilzomib meet this requirement, although in higher dosage, which can amplify undesired side effects. The determination of inhibition selectivity of all aldehyde terminated derivatives is presented in **Table 4**. Cell-based assays using fluorogenic substrates revealed that the compounds display preference towards the chymotrypsin-like β5 subunit.

Table 4: Antiproteasomal activities of novel salicylamides

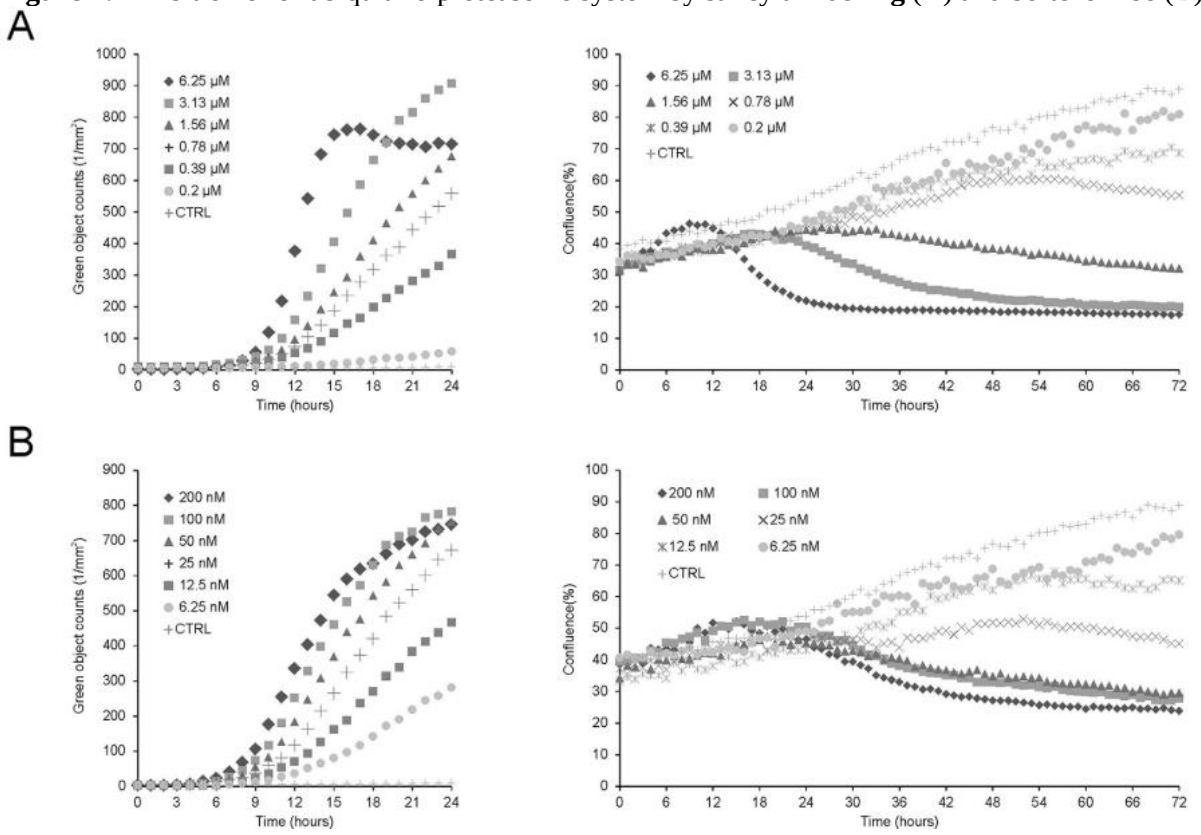


Prod.	n	R^1	Amino acids	X	Residual activity (%)[a] at 10μM		
					β1 caspaze	β2 trypsine	β5 chymotrypsine
18a	1	Bn	L-Phe	H	53,0	70,2	70,8
18b	1	Bn	L-Leu	H	68,2	57,5	68,8
22a	2	Bn	L-Phe-DL-Leu	H	39,9	92,0	95,0
22b	2	Bn	L-Leu-L-Phe	H	67,8	85,9	85,2
22c	2	Bn	L-Leu-DL-Leu	H	35,3	88,1	84,3
22d	2	Bn	L-Phe-L-Phe	H	71,5	78,4	97,3
24	2	H	L-Leu-L-Phe	H	≥100	81,0	≥100
27a	3	Bn	L-Leu-L-Leu-DL-Leu	H	39,2	64,2	10,4
27b	3	Bn	L-Leu-L-Leu-L-Leu	H	34,6	54,2	11,8
27c	3	Bn	L-Leu-L-Leu-D-Leu	H	35,5	74,3	18,0
27d	3	Bn	L-Leu-L-Leu-DL-Phe	H	66,2	48,4	27,9
27e	3	Bn	L-Leu-L-Phe-L-Leu	H	40,2	42,5	10,6
27f	3	Bn	L-Leu-L-Phe-D-Leu	H	19,7	≥100	20,7
27g	3	Bn	L-Leu-L-Phe-DL-Leu	H	74,6	81,8	13,4
27h	3	Bn	L-Leu-L-Phe-L-Phe	H	≥100	47,2	25,8
27i	3	Bn	L-Leu-L-Phe-DL-Phe	H	≥100	62,3	29,1
29a	3	H	L-Leu-L-Phe-L-Leu	H	28,9	72,6	27,9
29b	3	H	L-Leu-L-Phe-L-Phe	H	73,5	84,2	71,7

[a] Value is calculated from at least two measurments.

In order to confirm proteasome targeting by an independent method, a cell-based assay involving Green Fluorescence Protein (GFP) fused to a short degron was conducted.[10] Accumulation of GFP in cells treated with proteasome inhibitors was visualised and quantified by live-cell imaging. The results were compared with cells treated with bortezomib and untreated cells (**Figure 4 A,B**). As shown in **Figure 4A** the GFP signal in cells emerged approximately 9 h after exposing to the compound. The most potent compound (**27g**) inhibited the proteasome already at concentration 200 nM. The maximum concentration used (6,25 mM) was already toxic for cells after 16 h treatment. The proliferation of treated cells was monitored over 72 h. Results showed that compound **27g** can activate apoptotic machinery in a time-dependent manner.

