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DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION OF CXCR3 AND CXCR4 MODULATORS WITH PYRAZOLOPYRIDINE SCAFFOLD

NAČRTOVANJE, SINTEZA IN BIOLOŠKO VREDNOTENJE MODULATORJEV KEMOKINSKIH RECEPTORJEV CXCR3 IN CXCR4 S PIRAZOLOPIRIDINSKIM SKELETOM

Ljubljana, 2015

This master thesis was performed at the University of Ljubljana at the Faculty of Pharmacy, Department of Pharmaceutical Chemistry under the supervision of Assoc. Prof. Dr. Marko Anderluh and at the Friedrich Alexander University Erlangen-Nürnberg at the Department of Chemistry and Pharmacy, Division of medicinal chemistry under the supervision of Dr. Nuška Tschammer.

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Statement

I hereby declare that this Master's thesis was done by me under supervision of Assoc. Prof. Dr. Marko Anderluh and co-supervision of Dr. Nuška Tschammer.

Anja Kolarič

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ABSTRACT

The chemokine receptors CXCR3 and CXCR4 are G protein-coupled receptors, which convey extracellular signals into cells through conformational changes upon binding of their endogenous ligands chemokines CXCL11 and CXCL12. The overexpression of these receptors is linked to severe diseases such as inflammation, autoimmune diseases (e.g., multiple sclerosis, psoriasis, and rheumatoid arthritis), transplant rejection, cancer and HIV. The chemokine receptors can be regulated by allosteric modulators and the aim of this thesis is to develop CXCR3 and CXCR4 antagonists.

The first part of the thesis was performed at the Faculty of Pharmacy at University of Ljubljana, where the potentional antagonists were synthesized. They consist of pyrazolopiridine scaffold, connected via amide bond to four different linkers, which carry one or two aromatics that can be substitued. The second part of the thesis consisted of pharmacological profiling of the synthesized compounds and was performed at the Department of Chemistry and Pharmacy at the Friedrich-Alexander-University Erlangen-Nürnberg. The profiling was carried out in the β-arrestin 2 recruitment and BRET (The bioluminescence resonance energy transfer) based cAMP (Cyclic adenosine monophosphate) assays using HEK293T (Human embryonic kidney) cells expressing either CXCR3 or CXCR4.

We discovered several promising allosteric modulators, which displayed selective activity on each receptor. With linkers' modification and substitution patterns we have achieved negative and also positive allostery modulation. The negative modulators with piperidine linker and para substituted benzylamino group appeared as the most promising with equal inhibition of both receptors upon the activation with chemokines.

The thesis represents important findings in the field of allosteric modulators of CXCR3 and CXCR4 receptors. Discovered negative allosteric modulators presents valueable starting point for further development of potential lead compounds.

POVZETEK

Kemokinska receptorja CXCR3 in CXCR4 pripadata družini s proteinom G sklopljeni receptorji, katerih funkcija je prevajanje ekstracelularnega signala v notranjost celice po vezavi endogenih ligandov. Endogeni ligandi receptorjev CXCR3 in CXCR4 so kemokini, to so krajši proteini, ki so odgovorni za razvoj in vzdrževanje imunskega sistema, sodelujejo pa tudi v patofizioloških procesih. Receptor CXCR3 veže endogene ligande CXCL9, CXCL10 in CXCL11, medtem ko receptor CXCR4 veže kemokin CXCL12.

Prevelika ekspresija teh dveh receptorjev je bila ugotovljena pri velikem naboru bolezni, pri čemer je CXCR3 vpleten v vnetne bolezni, vključno z avtoimunimi boleznimi (revmatoidni artritis, multipla skleroza, luskavica…), zavrnitve presadkov, sodeluje pa tudi pri rakavih obolenjih (rak dojk, črevesja, pljuč, bezgavk…). Prav tako je tudi receptor CXCR4 vpleten v rakava obolenja (rak ovarijev, prostate, požiralnika, kože…), sodeluje pa tudi pri vstopu virusa HIV v telo.

Več antagonistov kemokinskih receptorjev je prišlo do klinične faze testiranj, vendar sta na trgu prisotna samo dva. To lahko pripišemo nedavnemu odkritju, da je mogoče funkcijo kemokinskih receptorjev uravnavati z alosteričnimi modulatorji, ki so bolj uporabni v terapiji od direktnih agonistov/antagonistov zaradi možnosti fine modulacije funkcije receptorjev. To so majhne molekule, ki se vežejo na alosterično vezavno mesto in vplivajo na afiniteto in/ali učinkovitost ortosteričnih ligandov tako, da jo povečajo, zmanjšajo ali pa se samo vežejo. Ker alosterični modulatorji lahko uravnavajo delovanje kemokinskih receptorjev, je naš namen razviti alosterične modulatorje receptorjev CXCR3 in CXCR4.

Prvi del naloge je bil izveden na Fakulteti za farmacijo, Univerze v Ljubljani, kjer smo sintetizirali potencialne antagoniste na podlagi izhodne spojine za katero je bilo ugotovljeno, da deluje kot negativni alosterični modulator receptorjev CXCR3 in CXCR4. Spojine sestavlja pirazolopiridinski skelet, ki je preko karbonilne skupine povezan s štirimi različnimi distančniki, 1,3-diaminopropan, piperazin in 4-aminopiperidin z različno orientacijo. Na distančnik je vezan en ali dva aromatska obroča (predvsem benzilni obroč), ki sta lahko substituirana s halogeni.

Drugi del naloge predstavlja karakterizacija sintetiziranih spojin na Oddelku za kemijo in farmacijo, Univerze Friedrich Alexander Erlangen-Nürnberg. Testiranje je potekalo s

testoma β-arrestin 2 recruitment in BRET (The bioluminescence resonance energy transfer) based cAMP (ciklični adenosin monofosfat) na HEK293T (humane embrionalne ledvične) celicah, katere izražajo receptor CXCR3 ali CXCR4. BRET based cAMP test je bil v tej nalogi prvič uporabljen kot test za karakterizacijo receptorjev CXCR3 in CXCR4. Prednosti tega testa pred do sedaj uporabljenim β-arrestin 2 recruitment so predvsem v hitrejši izvedbi in občutljivosti, kajti s tem testom uspeli testirati spojine za katere pri β-arrestin 2 recruitment nismo zaznali povečanja signala.

Odkrili smo nekaj obetavnih alosteričnih modulatorjev, ki so izrazili selektivno delovanje na enem izmed receptorjev. Z modifikacijami distančnika in različnimi vzorci substitucije smo dosegli negativno in tudi pozitivno modulacijo, vendar pa je ta bilo šibkejša. Kot najbolj obetajoči negativni modulatorji na obeh receptorjih se najbolj izkazali tisti s piperidinskim distančnikom in *para*-substituirano benzilaminsko skupino. Spojine z 1,3-diaminopropanskim distančnikom in dvema nesubstituiranima aromatskima skupinama so delovala selektivneje na CXCR4 receptorje, medtem ko so se spojine z 4-aminopiperidinskim distančnikom izkazale kot najslabši modulatorji.

Presenetila nas je ugotovitev, da spojina, katera nam je služila kot izhodišče in smo jo ponovno sintetizirali in ji določili biološki učinek *in vitro*, pokazalo samo šibak negativni alosterični učinek in še to samo na receptorju CXCR3. Razlog temu pripisujemo drugačni eksperimentalnim pogojem.

To delo predstavlja izvirna dognanja na področju alosterične modulacije receptorjev CXCR3 in CXCR4. Odkriti negativni alosterični modulatorji so izhodišče za nadaljnji razvoj potencialnih spojin s selektivnim modulatornim delovanjem na omenjena receptorja.

THE LIST OF ABBREVIATIONS

AcCN	Acetonitrile
BTEAC	Benzyltriethylammonium chloride
δ	Chemical shift
$CDCl_3$	Chloroform-d
J	Coupling constant
DCM	Dichloromethane
DMF	Dimethylformamide
TBTU	*O*-(benzotriazol-1-yl)-*N, N, N′, N′*-tetramethyluronium tetrafluoroborate
EtOAc	Ethyl acetate
HOBt	1-hydroxybenzotriazol
NMM	*N*-methylmorpholine
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
DMSO-d6	Dimethyl sulfoxide-d6
d	Doublet
dd	Doublet of doublets
Eq	Equivalent
HRMS	High resolution mass spectrometry
MS	Mass spectrometry
MF	Mobile phase
m	Multiplet
NMR	Nuclear magnetic resonance
Rf	Retention factor
q	Quartet
p	Quintet
s	Singlet
SEM	Standard error of mean
Boc	*Tert*-butil carbamate
Et_3N	Triethylamine
TFA	Trifluoroacetic acid
t	Triplet

1 INTRODUCTION

1.1 G protein-coupled receptors

G protein-coupled receptors (GPCRs), also known as seven transmembrane (7TM) receptors are the largest family of membrane proteins. Their function is to convey extracellular signal into cells through conformational changes upon binding of an agonist[1]. They are divided into three families that share seven-helix structure, but differ in the length of extracellular *N*-terminus and the location of agonists binding site. The largest subfamily is Group A or rhodopsin-like, followed by secretin/glucagon or B family and C or metabotropic glutamate subfamily [1, 2].

GPCRs consist of an extracellular *N*-terminus, seven helices connected with three extracellular and three intracellular loops, and an intracellular *C*-terminus. The third intracellular loop has the ability to bind trimeric guanine nucleotide binding protein (G-protein). From the three subunits α, β, and γ only α subunit possesses enzymatic activity. In the inactivated state all three subunits are bound together with the α subunit carrying guanosine diphosphate (GDP). Conformational changes caused by the agonist binding to the receptor induce the conformational changes of the trimeric G-protein. Upon GDP exchange with guanosine triphosphate (GTP), α subunit dissociates from the receptor and consequently activates various effectors, which then initiate different signaling cascades and cellular events. The βγ subunits stay together and activate separate set of effectors. GTP is then hydrolyzed trough the GTPase (guanosine triphosphatase) activity of the α subunit and the α subunit re-associates with the βγ subunit, which puts the receptor in the resting state (Figure 1) [1, 2].

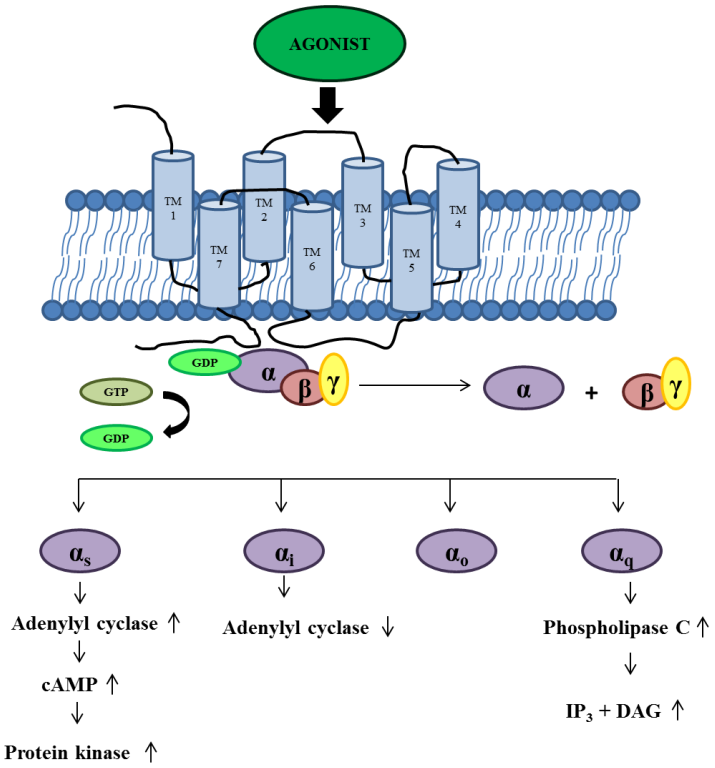


Figure 1: GPCR signaling. Binding of the agonist leads to exchange of GDP to GTP, causing dissociation of subunits, which activate various pathways [1].

1.2 Chemokines

Chemokines (chemoattractant cytokines) are a group of small (8–10 kDa) proteins responsible for the migration and response of immune cells. They convey their signal by interaction with their cognate chemokine receptors, which belong to the family of Group A GPCRs [3, 4, 5]. Functionally chemokines have two main roles: (1) homeostatic chemokines play an important role in development and maintenance of immune system, and (2) inflammatory chemokines are produced under infection or inflammation and play important role in various pathophysiological conditions [6]. Chemokines have four cysteines and are, based on the residues between the first two cysteines in their polypeptide chain, classified into four groups (CXC, CC, C and CX3C). The tertiary structure of chemokines is stabilized by two disulfide bonds, formed between the first and third and between the second and fourth cysteine [7]. The chemokine activity is initiated by the interaction between chemokine and its receptor by two-step mechanism. In the first step, the main domain of chemokine binds to the extracellular surface and the *N*-loop of the receptor, and in the second step conformational changes of chemokines' *N*-terminal

domain enable interaction with transmembrane domains and extracellular loops of the receptor that results in receptor activation [8].

1.3 CXCR3 and CXCR4 receptors

The CXCR3 receptor was first discovered in 1996 by Loetscher and colleagues during the cloning from a cDNA library derived from CD4+ T cells. CXCR3 is mostly expressed on activated Th1 cells, but it is also located on circulating blood T cells, B cells and on natural killer cells. Receptors' endogenous ligands are CXCL9, CXCL10, and CXCL11, of which CXCL11 is the dominant ligand, followed by CXCL10 [9].

CXCR4 was discovered by screening the chemokine receptor orphan genes for its endogenous ligand CXCL12 to find the receptor that can induce the intracellular Ca^{2+} efflux. It is expressed by different immune cells like monocytes, B cells and naive T cells. CXCR4 binds only the chemokine CXCL12 [6].

The endogenous ligands of CXCR3 and CXCR4 are produced and released by macrophages and lymphocytes [1]. Chemokine binding to CXCR3 and CXCR4 leads to activation of pertussis toxin-sensitive $G\alpha_i$ class of G proteins, which results in chemotaxis, inhibition of adenylate cyclase and thus lowering the concentration of cyclic adenosine monophosphate (cAMP), mobilization of Ca^{2+}, and activation of different kinases [9, 10]. Processes following the activation of the receptors lead to desensitization and internalization of receptors. Desensitization is initiated by G protein-coupled kinases (GRK), which phosphorylate the receptor at the C-terminal serine/threonine residues. This phosphorylation allows binding of β-arrestin, which is involved in the receptor internalization. After the internalization receptors can be recycled on the plasma membrane or degraded in lysosomes [10].

1.4 CXCR3 and CXCR4 as potential drug targets

Overexpression of CXCR3 and its endogenous ligands has been found to play an important role in a variety of inflammatory diseases including autoimmune diseases (e.g., multiple sclerosis, psoriasis, and rheumatoid arthritis), transplant rejection, and cancer [4, 9]. CXCR4 is often overexpressed in various cancers (e.g., breast cancer, prostate cancer, and colon cancer) and serves as co-entry receptor for HIV [11, 12].

Interplay between both receptors is reflected in the development and progression of solid tumors through interactions with CXCL11 and CXCL12. There is evidence for different expression and overexpression of these receptors in tumor endothelial and also in cancer stem cells, indicating their involvement in angiogenesis and metastasis [6]. CXCR4 is involved in more than 20 human tumor types like ovarian, prostate, esophageal, melanoma and renal cell carcinoma. CXCR3 is responsible for the promotion of melanoma, osteosarcoma and for metastasis of colon, breast, lymph nodes and lungs cancer [4, 11]. The investigations of the role of chemokines and their receptors in cancer are ongoing and represent opportunities for identification of novel drug targets.

Involvement of chemokines and their receptors in a number of disorders make them a very attractive therapeutic target, and it is no wonder that the interest of pharmaceutical companies in CXCR3 and CXCR4 has grown in the last years [4].

1.5 Chemokine receptor antagonists

Over 40 chemokine receptor antagonists have progressed into human clinical trials, but only two small molecules have succeeded to the market [8]. Maraviroc (Selzentry®) is allosteric inhibitor of the CCR5 receptor and it is used for the treatment of HIV infection. AMD-3100 (Mozobil®) has been found to inhibit CXCR4, which provides inhibition of stem cells mobilization and is used for treatment of non-Hodgkin lymphoma [8, 9]. The main cause for the failure of chemokine antagonists was attributed to inappropriate target selection, ineffective dosing, off-target effect and poor drug-like properties [8].