Figure 4: Inhibition of of ubiquitine-proteasome system by salicylamide **27g** (**A**) and bortezomibe (**B**)



CONCLUSION

Review part of presented thesis describes in detail several topics, which together form solid information foundation for the synthetical part. This part describes observed biological properties, methods of synthesis and the future perspective of salicylamides. The review in detail describes peptide synthesis, used reagents and additives suppressing racemization, their advantages and disadvantages. The following part describes the causes, development and options of analysis and quantification of racemization during peptide synthesis. Classical as well as modern methods of peptide synthesis like solid state synthesis or automated synthesis are discussed. The final part of review describes the function of proteasome and the consequences of proteasomal inhibition, which could be used in tumour cell treatment (breast or column cancer, multiple myeloma). Summary of FDA already approved and novel proteasome inhibitors is presented at the end of review.

At the beginning of the synthetic work massive racemization issue in compounds **5a-d** with two stereomeric centres was observed. By synthesis of an alternative line of compounds with different protective groups the influence of strong inorganic base present in one deprotection step was on the racemization was ruled out. The amidation conditions were determined as the crucial step. By suitable replacements of reagents and fine adjustments of reactant ratios a set of ideal amidation conditions with suppressed racemization was achieved. The final ratio of diastereoisomers is 96:4 (92 % ee). By the successfully conducted optimization the reaction time for one amidation step was reduced from 20 h to 1 h. The column chromatography purification is simpler due to lower amount of present side products. Thus, the yield of amidation raised from 65 % to 90 %. The possibility of scale-up was verified as well. **Table 1** summarizes the amidation conditions as well as achieved % ee for model compounds **5a-i-iv** and **9**.