1.6 Mechanism of allosteric modulation

Historical approach was oriented in development of GPCRs agonists and antagonists that bind to orthosteric receptor binding site. In the last few years it was discovered that a lot of ligands act as allosteric modulators. These are small molecules that bind to the receptor binding site distinct from the orthosteric site, with the ability to act as positive, negative or neutral allosteric modulators [13, 14]. When the orthosteric agonist binds to the GPCR receptor conformational changes occur, which leads to activation of the receptor and various effects. Positive allosteric modulators enhance the affinity and/or efficacy of the orthosteric agonist. On the other hand negative allosteric modulators decrease the affinity and/or efficacy of the orthosteric agonist. Neutral allosteric ligands do not affect affinity

and/or efficacy of the orthosteric agonist, but they prevent binding of other modulators [14].

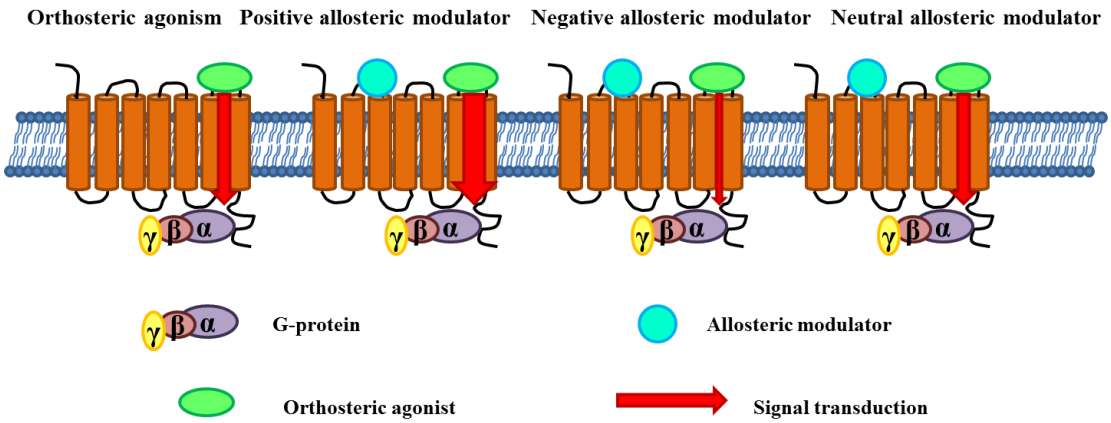


Figure 2: Allosteric modulators mode of action. Allosteric modulators modulate the agonistic signals in the presence of orthosteric agonist by binding to the distinct part of receptor.

Not only that the binding location of allosteric modulators differs from the orthosteric ligands, but there is also no structural relationship between allosteric modulators and endogenous agonists. In fact, endogenous agonist can interact with the receptor even in the presence of the allosteric modulator and their binding usually triggers fine modulation of agonist activity. This is not true for orthosteric ligands, which bind to receptor orthosteric site in a competitive mode with endogenous agonist/ligand, so a high affinity is a prerequisite for their potent activity or they have to be delivered in high concentration, both of which can be toxic [8]. This inherent advantage of allosteric modulators is the key feature in discovery of potential drugs for variety of diseases related to chemokine receptors, where most of potential drugs target allosteric rather than orthosteric binding site and mode of action.

1.7 Allosteric small molecule antagonists

Since allosteric modulators showed the ability to modulate the chemokine receptors, structurally diverse ligands for CXCR3 have been disclosed, including (aza)quinazolinones, pyrido-[1,2-a]pyrimidin-4-ones, piperazinylpiperidines, 1-aryl-3-piperidin-4-yl-ureas, 4-*N*-aryl-[1,4]diazepanylureas, 2-iminobenzimidazoles, bispiperidines and ergolines [15].

The most studied member of azaquinazolines is AMG487, which progressed to Phase II clinical trials for psoriasis and rheumatoid arthritis, but failed because of lack of efficacy [4]. AMG487 consists of azaquinazolinone scaffold connected with the C-stereogenic center, whose absolute configuration dictates affinity, and to other hydrophobic groups, such as phenylacetamide and pyridine to provide metabolic stability. The scaffold is also carrying substituted phenyl group (*p*-ethoxyphenyl grup), also responsible for affinity [15].

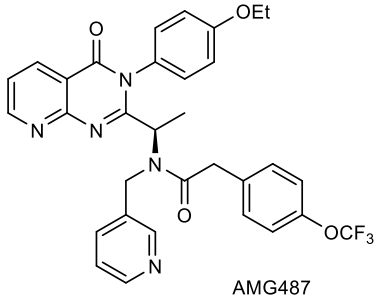


Figure 3: A representative of the class of azaquinazolines.

Azaquinazolinone bicycle core can be exchanged for a variety of heterocyclic groups to obtain the class of pyrido-[1,2-a]pyrimidin-4-ones. Unfortunately, this class of compounds is unstable *in vivo* because of the CYP induction [15].

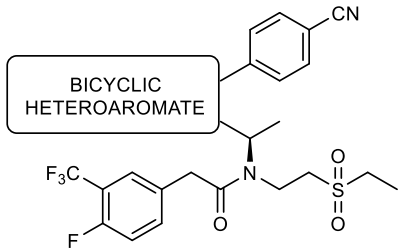


Figure 4: General structure of pyrido-[1,2-a]pyrimidin-4-ones.

Piperazinylpiperidines consist of a substituted benzyl unit, attached to piperazinyl-piperidine-scaffold and a polar group. Aryl substituents are usually halogens [15].

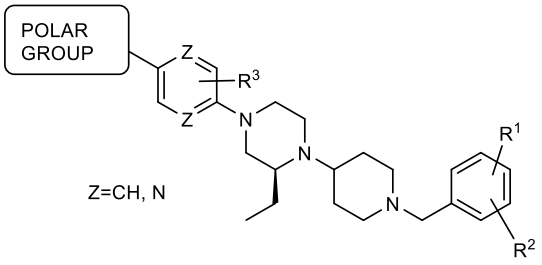


Figure 5: General structure of piperazinylpiperidines.

The class of 1-aryl-3-piperidin-4-yl-ureas is based on an initial rigid piperidinylurea scaffold, connected to the variety of highly lipophilic substituents. The urea group can be

replaced by hydantoin, imidazolinone, benzazol, arylazole group, to achieve microsomal activity and low cytochrome inhibition, or aminopiperidine spacer, which prevents oxidation *in vivo* [15].

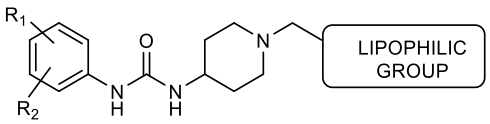


Figure 6: General structure of 1-aryl-3-piperidin-4-yl-ureas.

4-*N*-aryl-[1,4]diazepanylureas consist of phenethyl substituted with halogens (mostly 3,5-dichloro), azepane spacer and the urea unit, which are responsible for high affinity [15].

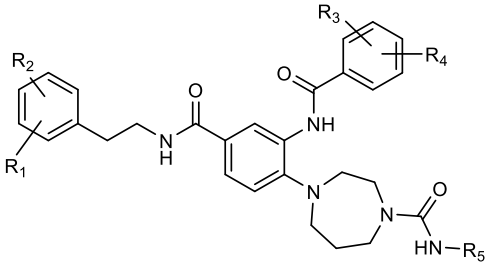


Figure 7: General structure of 4-*N*-aryl-[1,4]diazepanylureas.

2-Iminobenzimidazoles consist of a 2-acetyl-1*H*-benzimidazole, substituted by a 3-methyl group with variations of the benzophenone substituent at position 1, which improve both solubility and affinity [15].

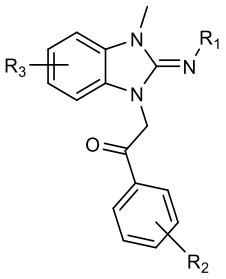


Figure 8: General structure of 2-iminobenzimidazoles.

3,4'-bispiperidine scaffold belongs to a group of bispiperidines, where one of the *N*-termini is linked to an amide or urea group or it can be also linked to carbonyl groups. Halogenated phenyl rings are attached to the scaffold to improve activity [15].

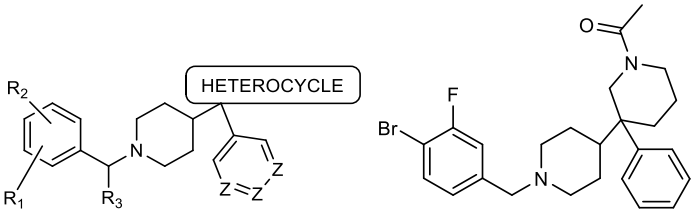


Figure 9: General structure of bispiperidines and one representative.

The replacement of *N*-Me group of LSD (Lysergic acid diethylamine) by an *N*-phenylcarbamate moiety causes potent binding, blocking of Ca^{2+} mobilization and chemotaxis of CXCR3 receptor. For the improvement of a group of ergolines the diethylamide moiety can be replaced [15].

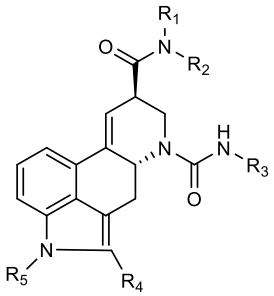


Figure 10: General structure of ergolines.

The crystal structure of CXCR3 is not known, so all of these classes were obtained by homology modeling [12].

As for CXCR4, the crystal structure has been determined [12]. There is a lot of molecules acting as CXCR4 antagonists, but the most known is the class of tetrahydroquinolines, among which AMD070 is the most known and it acts like inhibitor against HIV-1 replication. It consists of *N*-(1*H*-benzimidazol-2yl-methyl)-5,6,7,8-tetrahydro-8-quinolinamine and butylamine connected to the central N-atom, of responsible for the activity [21].

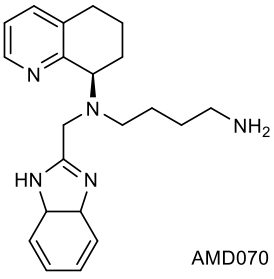


Figure 11: Structure of AMD070.

CXCR3 and CXCR4 receptors showed more than 50% of similarity, which is probably the key factor in dual binding. Recent studies have also shown potential selectivity sites, but more studies are needed for determination [12].

2 AIM OF THE WORK

The aim of this work is design, synthesis and biological characterization of the small-molecule chemokine CXCR3 and CXCR4 receptor antagonists. The work will be divided into two parts; the design and synthesis will be performed at the Department of Pharmaceutical Chemistry at the Faculty of Pharmacy at the University of Ljubljana under supervision of Assoc. Prof. Dr. Marko Anderluh, and biological assays at the Department of Chemistry and Pharmacy at the Friedrich-Alexander-University Erlangen-Nürnberg under supervision of Dr. Nuška Tschammer.

In Ljubljana the antagonists will be synthesized based on the "hit" compound, which was identified during the virtual screening as described by Schmidt et al (2014): *Identifying Modulators of CXC Receptors 3 and 4 with Tailored Selectivity Using Multi-Target Docking.* In this publication it has been discovered, that the lead compound acts like negative allosteric modulator. Based on this we assume that also our compounds will act like allosteric modulators.

The modification of the "hit" compound will include preservation of pyrazolopiridine scaffold that will be connected through carbonyl group with linkers, such as 1,3-diaminopropane, 4-aminopiperidine and piperazine. To the linker mostly anilino and/or benzylamino fragments will be attached and possibly substituted. The aniline fragments will be substituted only in two cases with 4-chloro and 4-metoxy, otherwise it will be unsubstituted. Benzylamino substituent will be developed via Topliss scheme, starting with unsubstituted benzylamino, followed by 4-chloro, 3,4-dichloro, 3,4-difluoro and 4-metoxy benzylamino fragment.

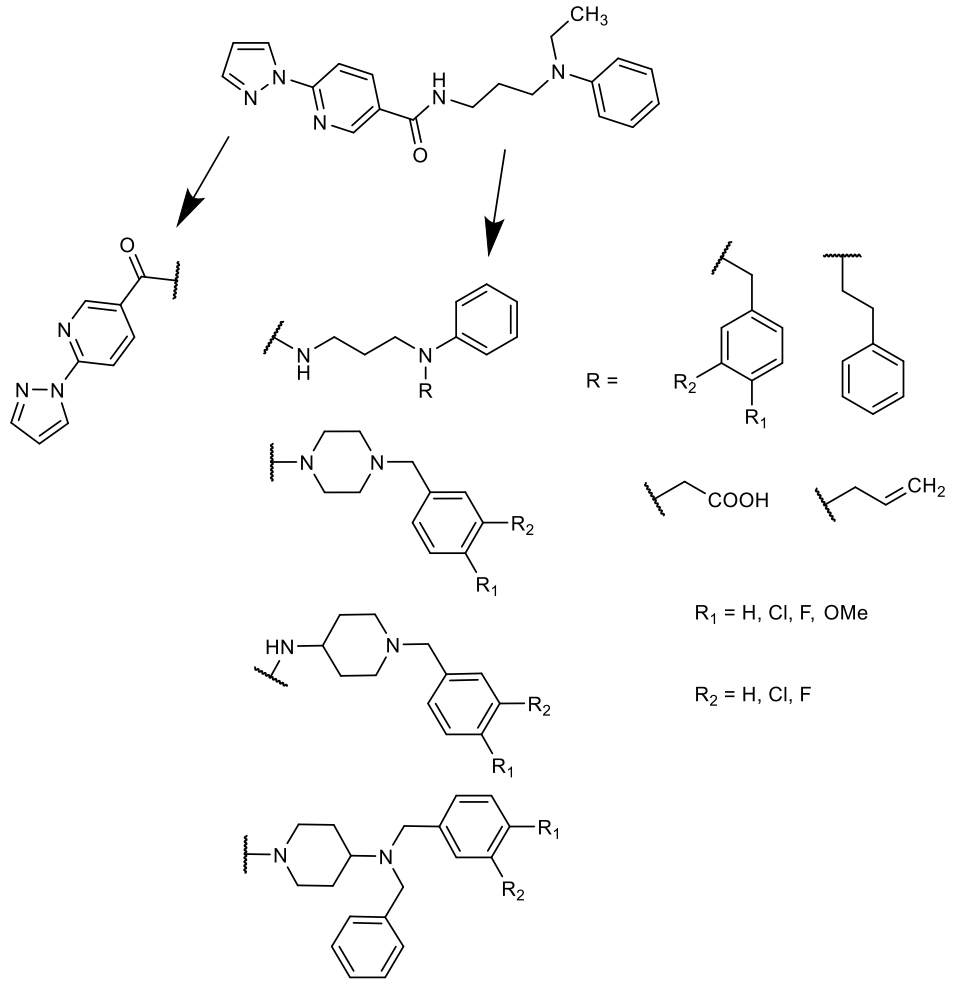


Figure 12: The development of antagonists.

The biological characterization of compounds will be performed at Department of Chemistry and Pharmacy at the Friedrich-Alexander-University Erlangen-Nürnberg with the main aim to pharmacologically characterize the compounds and estimate their selectivity, potency and efficacy in various functional assays. Synthesized compounds will be tested in β-arrestin 2 rectruitment and cAMP accumulation assay on CXCR3 and CXCR4. Based on these results we will get the information about structure activity relationship (SAR), how the structural changes affect the selectivity of receptors, the ability to activate or inhibit the receptor and which changes enable higher potency and efficacy. The major outcome of this project will be a novel series of allosteric modulators of CXCR3 and/or CXCR4, which might serve as an important starting point for potential drug candidates.

3 MATERIALS AND METHODS

3.1 Reagents, chemicals and solvents

The reagents, chemicals and solvents that we used in experimental part were manufactured by Maybridge, Acros, Apollo, Aldrich, Sigma Aldrich, Merck and Fluka. All the necessary anhydrous solvents were prepared by distillation and drying over molecular sieves. For the compound characterization we used reagents and mediums manufacturesd by Gibco Invitrogen, Mirus Bio, Sigma-Aldrich, and PeproTech. Cells were grown on petri dishes (Corning) and tests were carried out in half-area microtiter plates manufactured by Carl Roth.

3.2 Software

For the drawing and characterization of chemical structures we used the program ChemBioDraw Ultra 13.0. NMR spectrums were processed with MestRec 4.9.9. SciFinder Scholar was a helpful tool for finding compounds and synthesis procedures. The results for the compound characterization were processed with Microsoft® Office Excel 2010 and Prism 5.0 Graph Pad Software, LaJolla, CA, USA. Microsoft® Office PowerPoint 2010 was used for picture drawing.

3.3 Chromatographic methods

3.3.1 Thin layer chromatography (TLC)

The reactions and column chromatography were monitored with thin layer chromatography, using the plates made by Merck (TLC Silica gel 60 F254) with 0,20 mm thick layer of silicagel on aluminium carrier. For development of the chromatograms different mobile phases were used. For detection we used UV light (λ=254 nm) and ninhydrine staining reagent.

3.3.2 Column chromatography

For compound purification and separation we used column chromatography with different sizes of columns. Silicagel was purchased from Carl Roth and we always used the same paricle size, 0,040–0,063, while mobile phase composition differed depending on the compound physico-chemical properties.

3.4 Spectroscopic methods

3.4.1 Nuclear magnetic resonance (NMR)

NMR spectra (^{1}H and ^{13}C) were recorded at Faculty of Pharmacy on a Bruker AVANCE III 400 MHz instrument at 298K. For internal standard TMS (Tetramethylsilane) was used and the samples were diluted in DMSO-d6 or $CDCl_3$.

3.4.2 Mass spectroscopy (MS)

Mass spectra were recorded at the Centre of Mass Spectroscopy, Institut Jožef Stefan, Ljubljana. The used spectrometer was VG-Analytical Autospec Q with ESI and HRMS methods.

3.5 Melting point

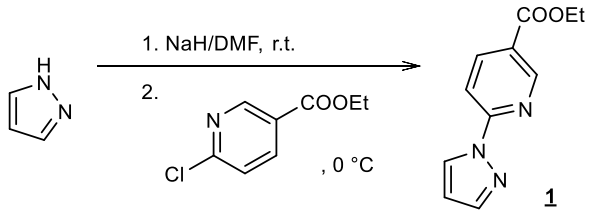
Melting points were determined by the microscope with a hot stage, made by Leica.

3.6 Compound characterization

The results were obtained at the Department of Chemistry and Pharmacy at the Friedrich-Alexander-University Erlangen-Nürnberg using microplate reader BMG Clariostar.

4 COMPOUND SYNTHESIS AND CHARACTERIZATION

4.1 Synthesis of 6-(1*H*-pyrazol-1-yl)nicotinic acid ethyl ester (1)



Pyrazol and NaH (60% in mineral oil) were dissolved in anhydrous DMF (20-60 mL) and stirred for 30 minutes under argon atmosphere at room temperature. The reaction mixture was cooled at 0 °C. Ethyl 6-chloronicotinate was dissolved in anhydrous DMF (10-20 mL), added dropwise to the reaction mixture and left stirring till the next day under the argon atmosphere at 0 °C. The reaction mixture was transferred into the ice cold water (50-100 g) wherein the white compound precipitated. The precipitate was filtered off and dried at 60 °C. The reaction was performed in triplicate with used quantities in the table I.

Table I: Reagents in synthesis of 6-(1*H*-pyrazol-1-yl)nicotinic acid ethyl ester.

Reagent	Amount	n (mmol)	Eq
Pyrazol	1.833 g	26.93	1.0
	3.666 g	53.86	
	5.50 g	80.82	
Ethyl 6-chloronicotinate	5.00 g	26.93	1.0
	10.00 g	53.86	
	15.00 g	80.82	
NaH (60% in mineral oil)	1.185 g	29.63	1.1
	2.370 g	58.72	
	3.550 g	88.90	

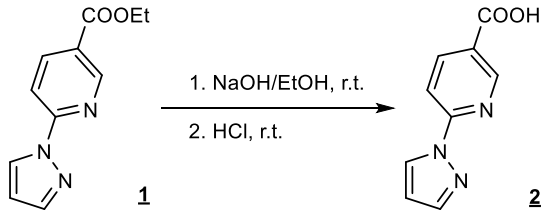
Description: white solid

Reaction yield: η_1 = 89.1% (5.213 g), η_2 = the compound was not completely dry at the time of weighting, η_3 = 74.0%

Rf = 0.63 (MF = EtOAc: hexane = 1:1)

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.35 (t, *J* = 7.1 Hz, 3H, $CH_2C\underline{H}_3$), 4.37 (q, *J* = 7.1 Hz, 2H, $C\underline{H}_2CH_3$), 6.65 (dd, *J* = 2.7, 1.7 Hz, 1H, Ar-H), 7.79 (dd, *J* = 1.7, 0.8 Hz, 1H, Ar-H), 8.05 (dd, *J* = 8.6, 0.8 Hz, 1H, Ar-H), 8.46 (dd, *J* = 8.6, 2.3 Hz, 1H, Ar-H), 8.70 (dd, *J* = 2.7, 0.8 Hz, 1H, Ar-H), 8.97 (dd, *J* = 2.3, 0.8 Hz, 1H, Ar-H) ppm.

4.2 Synthesis of 6-(1*H*-pyrazol-1-yl)nicotinic acid (2)



1 **2**

Compound **1** was dissolved in ethanol (40-80 mL). 3M $NaOH_{(aq)}$ was then added and the mixture stirred overnight. The solvent was evaporated under reduced pressure and the residue was dissolved in water (30-60 mL) and extracted with diethyl ether (20-40 mL) two times. pH of the water phase was adjusted with 2M HCl till 3 where white precipitate was formed. It was filtered off and dried on air. The used quantities are in the table II.

Table II: Reagents in synthesis of 6-(1*H*-pyrazol-1-yl)nicotinic acid.

The reagent	Amount	n (mmol)	Eq
Compound **1**	5.200 g	23.94	1.0
	10.257 g	47.88	
	8.662 g	40.43	
NaOH	23.94 mL	71.82	3.0
	47.88 mL	143.64	
	40.40 mL	121.30	

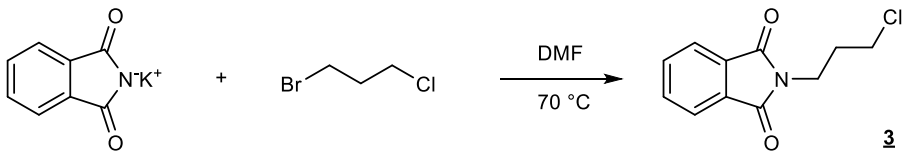
Description: white solid

Reaction yield: η_1 = 95.2% (4.313 g), η_2 = 87.0% (7.883 g), η_3 = 97.0% (7.422 g)

Rf = 0.06 (MF = DCM: methanol = 9:1), 0.02 (MF = DCM: methanol = 20:1)

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 6.65 (dd, *J* = 2.7, 1.6 Hz, 1H, Ar-H), 7.92 (dd, *J* = 1.6, 0.8 Hz, 1H, Ar-H), 8.04 (dd, *J* = 8.6, 0.8 Hz, 1H, Ar-H), 8.44 (dd, *J* = 8.6, 2.3 Hz, 1H, Ar-H), 8.70 (dd, *J* = 2.7, 0.8 Hz, 1H, Ar-H), 8.96 (dd, *J* = 2.3, 0.8 Hz, 1H, Ar-H), 13.44 (s, 1H, COOH).

4.3 Synthesis of 2-(3-chloropropyl)isoindoline-1,3-dione (3)



3

Phthalimide potassium salt was dissolved in anhydrous DMF (20-80 mL), 1-bromo-3-chloropropane was added and the reaction was stirred at 70 °C overnight, protected from

the moist with chlorcalcium tube. The reaction mixture was poured into an ice cold water (100-150 mL), which yielded white precipitate. The precipitate was filtered with vacuum and dried on air or under reduced pressure. The compound was taken into the next step without further purification and caracterization. The used quantities are in the table III.

Table III: Reagents in synthesis of 2-(3-chloropropyl)isoindoline-1,3-dione.

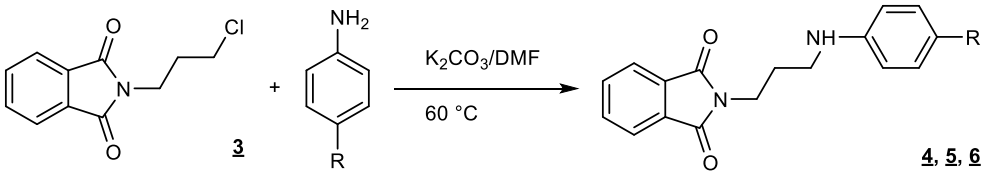
The reagent	Amount	n (mmol)	Eq
Phthalimide potassium salt	5.00 g	26.99	1.0
	10.00 g	53.99	
	20.00 g	107.98	
	20.00 g	107.98	
	15.61 g	84.26	
1-bromo-3-chloropropane	3.2 mL	32.39	1.2
	6.4 mL	64.79	
	12.8 mL	129.58	
	12.8 mL	129.58	
	10.0 mL	101.12	

Description: white-brown solid

Reaction yield: η_1 = 77.3% (4.664 g), η_2 = 80.9% (9.768 g), η_3 = 92.5% (22.345 g), η_4 = 86.6% (20.922 g) η_5 = 93.2% (17.564 g)

Rf = 0.41 (MF = EtOAc: hexane = 1:2), 0.63 (MF = DCM: methanol = 20:1)

4.4 Derivatives of 2-(3-(phenylamino)propyl)isoindoline-1,3-dione



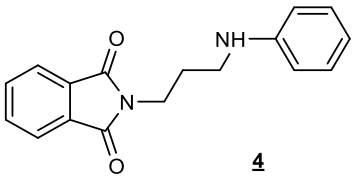
GENERAL SYNTHETIC PROCEDURE

Compound **3** was dissolved in anhydrous DMF (7.5-150 mL) and K_2CO_3 was added. Aniline derivatives were added and the reaction mixture was stirred at 120 °C overnight, equipped with chlorcalcium tube. The reaction mixture was poured into an ice cold water (50-200 mL), where precipitate of compound **5** formed. For compounds **4** and **6**, water phase was extracted three times with DCM (50-100 mL). The organic phase was dried over sodium sulphate, filtered and the solvent evaporated under reduced pressure. Compounds were purified by recristalization and/or column chromatography.

4.4.1 2-(3-(phenylamino)propyl)isoindoline-1,3-dione (4)

Column chromatography (MF = EtOAc: hexane = 3:1) was used to purify the compound. The final purification was performed by recrystallization using EtOAc and hexane, or performing another column chromatographic separation (MF = toluene: acetone = 10:1). The reaction was repeated four times and the used quantities are in the table IV.

Table IV: Reagents in synthesis of 2-(3-(phenylamino)propyl)isoindoline-1,3-dione.

R		The reagent	Amount	n (mmol)	Eq
H	 **4**	Compound **3**	2.000 g	8.94	1.0
			10.000 g	44.71	
			16.759 g	74.93	
			20.922 g	93.55	
		Aniline	0.98 mL	10.73	1.2
			4.08 mL	44.71	1.0
		Aniline sulfate	23.44 g	82.43	1.1
		Aniline	8.53 mL	93.55	1.0
		K_2CO_3	2.470 g	17.88	2.0
			12.360 g	89.42	
			41.430 g	299.73	4.0
			25.860 g	187.09	2.0

Description: yellow solid

Reaction yield: η_1 = 18.08% (0.453 g), η_2 = 16.8% (2.106 g), η_3 = 17.0% (3.581 g), η_4 = 22.5% (5.891 g)

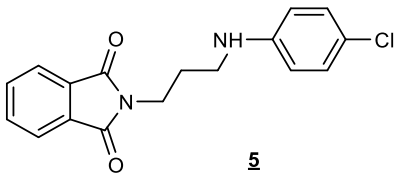
Rf = 0.49 (MF = toluene: acetone = 10:1), 0.38 (MF = EtOAc: hexane = 1:3)

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.87 (p, *J* = 7.0 Hz, 2H, $CH_2C\underline{H_2}CH_2$), 3.04 (m, *J* = 7.0, 5.8 Hz, 2H, $CH_2CH_2C\underline{H_2}$-NH-Ar), 3.65-3.72 (m, 2H, $C\underline{H_2}CH_2CH_2$-NH-Ar), 5.55 (t, *J* = 5.8 Hz, 1H, N<u>H</u>), 6.48-6.57 (m, 3H, Ar-<u>H</u>), 7.02-7.10 (m, 2H, Ar-<u>H</u>), 7-81-7.91 (m, 4H, Ar-<u>H</u>).

4.4.2 2-(3-((4-chlorophenyl)amino)propyl)isoindoline-1,3-dione (5)

The compound was purified by recrystallization using EtOAc and column chromatography (MF = EtOAc: hexane = 1:3). The used quantities are in the table V. The crude products were combined and purified together.

Table V: Reagents in synthesis of 2-(3-((4-chlorophenyl)amino)propyl)isoindoline-1,3-dione.

R		The reagent	Amount	n (mmol)	Eq
Cl	 **5**	Compound **3**	1.000 g	4.47	1.0
			1.200 g	5.37	
		4-chloroaniline	0.57 g	4.47	1.0
			0.68 g	5.37	
		K_2CO_3	1.24 g	8.94	2.0
			1.48 g	10.73	

Description: white solid

Reaction yield: η = 3.8% (0.118 g)

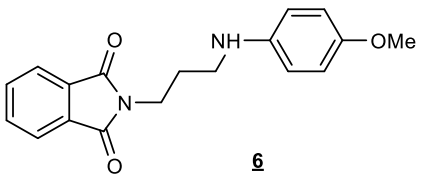
Rf = 0.73 (MF = DCM: methanole = 20: 1), 0.26 (MF = EtOAc: hexane = 1: 3)

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.82-1.93 (m, 2H, $CH_2C\underline{H_2}CH_2$), 2.97-3.10 (m, 2H, $CH_2CH_2C\underline{H_2}$-NH-Ar), 3.68 (t, *J* = 7.1 Hz, 2H, $C\underline{H_2}CH_2CH_2$-NH-Ar), 5.73-5.85 (m, 1H, NH), 6.54 (d, *J* = 8.9 Hz, 2H, Ar-H), 7.07 (d, *J* = 8.9 Hz, 2H, Ar-H), 7.80-7.91 (m, 4H, Ar-H).

4.4.3 2-(3-((4-methoxyphenyl)amino)propyl)isoindoline-1,3-dione (6)

Column chromatography was used for purification (MF = EtOAc: hexane = 1:1). The used quantities are in the table VI.

Table VI: Reagents in synthesis of 2-(3-((4-methoxyphenyl)amino)propyl)isoindoline-1,3-dione.

R		The reagent	Amount	n (mmol)	Eq
OMe	 **6**	Compound **3**	0.50 g	2.24	1.0
			0.50 g	2.24	
		4-methoxyaniline	0.28 g	2.24	1.0
			0.28 g	2.24	
		K_2CO_3	0.62 g	4.47	2.0
			0.62 g	4.47	

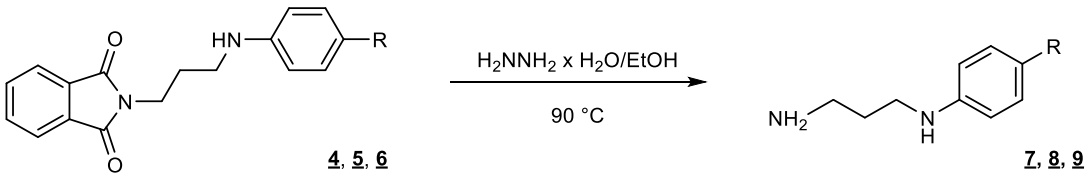
Description: brown solid

Reaction yield: η_1 = 38.4% (0.267 g), η_2 = 29.3% (0.204 g)

Rf = 0.48 (MF = EtOAc: hexane = 1:1)

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.86 (q, *J* = 6.9 Hz, 2H, $CH_2C\underline{H_2}CH_2$), 2.99 (q, *J* = 6.9 Hz, 2H, $CH_2CH_2C\underline{H_2}$-NH-Ar), 3.65-3.73 (m, 2H, $C\underline{H_2}CH_2CH_2$-NH-Ar), 5.12 (d, *J* = 5.8 Hz, 1H, NH), 6.48-6.56 (m, 2H, Ar-H), 6.67-6.76 (m, 2H, Ar-H), 7.81-7.93 (m, 4H, Ar-H).