Design of the first group of prepared compounds is subordinated to previous works, because the main goal is to observe the changes in biological activities according to certain structural changes in various combinations. The new compounds are to fill the logical gaps in the previous design. The synthetic method of new salicylamide derivatives with one amino acid terminated with aliphatic amides or (**10a-e, 11a-d**) was prepared by the gradual synthesis. When compared with the antiproliferative activities against leukemia cell lines (K562, THP-1, CEM) achieved by compounds prepared by Pratibha Magar these compounds achieve only higher mid-micromolar values (GI_{50} 14,4-93,3 μM). The substitution of anilide moiety for aliphatic amide had negative effect on antiproliferative activities against tested leukemia cell lines.

Design of the second group od prepared salicylamides applicable as proteasome inhibitors draws from the covalent peptide inhibitor terminated with aldehyde or epoxyketone functional group. The peptide chain consists of leucine, phenylalanine or their combinations exclusively. For the synthesis of the second group of derivatives two synthetic routes are designed based on the retrosynthetic analysis of target molecule. Both methods are experimentally verified and the superior is chosen for the synthesis of the whole series. First tested method is called the Building block method and the functional group is prepared separately on last amino acid to be joined to the rest of the salicylamide via optimized amidation protocol. The main goal of this procedure is to

synthesize the epoxyketone derivatives. Due to the problematic last step, which did not led to desired product, due to a large number of partial synthetic steps, problematic purification and low yields the total yield (9 %) of this method is unsatisfactory and unusable. Building block method was abandoned and not further optimized.

The second tested method is called the Gradual synthesis method and its strategy is to build the peptide chain step by step starting from O-Bn-5-chlorosalicylic acid (**1a**) and by repeating the amidation and carboxylic group deprotection step to build the peptide chain in desired composition and length. Optimized amidation protocol was successfully used to avoid racemization. The functional aldehyde group was created at the *C*-terminus of the peptide moiety. The reaction protocols were tested and optimized on simpler salicylamides with one amino acid and the with minor adjustments applied to synthesize di-/tripeptide salicylamide aldehydes (**22a-d**, **24**, **27a-i**, **29a,b**). By this method 46 original salicylamides with peptide chain made of leucine, phenylalanine or their combination were obtained and analysed by available methods. Prepared compounds were also analysed for their antiproliferative activities against two leukemia and one multiple myeloma cell lines. Some salicylamides wit aldehyde group achieved significant antiproliferative activity against multiple myeloma in nanomolar range. Connection between the proteasomal inhibition and antiproliferative activity was confirmed for all three tested cell lines. The most promising compounds (**27g**) achieved cellular effects corresponding to proteasomal inhibition and induced apoptosis against multiple myeloma.

One structural rule is valid for all molecules out of second group (potential proteasomal inhibitors). Molecules with protective benzyl group achieve better antiproliferative activities than their deprotected alternatives. The reason is probably the presence of highly polar phenolic hydroxyl group, which may interfere with proteasomal walls and complicate continuous entrance of the inhibitor inside of the proteasome or its coordination inside of the proteasome. Thus, derivatives with sterically larger but nonpolar benzyl group achieve better antiproliferative activities. Main structural aspect affecting the biological activities is the length of the peptide chain. Tripeptide derivatives achieve the best activities. Next aspect is the amino acid present at the last position in the peptide chain next to the functional group. If leucine occupies this position the compounds achieve better activities. The final rule valid for all molecules is the aldehyde functional group, which achieves the best results. Tested molecules inhibit all proteolytic sites but prefer the chymotrypsine-like site β5 subunit, which was observed by bortezomib and carfilzomib as well. Compound **27g** achieves in concentration 1,5 μM in 24 hours same antiproteasomal activities as bortezomib in concentration 50 nM.

The difference in activity is not an obstacle for compound **27g**. The proteasomal inhibition is still a new method of cancer treatment and even if there are already some FDA approved substances for multiple myeloma treatment, new and more severe side effect are observed more often. These side effects complicate the procedure, their lower the quality of patients life and might lead to interruption of started medication. That can lead to relapse of the illness, its strengthening or gaining immunity to common

treatment. These are the reasons why every newly discovered perspective compound might bring less severe side effects.

All synthesized compounds were characterised by NMR, MALDI-TOF, elementary analysis, infrared spectroscopy and, where the solid state of the product allowed, melting point measurement.

The results of this thesis were presented in several domestic and international scientific conferences and have been published in 2 scientific publications (both experimental).

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