4.5 Derivatives of *N1*-phenylpropane-1,3-diamine



4, **5**, **6** **7**, **8**, **9**

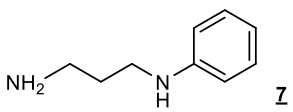
GENERAL SYNTHETIC PROCEDURE

Compounds **4**, **5** and **6** were dissolved in ethanol (30-330 mL), hydrazine hydrate was added and the reaction was stirred overnight (15h) at 90 °C. Ethanol was evaporated under reduced pressure. DCM (20-100 mL) and water (20-100 mL) were added to the residue, pH of the water phase was adjusted with 1M NaOH till 12, and the phases were separated. Aqueous phase was extracted with DCM (20-100 mL) two times, joined organic phases were dried over sodium sulphate, filtered and the solvent evaporated under reduced pressure.

4.5.1 *N1*-phenylpropane-1,3-diamine (7)

The used quantities are in the table VII.

Table VII: Reagents in synthesis of *N1*-phenylpropane-1,3-diamine.

R		The reagent	Amount	n (mmol)	Eq
H	 7	Compound **4**	0.489 g	1.74	1.0
			1.985 g	7.08	
			3.581 g	12.77	
			5.891 g	21.02	
		Hydrazine hydrate	0.27 mL	8.72	5.0
			1.11 mL	35.41	
			2.0 mL	63.87	
			3.29 mL	105.08	

Description: brown oil

Reaction yield: η_1 = 70.4% (0.184 g), η_2 = 71.3% (0.758 g), η_3 = 68.7% (1.318 g), η_4 = the compound was not completely dry at the time of weighting

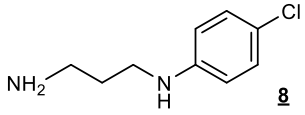
Rf = 0.07 (MF = DCM: methanol = 4:1)

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.61 (p, *J* = 6.7 Hz, 2H, $CH_2C\underline{H_2}CH_2$), 2.64 (t, *J* = 6.7 Hz, 2H, $CH_2CH_2C\underline{H_2}$-NH_2), 3.02 (q, *J* = 6.7 Hz, 2H, $C\underline{H_2}CH_2CH_2$-NH_2), 5.54 (s, 1H, N<u>H</u>), 6.46-6.52 (m, 1H, Ar-<u>H</u>), 6.52-6.58 (m, 2H, Ar-<u>H</u>), 6.97-7.09 (m, 2H, Ar-<u>H</u>). A peak for $N\underline{H_2}$ is missing due to H-bonding with the solvent and/or H_2O.

4.5.2 *N1*-(4-chlorophenyl)propane-1,3-diamine (<u>8</u>)

The used quantities are in the table VIII. The compound was used in the next reaction without purification and caracterization.

Table VIII: Reagents in synthesis of *N1*-(4-chlorophenyl)propane-1,3-diamine.

R		The reagent	Amount	n (mmol)	Eq
Cl	 <u>8</u>	Compound <u>5</u>	0.295 g	0.937	1.0
		Hydrazine hydrate	0.15 mL	4.686	5.0

Description: grey solid

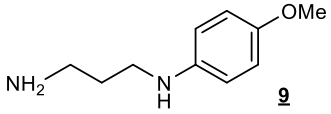
Reaction yield: η = 29.5% (0.51 g)

Rf = 0.02 (MF = DCM: methanol = 20:1)

4.5.3 *N1*-(4-methoxyphenyl)propane-1,3-diamine (<u>9</u>)

The compound was used in the next reaction without purification and caracterization. The used quantities are in the table IX.

Table IX: Reagents in synthesis of *N1*-(4-methoxyphenyl)propane-1,3-diamine.

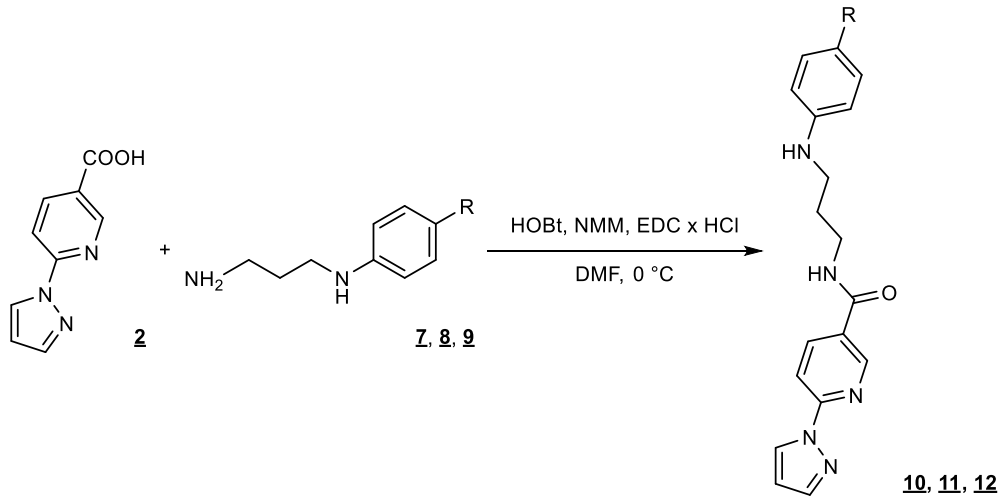
R		The reagent	Amount	n (mmol)	Eq
OMe	 <u>9</u>	Compound <u>6</u>	0.471 g	1.52	1.0
		Hydrazine hydrate	0.24 mL	7.59	5.0

Description: brown solid

Reaction yield: η = 85.0% (0.233 g)

Rf = 0.06 (MF = DCM: methanol = 20:1 + 1% Et_3N)

4.6 Derivatives of *N*-(3-(phenylamino)propyl)-6-(1*H*-pyrazol-1-yl) nicotinamine I



GENERAL SYNTHETIC PROCEDURE

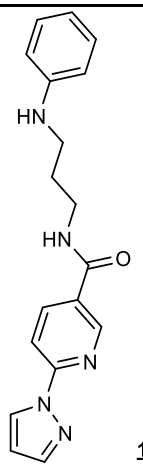
Compounds **2** and **7**, **8** or **9** were dissolved in anhydrous DMF (10-230 mL) and stirred at 0 °C. First HOBt, than NMM and after 10 minutes EDCxHCl were added and the reaction mixture was stirred overnight while allowing to warm up to the room temperature. Most of the DMF was evaporated under reduced pressure and to the residue EtOAc (30-100 mL) was added and extracted three times with distilled water (15-50 mL), three times with saturated $NaHCO_{3(aq)}$ (15-50 mL) and once with saturated $NaCl_{(aq)}$ (15-50 mL). The organic phase was dried over sodium sulphate, filtered and the solvent evaporated under reduced pressure. The compound was purified by column chromatography.

4.6.1 *N*-(3-(phenylamino)propyl)-6-(1*H*-pyrazol-1-yl)nicotinamide (10)

The compound was purified by column chromatography (MF = EtOAc: hexane = 1:1). The used quantities are in the table X.

Table X: Reagents in synthesis of *N*-(3-(phenylamino)propyl)-6-(1*H*-pyrazol-1-yl) nicotinamide.

R		The reagent	Amount	n (mmol)	Eq
H		Compound **2**	0.225 g	1.19	1.0
			1.310 g	6.91	
			2.741 g	14.49	
			4.210 g	22.25	

	 **10**	Compound **7**	0.188 g	1.25	1.05
			1.09	7.26	
			2.286 g	15.22	
			3.510 g	23.37	
		HOBt	0.209 g	1.55	1.3
			1.210 g	8.98	
			2.550 g	18.84	
			3.910 g	28.93	
		NMM	0.29 ml	2.62	2.2
			1.67 mL	15.20	
			3.51 mL	31.88	
			5.38 mL	48.96	
		EDCxHCl	0.320 g	1.67	1.4
			1.850 g	9.67	
			3.890 g	20.29	
			5.970 g	31.15	

Description: white solid

Reaction yield: η_1 = 46.8% (0.179 g), η_2 = 36.2% (0.804 g), η_3 = 41.8% (1.945 g), η_4 = 42.1% (3.012 g)

Rf = 0.22 (MF = EtOAc: hexane = 1:1), 0.56 (MF = DCM: methanol = 9:1)

Melting point = 135-138 °C

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.79-1.88 (m, 2H, $CH_2C\underline{H_2}CH_2$), 3.09 (q, *J* = 6.7 Hz, 2H,COO-NH-$C\underline{H_2}CH_2CH_2$), 3.38-3.44 (m, 2H, COO-NH-$CH_2CH_2C\underline{H_2}$), 5.59 (t, *J* = 5.7 Hz, 1H, Ar-N<u>H</u>), 6.49-6.54 (m, 1H, Ar-<u>H</u>), 6.56-6.59 (m, 2H, Ar-<u>H</u>), 6.63 (dd, *J* = 2.7, 1.7 Hz, 1H, Ar-<u>H</u>), 7.04-7.10 (m, 2H, Ar-<u>H</u>), 7.89 (dd, *J* = 1.7, 0.8 Hz, 1H, Ar-<u>H</u>), 8.01 (dd, *J* = 8.6, 0.8 Hz, 1H, Ar-<u>H</u>), 8.40 (dd, *J* = 8.6, 2.3 Hz, 1H, Ar-<u>H</u>), 8.69 (dd, *J* = 2.7, 0.8 Hz, 1H, Ar-<u>H</u>), 8.75 (t, *J* = 5.5 Hz, 1H, Ar-CO-N<u>H</u>), 8.90 (dd, *J* = 2.3, 0.8 Hz, 1H, Ar-<u>H</u>).

^{13}C (100 MHz, $CDCl_3$) δ(ppm) = 28.66 ($CH_2\underline{C}H_2CH_2$), 38.21 (COO-NH-$\underline{C}H_2CH_2CH_2$), 42.65 (COO-NH-$CH_2CH_2\underline{C}H_2$), 108.48 (Ar-<u>C</u>), 111.86 (2×Ar-<u>C</u>), 114.13 (2×Ar-<u>C</u>), 119.11 (Ar-<u>C</u>), 127.40 (Ar-<u>C</u>), 127.60 (Ar-<u>C</u>), 129.53 (2×Ar-<u>C</u>), 137.60 (Ar-<u>C</u>), 142.90 (Ar-<u>C</u>), 147.26 (Ar-<u>C</u>), 153.15 (Ar-<u>C</u>), 165.29 (Ar-<u>C</u>O).

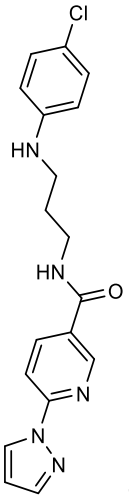
MS (ES^+) *m/z* = 322.2 (MH^+)

HRMS (ES^+) *m/z* for $C_{18}H_{19}N_5O$ calculated: 322.1668, found: 322.1661

4.6.2 *N*-(3-((4-chlorophenyl)amino)propyl)-4-(1*H*-pyrazol-1-yl)benzamide (**11**)

The compound was purified by column chromatography (MF = EtOAc: hexane = 1:1). The used quantities are in the table XI.

Table XI: Reagents in synthesis of *N*-(3-((4-chlorophenyl)amino)propyl)-4-(1*H*-pyrazol-1-yl)benzamide.

R		The reagent	Amount	n (mmol)	Eq
Cl	 **11**	Compound **2**	0.050 g	0.263	1.0
		Compound **8**	0.051 g	0.276	1.05
		HOBt	0.046 g	0.342	1.3
		NMM	0.06 mL	0.579	2.2
		EDC	0.071 g	0.368	1.4

Description: white solid

Reaction yield: η = 58.9% (0.055 g)

Rf = 0.28 (MF = EtOAc: hexane = 1: 1), 0.68 (MF = DCM: methanol = 9: 1)

Melting point = 170-173 °C

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.78-1.86 (m, 2H, $CH_2C\underline{H_2}CH_2$), 3.07 (q, *J* = 4.9 Hz, 2H,COO-NH-C$\underline{H_2}CH_2CH_2$), 3.39 (t, *J* = 4.9 Hz, 2H, COO-NH-C$\underline{H_2}CH_2CH_2$), 5.84 (t, *J* = 5.7 Hz, 1H, Ar-N$\underline{H}$), 6.56-6.60 (m, 2H, Ar-$\underline{H}$), 6.63 (dd, *J* = 2.6, 1.7, Hz, 2H, Ar-$\underline{H}$), 7.07-7.11 (m, 2H, Ar-$\underline{H}$), 7.89 (dd, *J* = 1.7, 0.8 Hz, 1H, Ar-$\underline{H}$), 8.01 (dd, *J* = 8.6, 0.8 Hz, 1H, Ar-$\underline{H}$), 8.39 (dd, *J* = 8.6, 2.3 Hz, 1H, Ar-$\underline{H}$), 8.69 (dd, *J* = 2.6, 0.8 Hz, 1H, Ar-$\underline{H}$), 8.74 (t, *J* = 5.6 Hz, 1H, Ar-CO-N$\underline{H}$), 8.90 (dd, *J* = 2.3, 0.8 Hz, 1H, Ar-$\underline{H}$).

^{13}C (100 MHz, $CDCl_3$) δ(ppm) = 28.77 ($CH_2\underline{C}H_2CH_2$), 37.91 (COO-NH-$\underline{C}H_2CH_2CH_2$ and COO-NH-$CH_2CH_2\underline{C}H_2$), 108.54 (Ar-$\underline{C}$), 111.94 (2×Ar-$\underline{C}$), 114.82 (3×Ar-$\underline{C}$), 127.34 (Ar-$\underline{C}$), 127.62 (Ar-$\underline{C}$), 129.29 (4×Ar-$\underline{C}$), 137.64 (Ar-$\underline{C}$), 142.96 (Ar-$\underline{C}$), 147.14 (Ar-$\underline{C}$), 165.43 (Ar-$\underline{C}$O).

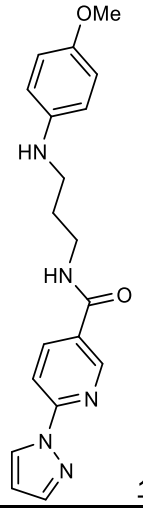
MS (ES^+) *m/z* = 356.1 (MH^+)

HRMS (ES^+) *m/z* for $C_{19}H_{19}ClN_5O$ calculated: 356.1278, found: 356.1273

4.6.3 *N*-(3-((4-methoxyphenyl)amino)propyl)-4-(1*H*-pyrazol-1-yl)benzamide (12)

The compound was purified by column chromatography (MF = EtOAc: hexane = 2:1). The used quantities are in the table XII.

Table XII: Reagents in synthesis of *N*-(3-((4-methoxyphenyl)amino)propyl)-4-(1*H*-pyrazol-1-yl)benzamide.

R		The reagent	Amount	n (mmol)	Eq
OMe	 12	Compound **2**	0.233 g	1.23	1.0
		Compound **9**	0.233 g	1.29	1.05
		HOBt	0.216 g	1.30	1.3
		NMM	0.30 mL	2.70	2.2
		EDC	0.330 g	1.72	1.4

Description: beige solid

Reaction yield: η = 69.2% (0.299 g)

Rf = 0.45 (MF = EtOAc: hexane = 1:1), 0.46 (MF = DCM: methanol = 9:1)

Melting point = 140-143 °C

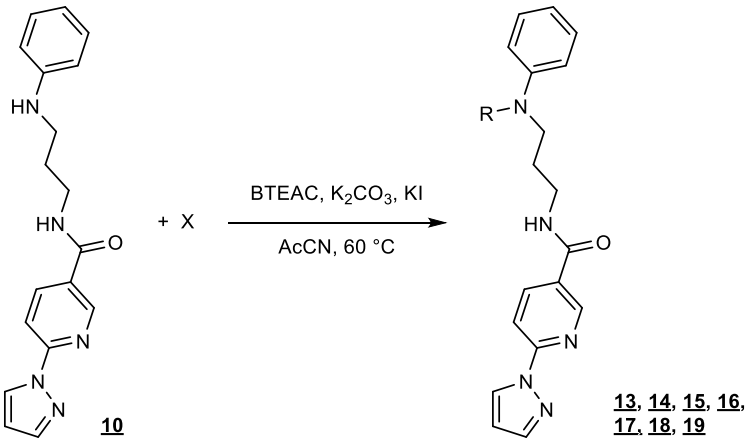
^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.82 (p, *J* = 6.8 Hz, 2H, $CH_2CH_2CH_2$), 3.04 (q, *J* = 6.8 Hz, 2H, COO-NH-$CH_2CH_2CH_2$), 3.40 (q, *J* = 6.8 Hz, 2H, COO-NH-$CH_2CH_2CH_2$), 3.63 (s, 3H, Ar-OCH_3), 5.12-5.20 (m, 1H, Ar-NH), 6.51-6.57 (m, 2H, Ar-H), 6.63 (dd, *J* = 2.7, 1.7, Hz, 1H, Ar-H), 6.69-6.75 (m, 2H, Ar-H), 7.89 (dd, *J* = 1.7, 0.8 Hz, 1H, Ar-H), 8.01 (dd, *J* = 8.6, 0.8 Hz, 1H, Ar-H), 8.40 (dd, *J* = 8.6, 2.3 Hz, 1H, Ar-H), 8.69 (dd, *J* = 2.7, 0.8 Hz, 1H, Ar-H), 8.74 (t, *J* = 5.5 Hz, 1H, Ar-CO-NH), 8.91 (dd, *J* = 2.3, 0.8 Hz, 1H, Ar-H).

^{13}C (100 MHz, DMSO-d6) δ(ppm) = 28.65 ($CH_2CH_2CH_2$), 37.39 (COO-NH-$CH_2CH_2CH_2$), 41.34 (COO-NH-$CH_2CH_2CH_2$), 55.25 (OCH_3), 108.77 (Ar-C), 111.26 (Ar-C), 113.07 (2×Ar-C), 114.56 (2×Ar-C), 127.51 (Ar-C), 127.97 (Ar-C), 138.47 (Ar-C), 142.90 (Ar-C), 143.15 (Ar-C), 147.66 (Ar-C), 150.55 (Ar-C), 152.06 (Ar-C), 163.98 (Ar-CO).

MS (ES^{+}) *m/z* = 352.2 (MH^{+})

HRMS (ES^{+}) *m/z* for $C_{19}H_{21}N_5O_2$ calculated: 352.1774, found: 352.1777

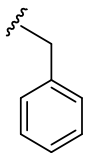
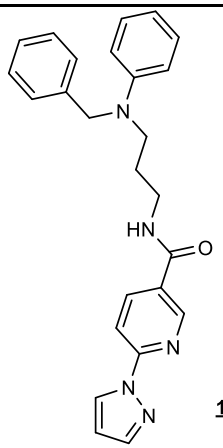
4.7 Derivatives of *N*-(3-(phenylamino)propyl)-6-(1*H*-pyrazol-1-yl) nicotinamide II

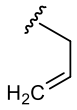
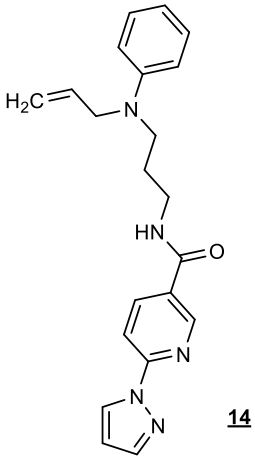
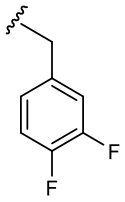
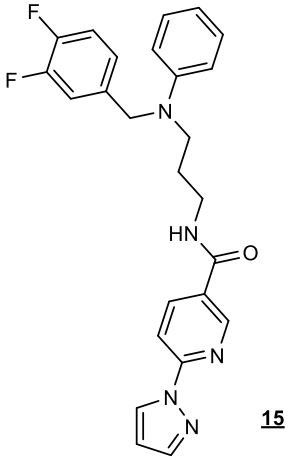
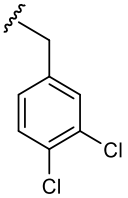
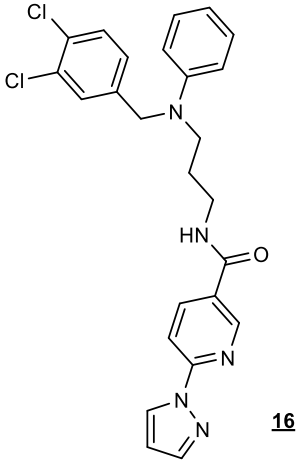


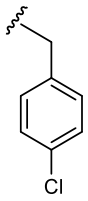
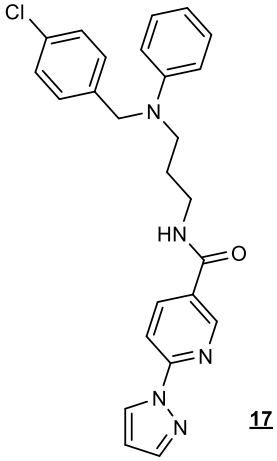
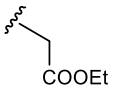
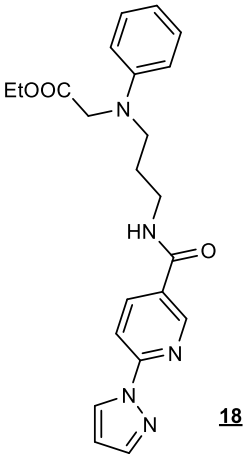
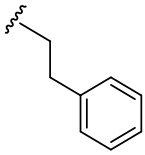
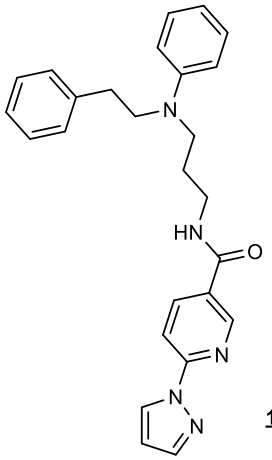
GENERAL SYNTHETIC PROCEDURE

Compound **10** (0.150 g, 0.47 mmol, 1.0 eq), BTEAC (0.011 g, 0.047 mmol, 0.1 eq), KI (0.011 g, 0.047 mmol, 0.1 eq) and K_2CO_3 (0.387 g, 2.80 mmol, 6.0 eq) were dissolved in acetonitrile (10 mL). Various reagents (benzyl bromide, allyl bromide, 3,4-difluorobenzyl bromide, 3,4-dichlorobenzyl bromide, 4-chlorobenzyl chloride, Ethyl 2-bromoacetate and 2-bromoethyl benzene) were added and stirred under argon atmosphere at 60 °C overnight. The reaction mixture was filtered to remove K_2CO_3, the solvent was evaporated under reduced pressure and the compounds were purified by column chromatography using EtOAc/hexane = 1:1. Only in the case of compound **15** DCM/methanol = 20:1 was used as a mobile phase for purification.

Table XIII: Derivatives of *N*-(3-(phenylamino)propyl)-6-(1*H*-pyrazol-1-yl)nicotinamide II and their reagent.

R	Final compound	X	Amount	n (mmol)	Eq
	 13	Benzyl bromide	0.22 mL	1.87	4.0

	 **14**	Allyl bromide	0.16 mL	1.89	4.0
	 **15**	3,4-difluorobenzyl bromide	0.24 mL	1.87	4.0
	 **16**	3,4-dichlorobenzyl bromide	0.224 g	0.93	2.0

	 **17**	4-chlorobenzyl chloride	0.150 g	0.93	2.0
	 **18**	Ethyl 2-bromoacetate	0.10 mL	0.93	2.0
			0.16 mL	1.40	3.0
	 **19**	2-bromoethyl benzene	0.13 mL	0.93	2.0

4.7.1 *N*-(3-(benzyl(phenyl)amino)propyl)-6-(1*H*-pyrazol-1-yl)nicotinamide (13)

Description: yellow-green solid

Reaction yield: η = 91.5% (0.177 g)

Rf = 0.43 (MF = EtOAc: hexane = 1:1)

Melting point = 121-124 °C

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.84-1.94 (m, 2H, $CH_2C\underline{H_2}CH_2$), 3.38-3.42 (m, 2H, COO-NH-$C\underline{H_2}CH_2CH_2$), 3.47-3.54 (m, 2H, COO-NH-$CH_2CH_2C\underline{H_2}$), 4.58 (s, 2H,

Ar-C$\underline{H_2}$), 6.55-6.59 (m, 1H, Ar-$\underline{H}$), 6.63 (dd, *J* = 2.7, 1.7 Hz, 1H, Ar-$\underline{H}$), 6.65-6.70 (m, 2H, Ar-$\underline{H}$), 7.06-7.14 (m, 2H, Ar-$\underline{H}$), 7.18-7.26 (m, 3H, Ar-$\underline{H}$), 7.26-7.34 (m, 3H, Ar-$\underline{H}$), 7.89 (dd, *J* = 1.7, 0.8 Hz, 1H, Ar-$\underline{H}$), 8.01 (dd, *J* = 8.6, 0.8 Hz, 1H, Ar-$\underline{H}$), 8.39 (dd, *J* = 8.6, 2.3 Hz, 1H, Ar-$\underline{H}$), 8.69 (dd, *J* = 2.7, 0.8 Hz, 1H, Ar-$\underline{H}$), 8.76 (t, *J* = 5.6 Hz, 1H, Ar-CO-N$\underline{H}$), 8.90 (dd, *J* = 2.3, 0.8 Hz, 1H, Ar-$\underline{H}$).

^{13}C (100 MHz, $CDCl_3$) δ(ppm) = 26.81 ($CH_2\underline{C}H_2CH_2$), 37.18 (COO-NH-$\underline{C}H_2CH_2CH_2$), 48.60 (COO-NH-$CH_2CH_2\underline{C}H_2$), 53.63 (Ar-$\underline{C}H_2$), 108.78 (Ar-$\underline{C}$), 111.28 (Ar-$\underline{C}$), 111.92 (2×Ar-$\underline{C}$), 115.54 (Ar-$\underline{C}$), 126.53 (Ar-$\underline{C}$), 127.99 (Ar-$\underline{C}$), 128.38 (2×Ar-$\underline{C}$), 128.98 (2×Ar-$\underline{C}$), 138.48 (Ar-$\underline{C}$), 139.23 (Ar-$\underline{C}$), 142.48 (Ar-$\underline{C}$), 147.66 (Ar-$\underline{C}$), 147.48 (Ar-$\underline{C}$), 152.07 (Ar-$\underline{C}$), 164.01 (Ar-$\underline{C}$O).

MS (ES^+) *m/z* = 412.2 (MH^+)

HRMS (ES^+) *m/z* for $C_{25}H_{25}N_5O$ calculated: 412.2137, found: 412.2126.

4.7.2 *N*-(3-(allyl(phenyl)amino)propyl)-6-(1*H*-pyrazol-1-yl)nicotinamide (<u>14</u>)

Description: light-pink solid

Reaction yield: η = 88.9% (0.151 g)

Rf = 0.51 (MF = EtOAc: hexane = 1:1)

Melting point = 111-112 °C

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.78-1.89 (m, 2H, $CH_2C\underline{H_2}CH_2$), 3.36 (s, 4H, COO-NH-$CH_2CH_2C\underline{H_2}$ and COO-NH-$C\underline{H_2}CH_2CH_2$)*, 3.93-9.95 (m, 2H, N-$C\underline{H_2}$-$CH=CH_2$), 5.07-5.15 (m, 2H, N-CH_2-$CH=C\underline{H_2}$), 5.77-5.88 (m, 1H, N-CH_2-$C\underline{H}=CH_2$), 6.55-6.59 (m, 1H, Ar-$\underline{H}$), 6.63 (dd, *J* = 2.6, 1.7 Hz, 1H, Ar-$\underline{H}$), 6.65-6.71 (m, 2H, Ar-$\underline{H}$), 7.08-7.16 (m, 2H, Ar-$\underline{H}$), 7.89 (dd, *J* = 1.7, 0.7 Hz, 1H, Ar-$\underline{H}$), 8.02 (dd, *J* = 8.6, 0.8 Hz, 1H, Ar-$\underline{H}$), 8.40 (dd, *J* = 8.6, 2.3 Hz, 1H, Ar-$\underline{H}$), 8.69 (dd, *J* = 2.6, 0.7 Hz, 1H, Ar-$\underline{H}$), 8.76 (t, *J* = 5.5 Hz, 1H, Ar-CO-N$\underline{H}$), 8.91 (dd, *J* = 2.3, 0.8 Hz, 1H, Ar-$\underline{H}$). *Signal partially overlaps with that of H_2O in DMSO.

^{13}C (100 MHz, $CDCl_3$) δ(ppm) = 27.13 ($CH_2\underline{C}H_2CH_2$), 38.60 (COO-NH-$\underline{C}H_2CH_2CH_2$), 48.75 (COO-NH-$CH_2CH_2\underline{C}H_2$ and N-$\underline{C}H_2$-$CH=CH_2$), 108.45 (Ar-$\underline{C}$), 111.83 (N-CH_2-$CH=\underline{C}H_2$ and Ar-$\underline{C}$), 113.22 (3×Ar-$\underline{C}$), 127.59 (Ar-$\underline{C}$), 129.48 (3×Ar-$\underline{C}$), 137.55 (N-CH_2-$\underline{C}H=CH_2$), 142.87 (2×Ar-$\underline{C}$), 147.20 (2×Ar-$\underline{C}$), 153.09 (Ar-$\underline{C}$), 164.97 (Ar-$\underline{C}$O).

MS (ES^+) *m/z* = 362.2 (MH^+)

HRMS (ES^+) *m/z* for $C_{21}H_{23}N_5O$ calculated: 362.1981, found: 362.1980

4.7.3 *N*-(3-((3,4-difluorobenzyl)(phenyl)amino)propyl)-6-(1*H*-pyrazol-1-yl) nicotinamide (15)

Description: white solid

Reaction yield: η = 65.6% (0.138 g)

Rf = 0.37 (MF = EtOAc: hexane = 1:1)

Melting point = 123-124 °C

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.88 (p, *J* = 7.3 Hz, 2H, $CH_2C\underline{H_2}CH_2$), 3.36-3.41 (m, 2H, COO-NH-$C\underline{H_2}CH_2CH_2$), 3.51 (t, *J* = 7.3 Hz, 2H, COO-NH-$CH_2CH_2C\underline{H_2}$), 4.56 (s, 2H, Ar-$C\underline{H_2}$), 6.57-6.62 (m, 1H, Ar-H), 6.63 (dd, *J* = 2.6, 1.7 Hz, 1H, Ar-H), 6.65-6.71 (m, 2H, Ar-H), 7.02-7.08 (m, 1H, Ar-H), 7.08-7.15 (m, 2H, Ar-H), 7.20-7.25 (m, 1H, Ar-H), 7.33-7.40 (m, 1H, Ar-H), 7.89 (dd, *J* = 1.7, 0.7 Hz, 1H, Ar-H), 8.01 (dd, *J* = 8.6, 0.8 Hz, 1H, Ar-H), 8.38 (dd, *J* = 8.6, 2.3 Hz, 1H, Ar-H), 8.69 (dd, *J* = 2.6, 0.7 Hz, 1H, Ar-H), 8.75 (t, *J* = 5.5 Hz, 1H, Ar-CO-NH), 8.90 (dd, *J* = 2.3, 0.8 Hz, 1H, Ar-H)

^{13}C (100 MHz, $CDCl_3$) δ(ppm) = 26.80 ($CH_2\underline{C}H_2CH_2$), 37.14 (COO-NH-$\underline{C}H_2CH_2CH_2$), 48.62 (COO-NH-$CH_2CH_2\underline{C}H_2$), 52.77 (Ar-$\underline{C}H_2$), 108.77 (Ar-C), 111.27 (Ar-C), 112.11 (2×Ar-C), 115.33 (Ar-C), 115.94 (Ar-C), 117.41 (Ar-C), 122.96 (Ar-C), 127.51 (2×Ar-C), 127.95 (Ar-C), 129.06 (2×Ar-C), 137.87 (2×Ar-C), 142.91 (Ar-C), 148.92 (4×Ar-C), 164.01 (Ar-CO).

MS (ES^+) *m/z* = 448.2 (MH^+)

HRMS (ES^+) *m/z* for $C_{25}H_{23}F_2N_5O$ calculated: 448.1949, found: 448.1953

4.7.4 *N*-(3-((3,4-dichlorobenzyl)(phenyl)amino)propyl)-6-(1*H*-pyrazol-1-yl) nicotinamide (16)

Description: yellow-green solid

Reaction yield: η = 46.9% (0.106 g)

Rf = 0.25 (MF = EtOAc: hexane = 1:1)

Melting point = 149-152 °C

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.88 (p, *J* = 7.3 Hz, 2H, $CH_2C\underline{H_2}CH_2$), 3.38-3.42 (m, 2H, COO-NH-$C\underline{H_2}CH_2CH_2$), 3.52 (t, *J* = 7.3 Hz, 2H, COO-NH-$CH_2CH_2C\underline{H_2}$), 4.59 (s, 2H, Ar-$C\underline{H_2}$), 6.58-6.60 (m, 1H, Ar-H), 6.63 (dd, *J* = 2.7, 1.7 Hz, 1H, Ar-H), 6.65-6.71 (m, 2H, Ar-H), 7.09-7.16 (m, 2H, Ar-H), 7.19 (dd, *J* = 8.3, 2.0 Hz, 1H, Ar-H), 7.44 (d, *J* = 2.0 Hz, 1H, Ar-H), 7.57 (d, *J* = 8.3 Hz, 1H, Ar-H), 7.89 (dd, *J* = 1.7, 0.7 Hz, 1H, Ar-H), 8.01 (dd, *J* = 8.6, 0.8 Hz, 1H, Ar-H), 8.38 (dd, *J* = 8.6, 2.3 Hz, 1H, Ar-H), 8.69 (dd,

J = 2.7, 0.7 Hz, 1H, Ar-H), 8.75 (t, *J* = 5.5 Hz, 1H, Ar-CO-NH), 8.89 (dd, *J* = 2.3, 0.8 Hz, 1H, Ar-H).

^{13}C (100 MHz, $CDCl_3$) δ(ppm) = 27.25 ($CH_2CH_2CH_2$), 38.19 (COO-NH-$CH_2CH_2CH_2$), 48.63 (COO-NH-$CH_2CH_2CH_2$), 53.94 (Ar-CH_2), 108.50 (Ar-C), 111.90 (Ar-C), 113.27 (3×Ar-C), 127.61 (2×Ar-C), 129.59 (5×Ar-C), 130.66 (2×Ar-C), 132.78 (Ar-C), 137.56 (2×Ar-C), 142.93 (3×Ar-C), 164.96 (Ar-CO).

MS (ES^+) *m/z* = 480.1 (MH^+)

HRMS (ES^+) *m/z* for $C_{25}H_{23}Cl_2N_5O$ calculated: 480.1358, found: 480.1349

4.7.5 *N*-(2-((4-chlorobenzyl)(phenyl)amino)ethyl)-6-(1*H*-pyrazol-1-yl)nicotinamide (17)

Description: white solid

Reaction yield: η = 52.2% (0.106 g)

Rf = 0.32 (MF = EtOAc: hexane = 1:1)

Melting point = 80-84 °C

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.87 (q, *J* = 7.4 Hz, 2H, $CH_2CH_2CH_2$), 3.36-3.41 (m, 2H, COO-NH-$CH_2CH_2CH_2$), 3.50 (t, *J* = 7.4 Hz, 2H, COO-NH-$CH_2CH_2CH_2$), 4.57 (s, 2H, Ar-CH_2), 6.56-6.60 (m, 1H, Ar-H), 6.63 (dd, *J* = 2.6, 1.6 Hz, 1H, Ar-H), 6.64-6.69 (m, 2H, Ar-H), 7.07-7.14 (m, 2H, Ar-H), 7.19-7.25 (m, 2H, Ar-H), 7.33-7.39 (m, 2H, Ar-H), 7.89 (dd, *J* = 1.6, 0.7 Hz, 1H, Ar-H), 8.01 (dd, *J* = 8.6, 0.8 Hz, 1H, Ar-H), 8.38 (dd, *J* = 8.6, 2.3 Hz, 1H, Ar-H), 8.69 (dd, *J* = 2.6, 0.7 Hz, 1H, Ar-H), 8.75 (t, *J* = 5.5 Hz, 1H, Ar-CO-NH), 8.89 (dd, *J* = 2.3, 0.8 Hz, 1H, Ar-H).

^{13}C (100 MHz, $CDCl_3$) δ(ppm) = 38.28 ($CH_2CH_2CH_2$, COO-NH-$CH_2CH_2CH_2$, COO-NH-$CH_2CH_2CH_2$), 77.23 (Ar-CH_2), 108.48 (2×Ar-C), 111.87 (Ar-C), 113.35 (2×Ar-C), 127.60 (2×Ar-C), 128.23 (4×Ar-C), 128.81 (3×Ar-C), 129.52 (2×Ar-C), 142.91 (4×Ar-C), 163.90 (Ar-CO).

MS (ES^+) *m/z* = 446.2 (MH^+)

HRMS (ES^+) *m/z* for $C_{25}H_{24}ClN_5O$ calculated: 446.1748, found: 446.1759

4.7.6 Ethyl 2-((3-(6-(1*H*-pyrazol-1-yl)nicotinamido)propyl)(phenyl)amino)acetate (18)

Description: white solid

Reaction yield: η = 91.4% (0.350 g)

Rf = 0.25 (MF = DCM: methanol = 20:1)

Melting point = 123-126 °C

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.19 (t, *J* = 7.1 Hz, 3H, COO-CH_2-$C\underline{H}_3$), 1.86 (p, *J* = 7.2 Hz, 2H, $CH_2C\underline{H}_2CH_2$), 3.37-3.40 (m, 2H, COO-NH-$C\underline{H}_2CH_2CH_2$), 3.44 (t, *J* = 7.2 Hz, 2H, COO-NH-$CH_2CH_2C\underline{H}_2$), 4.11 (q, *J* = 7.1 Hz, 2H, COO-$C\underline{H}_2$-CH_3), 4.18 (s, 2H, Ar-$C\underline{H}_2$), 6.58-6.65 (m, 4H, Ar-H), 7.11-7.18 (m, 2H, Ar-H), 7.89 (dd, *J* = 1.6, 0.7 Hz, 1H, Ar-H), 8.02 (dd, *J* = 8.6, 0.8 Hz, 1H, Ar-H), 8.39 (dd, *J* = 8.6, 2.3 Hz, 1H, Ar-H), 8.69 (dd, *J* = 2.6, 0.7 Hz, 1H, Ar-H), 8.75 (t, *J* = 5.5 Hz, 1H, Ar-CO-NH), 8.91 (dd, *J* = 2.3, 0.8 Hz, 1H, Ar-H).

^{13}C (100 MHz, $CDCl_3$) δ(ppm) = 14.21 ($C\underline{H}_2C\underline{H}_3$), 26.97 ($CH_2\underline{C}H_2CH_2$), 39.01 (COO-NH-$\underline{C}H_2CH_2CH_2$), 51.09 (COO-NH-$CH_2CH_2\underline{C}H_2$), 55.57 ($CH_3CH_2$-COO-$\underline{C}H_2$), 61.67 ($\underline{C}$OO), 108.48 (2×Ar-C), 111.38 (Ar-C), 113.60 (2×Ar-C), 118.65 (Ar-C), 127.52 (Ar-C), 127.82 (Ar-C), 129.39 (2×Ar-C), 137.56 (Ar-C), 142.64 (Ar-C), 147.58 (Ar-C), 152.67 (Ar-C), 165.11 (Ar-C), 172.60 (Ar-$\underline{C}$O).

MS (ES^+) *m/z* = 408.2 (MH^+)

HRMS (ES^+) *m/z* for $C_{22}H_{25}N_5O_3$ calculated: 408.2036, found: 408.2031

4.7.7 *N*-(3-(phenethyl(phenyl)amino)propyl)-6-(1*H*-pyrazol-1-yl)nicotinamide (19)

Description: yellow-brown solid

Reaction yield: η = 70.0% (0.140 g)

Rf = 0.44 (MF = EtOAc: hexane = 1:1)

Melting point = 95-98 °C

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.81 (p, *J* = 7.1 Hz, 2H, $CH_2C\underline{H}_2CH_2$), 2.77-2.83 (m, 2H, Ar-$C\underline{H}_2$-CH_2), 3.31-3.35 (m, 4H, COO-NH-$C\underline{H}_2CH_2CH_2$ and Ar-CH_2-$C\underline{H}_2$), 3.47-3.54 (m, 2H, COO-NH-$CH_2CH_2C\underline{H}_2$), 6.57-6.61 (m, 1H, Ar-H), 6.63 (dd, *J* = 2.6, 1.7 Hz, Ar-H), 6.72-6.76 (m, 2H, Ar-H), 7.14-7.23 (m, 4H, Ar-H), 7.25-7.32 (m, 5H, Ar-H), 7.90 (dd, *J* = 1.7, 0.7 Hz, 1H, Ar-H), 8.02 (dd, *J* = 8.6, 0.8 Hz, 1H, Ar-H), 8.39 (dd, *J* = 8.6, 2.3 Hz, 1H, Ar-H), 8.70 (dd, *J* = 2.6, 0.7 Hz, 1H, Ar-H), 8.75 (t, *J* = 5.5 Hz, 1H, Ar-CO-NH), 8.91 (dd, *J* = 2.3, 0.8 Hz, 1H, Ar-H).

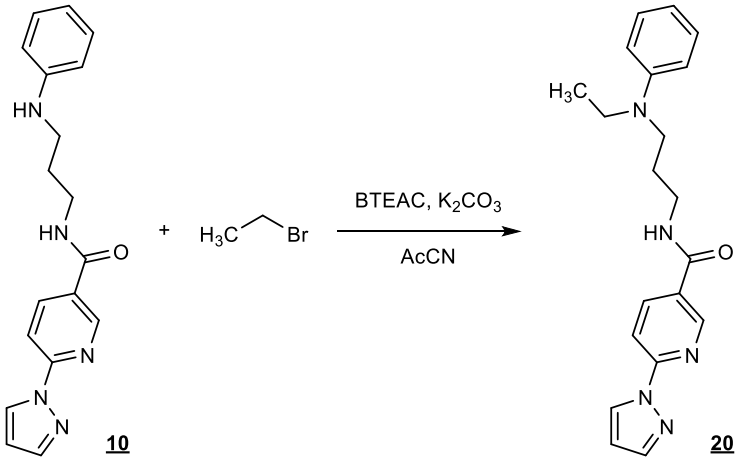
^{13}C (100 MHz, $CDCl_3$) δ(ppm) = 27.19 ($CH_2\underline{C}H_2CH_2$), 33.22 (Ar-$\underline{C}H_2CH_2$), 38.64 (COO-NH-$\underline{C}H_2CH_2CH_2$), 49.37 (COO-NH-$CH_2CH_2\underline{C}H_2$), 53.71 (Ar-$CH_2\underline{C}H_2$), 108.44 (Ar-C), 111.81 (2×Ar-C), 113.11 (2×Ar-C), 117.07 (Ar-C), 126.34 (Ar-C), 127.59 (Ar-C), 128.60

(3×Ar-C), 128.81 (2×Ar-C), 129.05 (Ar-C), 129.65 (2×Ar-C), 137.52 (Ar-C), 142.87 (Ar-C), 147.17 (Ar-C), 153.08 (Ar-C), 164.91 (Ar-CO).

MS (ES^+) *m/z* = 426.2 (MH^+)

HRMS (ES^+) *m/z* for $C_{26}H_{27}N_5O$ calculated: 426.2294, found: 426.2288

4.8 Synthesis of *N*-(3-(ethyl(phenyl)amino)propyl)-6-(1*H*-pyrazol-1-yl) nicotinamide (20)



In a 10 mL vial compound **10** (0.150 g, 0.47 mmol, 1.0 eq), K_2CO_3 (0.193 g, 1.40 mmol, 3.0 eq), BTEAC (0.011 g, 0.05 mmol, 0.1 eq), and bromoethane (0.105 mL, 1.40 mmol, 3 eq) were dissolved in AcCN (3 mL). The reaction was carried out in microwave reactor under the following conditions: 100 °C, 15 bar for 20 minutes. Because there was still a lot of compound **10**, bromoethane (0.350 mL, 4.67 mmol, 10 eq) and BTEAC (0.011 g, 0.05 mmol, 0.1 eq) were added and the reaction was carried out again in microwave reactor for 20 minutes under the same conditions. After that the reaction mixture was filtered to remove K_2CO_3, the solvent was evaporated under reduced pressure and the compound was purified by column chromatography using EtOAc/hexane = 1:1.

Description: white solid

Reaction yield: η = 59.7% (0.098 g)

Rf = 0.36 (MF = EtOAc: hexane = 1:1)

Melting point = 105-106 °C

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.08 (t, *J* = 7.0 Hz, 3H, CH_2CH_3), 1.77-1.86 (m, 2H, COO-NH-$CH_2CH_2CH_2$), 2.30-2.47(m, 2H, COOH-NH-$CH_2CH_2CH_2$), 3.35-3.41 (m, 4H, CH_2CH_3 and COO-NH-$CH_2CH_2CH_2$), 6.53-6.58 (m, 2H, Ar-H), 6.63 (dd, *J* = 2.6, 1.7 Hz, 1H, Ar-H), 6.67 (dd, *J* = 8.8, 0.8 Hz, 2H, Ar-H), 7.09-7.16 (m, 2H, Ar-H), 7.90

(dd, *J* = 1.7, 0.7 Hz, 1H, Ar-H), 8.02 (dd, *J* = 8.6, 0.8 Hz, 1H, Ar-H), 8.40 (dd, *J* = 8.6, 2.3 Hz, 1H, Ar-H), 8.69 (dd, *J* = 2.6, 0.7 Hz, 1H, Ar-H), 8.76 (t, *J* = 5.4 Hz, 1H, Ar-CO-NH), 8.91 (dd, *J* = 2.3, 0.8 Hz, 1H, Ar-H).

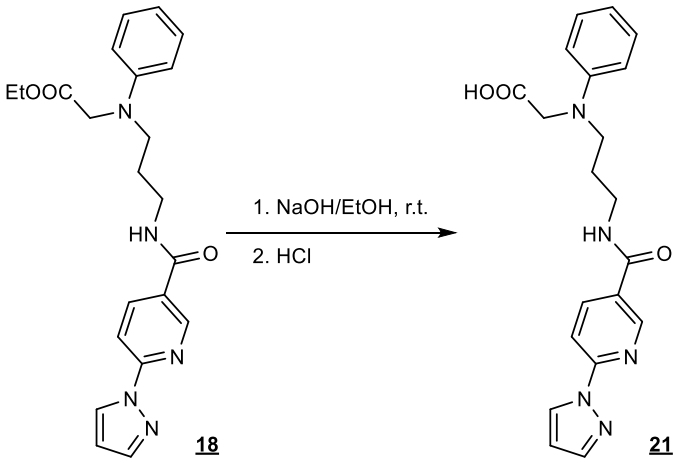
^{13}C (100 MHz, $CDCl_3$) δ(ppm) = 11.86 (CH_3CH_2), 27.05 ($CH_2CH_2CH_2$), 38.89 (COO-NH-$CH_2CH_2CH_2$), 48.79 (COO-NH-$CH_2CH_2CH_2$ and CH_3CH_2), 108.43 (Ar-C), 111.79 (2×Ar-C), 113.45 (2×Ar-C), 127.58 (2×Ar-C), 129.58 (3×Ar-C), 137.49 (Ar-C), 142.85 (Ar-C), 147.25 (Ar-C), 153.05 (Ar-C), 164.91 (Ar-CO).

MS (ES^+) *m/z* = 350.2 (MH^+)

HRMS (ES^+) *m/z* for $C_{20}H_{23}N_5O$ calculated: 350.1981, found: 350.1982

This compound is already known [12].

4.9 Synthesis of 2-((3-(6-(1*H*-pyrazol-1-yl)nicotinamido)propyl)(phenily) amino) acetic acid (21)



18 21

Compound **18** (0.175 g, 0.43 mmol, 1.0 eq) was dissolved in ethanol (5 mL), 3M $NaOH_{(aq)}$ (0.43 mL, 1.29 mmol, 3.0 eq) was added, and the mixture stirred for 2 hours at room temperature. Ethanol was evaporated under reduced pressure; 20 mL of distillated water was added to the residue and extracted with 10 mL of diethyl ether two times. pH of the water phase was adjusted with 2M HCl till pH = 3 to obtain white precipitate. The precipitate was filtered with vacuum and dried at room temperature.

Description: white-gray solid

Reaction yield: η = 84.0% (0.137 g)

Rf = 0.02 (MF = EtOAc: hexane = 1:1)

Melting point = 127-130 °C

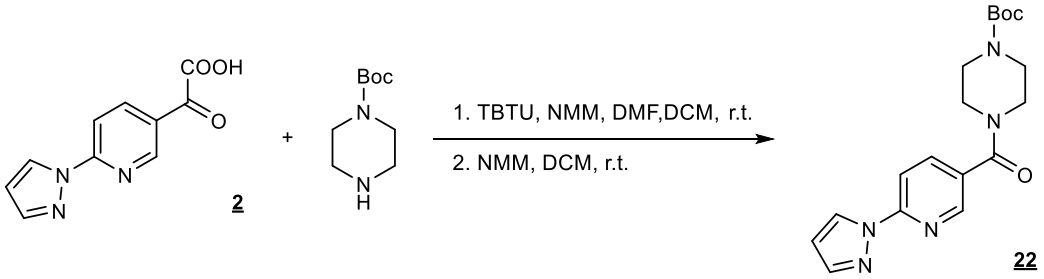
^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.86 (p, *J* = 7.2 Hz, 2H, $CH_2C\underline{H_2}CH_2$), 3.36-6.41 (m, 2H, COO-NH-$C\underline{H_2}CH_2CH_2$), 3.44 (t, *J* = 7.2 Hz, 2H, COO-NH-$CH_2CH_2C\underline{H_2}$), 4.08 (s, 2H, $C\underline{H_2}$-COOH), 6.60 (m, 3H, Ar-<u>H</u>), 6.63 (dd, *J* = 2.6, 1.7 Hz, 1H, Ar-<u>H</u>), 7.11-7.17 (m, Hz, 2H, Ar-<u>H</u>), 7.90 (dd, *J* = 1.7, 0.7 Hz, 1H, Ar-<u>H</u>), 8.01 (dd, *J* = 8.6, 0.8 Hz, 1H, Ar-<u>H</u>), 8.39 (dd, *J* = 8.6, 2.3 Hz, 1H, Ar-<u>H</u>), 8.69 (dd, *J* = 2.6, 0.7 Hz, 1H, Ar-<u>H</u>), 8.76 (t, *J* = 5.5 Hz, 1H, Ar-CO-N<u>H</u>), 8.90 (dd, *J* = 2.3, 0.8 Hz, 1H, Ar-<u>H</u>), 12.59 (s, 1H, COO<u>H</u>).

^{13}C (100 MHz, $CDCl_3$) δ(ppm) = 27.02 ($CH_2\underline{C}H_2CH_2$), 37.16 (COO-NH-$\underline{C}H_2CH_2CH_2$), 49.03 (COO-NH-$CH_2CH_2\underline{C}H_2$), 108.78 (Ar-<u>C</u>), 111.27 (Ar-<u>C</u>), 111.36 (2×Ar-<u>C</u>), 115.74 (Ar-<u>C</u>), 127.52 (Ar-<u>C</u>), 128.02 (Ar-<u>C</u>), 128.95 (2×Ar-<u>C</u>), 138.49 (Ar-<u>C</u>), 142.91 (Ar-<u>C</u>), 147.65 (Ar-<u>C</u>), 147.78 (Ar-<u>C</u>), 152.06 (Ar-<u>C</u>), 164.03 (<u>C</u>OOH), 172.38 (Ar-<u>C</u>O).

MS (ES^{+}) *m/z* = 378.2 (MH^{-})

HRMS (ES^{+}) *m/z* for $C_{20}H_{21}N_5O_3$ calculated: 378.1566, found: 378.1560

4.10 Synthesis of *tert*-butyl 4-(6-(1*H*-pyrazol-1-yl)nicotinoyl)piperazine-1-carboxylate (<u>22</u>)



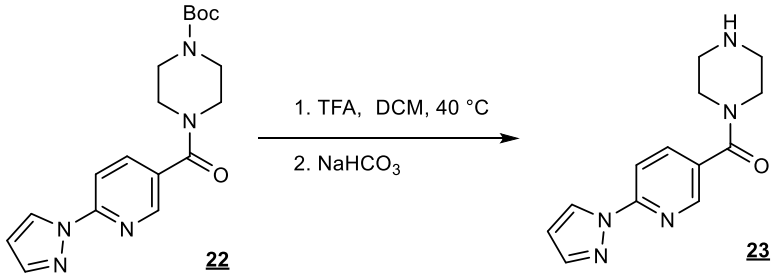
Compound **<u>2</u>** (0.50 g, 2.64 mmol, 1.0 eq) and TBTU (1.10 g, 3.43 mmol, 1.3 eq) were added into the mixture of DMF (5 mL) and DCM (5 mL), and stirried under the argon atmosphere at room temperature. After few minutes, NMM (0.87 mL, 7.92 mmol, 3.0 eq) was added, leaving the reaction for 40 minutes. 1-Boc piperazine (0.49 g, 2.64 mmol, and 1.0 eq) was diluted in DCM (5 mL) and added dropwise. In the end NMM (0.29 mL, 2.64 mmol, and 1.0 eq) was added and the reaction was left stirring overnight. The next day the reaction mixture was evaporated and diluted in 40 mL of EtOAc and then extracted with 3 × 20 mL of distilled water, 3 × 20 mL of saturated $NaHCO_{3(aq)}$ and 1 × 20 mL of saturated $NaCl_{(aq)}$. The organic phase was dried over sodium sulphate, filtered and the solvent evaporated under reduced pressure. Reaction was repeated with the same amount of substances. The compound was taken to the next step withouth further characterization.

Description: white solid

Reaction yield: η_1 = 100.0% (0.950 g), η_2 = 84.0% (0.793 g)

Rf = 0.26 (MF = DCM: methanol = 20:1), 0.36 (MF = EtOAc: hexane = 1:1)

4.11 Synthesis of (6-(1*H*-pyrazol-1-yl)pyridin-3-yl)(piperazin-1-yl) methanone (23)



Compound **22** was diluted in redistilled DCM (20 mL). To that trifluoroacetic acid was added and stirred at 40 °C under the argon atmosphere overnight. 20 mL of DCM was added and extracted with 3 × 15 mL of saturated $NaHCO_{3(aq)}$. Because the substance was still in aqueous phase, it was extracted with 4 × 40 mL of DCM. Organic phase was then washed with saturated $NaCl_{(aq)}$, dried over sodium sulphate, filtered and evaporated under reduced pressure. The used quantities are in the table XIV.

Table XIV: Reagents in synthesis of (6-(1*H*-pyrazol-1-yl)pyridin-3-yl)(piperazin-1-yl) methanone.

The reagent	Amount	n (mmol)	Eq
Compound **22**	0.950 g	2.658	1.0
	0.793 g	2.219	
Trifluoroacetic acid	1.18 mL + 0.79 mL	15.95 + 10.63	6.0 + 4.0
	1.65 mL	22.19	10.0

Description: white solid

Reaction yield: η_1 = 79.5% (0.544 g), η_2 = 84.9% (0.485 g)

Rf = 0.33 (MF = dichloromethane: methanol = 20:1)

Melting point = 122 °C

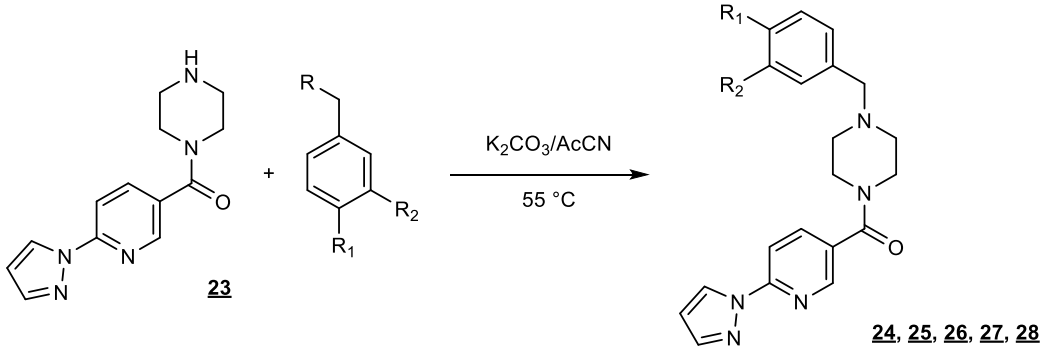
^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 2.70 (s, 4H, $N(CH_2C\underline{H_2})_2NH$), 3.34-3.58 (2×s, 5H, $N(C\underline{H_2}CH_2)_2NH$ and $N(CH_2CH_2)_2N\underline{H}$)), 6.62 (dd, *J* = 2.6, 1.7 Hz, 1H, Ar-H), 7.88 (dd, *J* = 1.7, 0.7 Hz, 1H, Ar-H), 7.98 (dd, *J* = 8.4, 0.9, Hz, 1H, Ar-H), 8.03 (dd, *J* = 8.4,

2.2 Hz, 1H, Ar-H), 8.51 (dd, *J* = 2.2, 0.9 Hz, 1H, Ar-H), 8.66 (dd, *J* = 2.6, 0.7 Hz, 1H, Ar-H).

MS (ES^+) *m/z* = 258.1 (MH^+)

HRMS (ES^+) *m/z* for $C_{13}H_{15}N_5O$ calculated: 258.1355, found: 258.1354

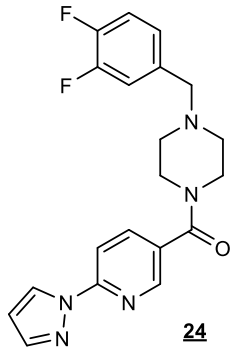
4.12 Derivatives of (6-(1*H*-pyrazol-1-yl)pyridin-3-yl)(piperazin-1-yl) methanone

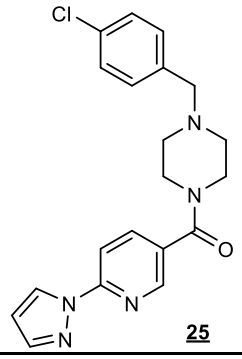
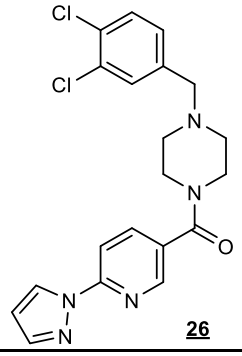
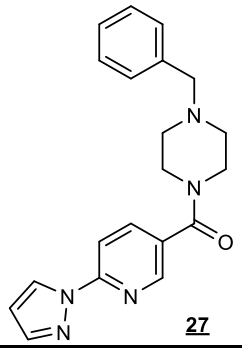
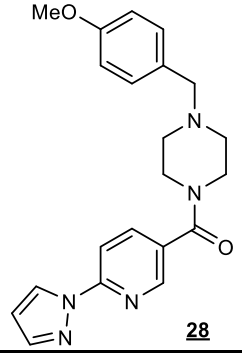


GENERAL SYNTHETIC PROCEDURES

Compound **23** (1.0 eq) and K_2CO_3 (2.5 eq) were diluted in acetonitrile (15 mL). To that various benzyl halydes (1.2 eq) were added and stirred overnight at 55 °C, equipped with chlorcalcium tube. K_2CO_3 was removed with filtration and the compounds were purified by column chromatography or recrystallization.

Table XV: Derivatives of (6-(1*H*-pyrazol-1-yl)pyridin-3-yl)(piperazin-1-yl)methanone and their reagents.

R	Final compound	The reagent	Amount	n (mmol)
R = Br $R_1 = R_2 = F$		Compound **23**	0.250 g	0.97
		3,4-difluorobenzyl bromide	0.15 mL	1.17
		K_2CO_3	0.335 g	2.43

R = Cl R_1 = Cl R_2 = H	 **25**	Compound **23**	0.200 g	0.78
		4-chlorobenzyl chloride	0.150 g	0.93
		K_2CO_3	0.270 g	1.95
R_1 = Br R_2 = R_3 = Cl	 **26**	Compound **23**	0.200 g	0.78
		3,4-dichlorobenzyl bromide	0.187 g	0.93
		K_2CO_3	0.270 g	1.95
R_1 = Cl R_2 = R_3 = H	 **27**	Compound **23**	0.120 g	0.47
		Benzyl chloride	0.07 mL	0.56
		K_2CO_3	0.161 g	1.17
R_1 = Cl R_2 = OMe R_3 = H	 **28**	Compound **23**	0.200 g	0.78
		4-methoxybenzyl chloride	0.13 mL	0.93
		K_2CO_3	0.270 g	1.95

4.12.1 (6-(1*H*-pyrazol-1-yl)pyridin-3-yl)(4-(3,4-difluorobenzyl)piperazin-1-yl) methanone (24)

The compound was purified by recrystallization, using EtOAc and hexane.

Description: white-silver solid

Reaction yield: η = 67.2% (0.250 g)

Rf = 0.23 (MF = DCM: methanol = 20:1)

Melting point = 122-124 °C

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 2.36-2.47 (m , 4H, N(CH_2CH_2)$_2$N-CH_2), 3.41 (d, *J* = 13.3 Hz, 2H, N(C$H_2$$CH_2$)$_2$N-$CH_2$), 3.52 (s, 2H, N(C$H_2$$CH_2$)$_2$N-$CH_2$), 3.66 (s, 2H, N-C$H_2$-Ar), 6.62 (dd, *J* = 2.6, 1.7 Hz, 1H, Ar-H), 7.16-7.20 (m, 1H, Ar-H), 7.34-7.43 (m, 2H, Ar-H), 7.88 (dd, *J* = 1.7, 0.7 Hz, 1H, Ar-H), 7.97 (dd, *J* = 8.5, 0.9 Hz, 1H, Ar-H), 8.03 (dd, *J* = 8.5, 2.3 Hz, 1H, Ar-H), 8.51 (dd, *J* = 2.3, 0.9 Hz, 1H, Ar-H), 8.65 (dd, *J* = 2.6, 0.7 Hz, 1H, Ar-H).

^{13}C (100 MHz, DMSO-d6) δ(ppm) = 40.14 (N(C$H_2$$CH_2$)$_2$N-$CH_2$), 60.29 (N($CH_2CH_2$)$_2$N-C$H_2$,), 108.68 (Ar-C), 111.48 (Ar-C), 117.29 (3×Ar-C), 127.30 (Ar-C), 129.50 (Ar-C), 138.56 (2×Ar-C), 142.77 (2×Ar-C), 146.89 (2×Ar-C), 151.05 (Ar-C), 166.09 (Ar-CO).

MS (ES$^+$) *m/z* = 384.2 (MH^+)

HRMS (ES$^+$) *m/z* for $C_{20}H_{19}F_2N_5O$ calculated: 384.1636, found: 384.1639

4.12.2 (6-(1*H*-pyrazol-1-yl)pyridin-3-yl)(4-(4-chlorobenzyl)piperazin-1-yl)methanone (25)

The compound was purified by column chromatography (MF = EtOAc: hexane = 2:1).

Description: white solid

Reaction yield: η = 80.6% (0.240 g)

Rf = 0.29 (MF = EtOAc: hexane = 2:1)

Melting point = 135-138 °C

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 2.31-2.48 (m, 4H, N(CH_2CH_2)$_2$N-CH_2), 3.42 and 3.64 (2×s, 4H, N(C$H_2$$CH_2$)$_2$N-$CH_2$), 3.51 (s, 2H, N-C$H_2$-Ar), 6.62 (dd, *J* = 2.6, 1.7 Hz, 1H, Ar-H), 7.31-7.42 (m, 4H, Ar-H), 7.88 (dd, *J* = 1.7, 0.7 Hz, 1H, Ar-H), 7.97 (dd, *J* = 8.5, 0.9 Hz, 1H, Ar-H), 8.03 (dd, *J* = 8.5, 2.2 Hz, 1H, Ar-H), 8.51 (dd, *J* = 2.2, 0.9 Hz, 1H, Ar-H), 8.65 (dd, *J* = 2.6, 0.7 Hz, 1H, Ar-H).

^{13}C (100 MHz, DMSO-d6) δ(ppm) = 39.15 (N(C$H_2$$CH_2$)$_2$N-$CH_2$), 39.38 (N($CH_2CH_2$)$_2$N-$CH_2$), 60.78 (N($CH_2$$CH_2$)$_2$N-C$H_2$), 108.67 (Ar-C), 111.48 (Ar-C), 127.30 (Ar-C), 128.17 (2×Ar-C), 129.50 (Ar-C), 130.62 (2×Ar-C), 131.52 (Ar-C), 136.87 (Ar-C), 138.56 (Ar-C), 142.76 (Ar-C), 146.89 (Ar-C), 151.05 (Ar-C), 166.09 (Ar-CO).

MS (ES$^+$) *m/z* = 382.1 (MH^+)

HRMS (ES$^+$) *m/z* for $C_{20}H_{20}ClN_5O$ calculated: 382.1435, found: 382.1428

The compound can be purchased [20].

4.12.3 (6-(1*H*-pyrazol-1-yl)pyridin-3-yl)(4-(3,4-dichlorobenzyl)piperazin-1-yl) methanone (26)

The compound was purified by column chromatography (MF = EtOAc: hexane = 2: 1).

Description: white-yellow solid

Reaction yield: η = 73.3% (0.238 g)

Rf = 0.15 (MF = EtOAc: hexane = 2:1)

Melting point = 129-132 °C

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 2.32-2.48 (m, 4H, N($CH_2C\underline{H_2}$)N-CH_2), 3.43 and 3.65 (2×s, 4H, N($C\underline{H_2}CH_2$)N-CH_2), 3.53 (s, 2H, $NC\underline{H_2}$Ar), 6.62 (dd, *J* = 2.6, 1.7 Hz, 1H, Ar-H), 7.33 (dd, *J* = 8.2, 2.0 Hz, 1H, Ar-H), 7.57-7.62 (m, 2H, Ar-H), 7.88 (dd, *J* = 1.7, 0.7 Hz, 1H, Ar-H), 7.97 (dd, *J* = 8.5, 0.9 Hz, 1H, Ar-H), 8.03 (dd, *J* = 8.5, 2.2 Hz, 1H, Ar-H), 8.51 (dd, *J* = 2.2, 0.9 Hz, 1H, Ar-H), 8.65 (dd, *J* = 2.6, 0.7 Hz, 1H, Ar-H).

^{13}C (100 MHz, DMSO-d6) δ(ppm) = 39.94 (N($\underline{C}H_2CH_2)_2$N-CH_2), 40.15 (N($CH_2\underline{C}H_2)_2$N-CH_2), 60.10 (N$(CH_2CH_2)_2$N-$\underline{C}H_2$), 108.67 (Ar-C), 111.48 (Ar-C), 127.30 (Ar-C), 129.07 (Ar-C), 129.48 (Ar-C), 130.38 (2×Ar-C), 130.56 (2×Ar-C), 130.85 (Ar-C), 138.57 (Ar-C), 139.28 (Ar-C), 142.77 (Ar-C), 146.89 (Ar-C), 151.05 (Ar-C), 166.09 (Ar-CO).

MS (ES$^+$) *m/z* **=** 416.1 (MH^+)

HRMS (ES$^+$) ***m/z*** for $C_{20}H_{19}Cl_2N_5O$ calculated: 416.1045, found: 416.1041

4.12.4 (6-(1*H*-pyrazol-1-yl)pyridin-3-yl)(4-benzylpiperazin-1-yl)methanone (27)

The compound was purified by column chromatography (MF = EtOAc:hexane = 3:1).

Description: white solid

Reaction yield: η = 72.9% (0.118 g)

Rf = 0.30 (MF = EtOAc: hexane = 3:1)

Melting point = 108-110 °C

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 2.41 (d, *J* = 14.6 Hz, 4H, N($CH_2C\underline{H_2}$)N-CH_2), 3.42 and 3.65 (2×s, 4H, N($C\underline{H_2}CH_2$)N-CH_2), 3.52 (s, 2H, N-$C\underline{H_2}$-Ar), 6.61 (dd, *J* = 2.6, 1.7 Hz, 1H, Ar-H), 7.23-7.29 (m, 1H, Ar-H), 7.29-7.37 (m, 4H, Ar-H), 7.87 (dd, *J* = 1.7, 0.7 Hz, 1H, Ar-H) 7.97 (dd, *J* = 8.5, 0.9 Hz, 1H, Ar-H), 8.03 (dd, *J* = 8.5, 2.2 Hz, 1H, Ar-H), 8.51 (dd, *J* = 2.2, 0.9 Hz, 1H, Ar-H), 8.65 (dd, *J* = 2.6, 0.7 Hz, 1H, Ar-H).

^{13}C (100 MHz, DMSO-d6) δ(ppm) = 39.93 (N($\underline{C}H_2CH_2)_2$N-CH_2), 39.94 (N($CH_2\underline{C}H_2)_2$N-CH_2), 61.76 (N$(CH_2CH_2)_2$N-$\underline{C}H_2$), 108.67 (Ar-C), 111.48 (Ar-C), 127.02 (Ar-C), 127.30

(Ar-C), 128.20 (2×Ar-C), 128.85 (2×Ar-C), 129.53 (Ar-C), 137.76 (Ar-C), 138.55 (Ar-C), 142.76 (Ar-C), 146.88 (Ar-C), 151.04 (Ar-C), 166.08 (Ar-CO).

MS (ES^+) *m/z* = 348.2 (MH^+)

HRMS (ES^+) *m/z* for $C_{20}H_{21}N_5O$ calculated: 348.1824, found: 348.1818

The compound can be purchased [20].

4.12.5 (6-(1*H*-pyrazol-1-yl)pyridin-3-yl)(4-(4-methoxybenzyl)piperazin-1-yl) methanone (28)

The compound was purified by column chromatography (MF = EtOAc: hexane = 3:1).

Description: yellow solid

Reaction yield: η = 76.1% (0.224 g)

Rf = 0.14 (MF = EtOAc: hexane = 3:1)

Melting point = 104-105 °C

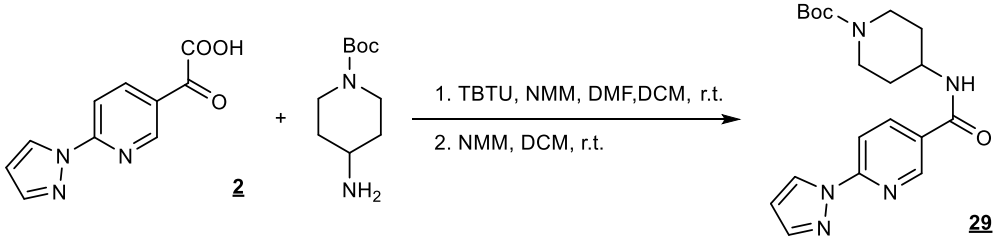
^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 2.30-2.46 (m, 4H, $N(CH_2CH_2)_2N$-CH_2), 3.40 and 3.63 (2×s, 4H, $N(CH_2CH_2)_2N$-CH_2), 3.44 (s, 2H, N-CH_2-Ar), 3.73 (s, 3H, Ar-OCH_3), 6.61 (dd, *J* = 2.6, 1.7 Hz, 1H, Ar-H), 6.86-6.91 (m, 2H, Ar-H), 7.19-7.25 (m, 2H, Ar-H), 7.87 (dd, *J* = 1.7, 0.7 Hz, 1H, Ar-H), 7.97 (dd, *J* = 8.5 Hz, 0.9 Hz, 1H, Ar-H), 8.02 (dd, *J* = 8.5, 2.2 Hz, 1H, Ar-H), 8.51 (dd, *J* = 2.2, 0.9 Hz, 1H, Ar-H), 8.65 (dd, *J* = 2.6, 0.7 Hz, 1H, Ar-H).

^{13}C (100 MHz, DMSO-d6) δ(ppm) = 39.15 ($N(CH_2CH_2)_2N$-CH_2), 54.95 (OCH3), 61.16 ($N(CH_2CH_2)_2N$-CH_2 and $N(CH_2CH_2)_2N$-CH_2), 108.67 (Ar-C), 111.48 (Ar-C), 113.55 (Ar-C), 127.29 (Ar-C), 129.50 (2×Ar-C), 129.54 (Ar-C), 130.10 (3×Ar-C), 138.55 (Ar-C), 142.76 (Ar-C), 146.88 (Ar-C), 151.04 (Ar-C), 158.30 (Ar-C), 166.07 (Ar-CO).

MS (ES^+) *m/z* = 378.2 (MH^+)

HRMS (ES^+) *m/z* for $C_{21}H_{23}N_5O_2$ calculated: 378.1930, found: 378.1926

4.13 Synthesis of *tert*-butyl 4-(6-(1*H*-pyrazol-1-yl)nicotinamido) piperidine-1-carboxylate (29)



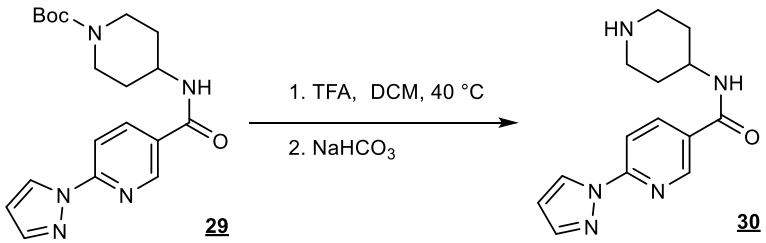
Compound **2** (0.944 g, 4.99 mmol, 1.0 eq), and TBTU (2.084 g, 6.49 mmol, 1.3 eq) were added into the mixture of DMF (10 mL) and DCM (10 mL), stirred under the argon atmosphere at room temperature. After few minutes NMM (1.65 mL, 15.0 mmol, 3.0 eq) was added, leaving the reaction for 40 minutes. 4-Amino-1-Boc-piperidine (1.00 g, 4.99 mmol, 1.0 eq) was diluted in DCM (10 mL) and added dropwise. In the end NMM (0.50 mL, 4.99 mmol, 1.0 eq) was added and the reaction was left stirring overnight. On the next day the reaction mixture was evaporated and diluted in 70 mL of EtOAc and then extracted with 3 × 30 mL of distilled water, 3 × 30 mL of saturated $NaHCO3_{(aq)}$ and 1 × 30 mL of saturated $NaCl_{(aq)}$. The organic phase was dried over sodium sulphate, filtered and the solvent evaporated under reduced pressure. The compound was taken to the next step withouth further characterization.

Description: white solid

Reaction yield: the compound was not completely dry at the time of weighting

Rf = 0.17 (MF = DCM: methanol = 20:1)

4.14 Synthesis of *N*-(piperidin-4-yl)-6-(1*H*-pyrazol-1-yl)nicotinamide (30)



Compound **29** (1.679 g, 4.54 mmol, 1.0 eq) was diluted in distilled DCM (30 mL). To that trifluoroacetic acid (3.38 mL, 45.5 mmol, 10.0 eq) was added and stirred at 40 °C under the argon atmosphere overnight. 30 mL of DCM and 30 mL of saturated $NaHCO_{3\ (aq)}$ were added and stirred on magnetic stirrer to determine pH. The pH was adjusted till 12 with 4M NaOH. Organic phase was than extracted with 2 × 30 mL of saturated $NaHCO_{3\ (aq)}$. Because the substance was still in aqueous phase, it was extracted with DCM and EtOAc. Organic phase was dried over sodium sulphate, filtered and evaporated under reduced pressure.

Description: white solid

Reaction yield: η = 93.9% (1.157 g)

Rf = 0.02 (MF = DCM: methanol = 9:1)

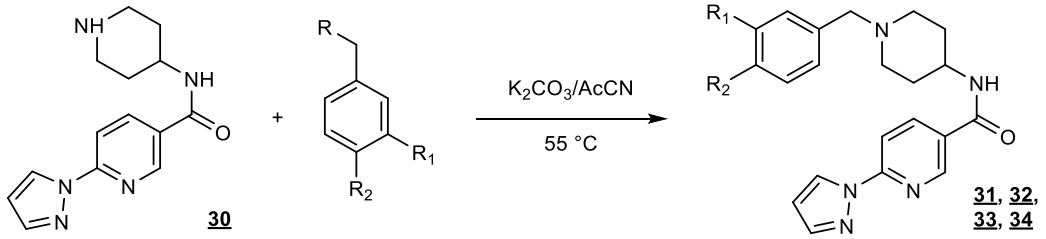
Melting point = 195-198 °C

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.47 (m, 2H, NHCH(C<u>H$_2$</u>CH$_2$)$_2$NH), 1.72-1.88 (m, 2H, NHCH(C<u>H$_2$</u>CH$_2$)$_2$NH), 2.58 (q, *J* = 14.3, 13.1 Hz, 2H, NHCH(CH$_2$C<u>H$_2$</u>)$_2$NH), 3.02 (d, *J* = 12.3 Hz, 2H, NHCH(CH$_2$C<u>H$_2$</u>)$_2$NH), 3.15-3.78 (m, 1H, NHCH(CH$_2$CH$_2$)$_2$N<u>H</u>)*, 3.88 (s, 1H, NHC<u>H</u>(CH$_2$CH$_2$)$_2$NH), 6.62 (dd, *J* = 2.6, 1.7 Hz, 1H, Ar-<u>H</u>), 7.89 (dd, *J* = 1.7, 0.7 Hz, 1H, Ar-<u>H</u>), 7.99 (dd, *J* = 8.6, 0.8 Hz, 1H, Ar-<u>H</u>), 8.40 (dd, *J* = 8.6, 2.4 Hz, 1H, Ar-<u>H</u>), 8.53 (d, *J* = 7.7 Hz, 1H, Ar-<u>H</u>), 8.68 (dd, *J* = 2.6, 0.7 Hz, 1H, Ar-<u>H</u>), 8.89 (dd, *J* = 2.4, 0.8 Hz, 1H, Ar-<u>H</u>). *Signal partially overlaps with that of H_2O in DMSO.

MS (ES$^+$) *m/z* = 272.1 (MH$^+$)

HRMS (ES$^+$) *m/z* for $C_{14}H_{17}N_5O$ calculated: 272.1511, found: 272.1515

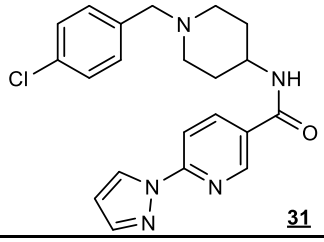
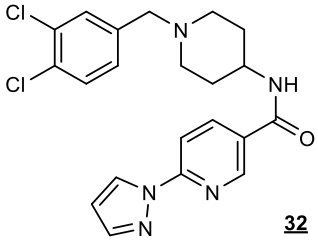
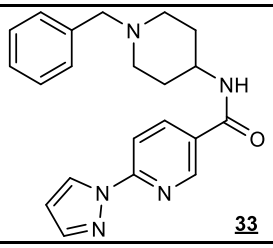
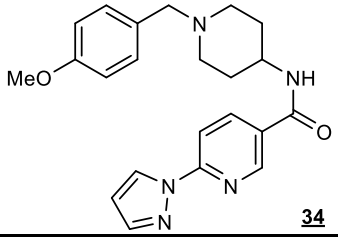
4.15 Derivatives of *N*-(piperidin-4-yl)-6-(1*H*-pyrazol-1-yl)nicotinamide



GENERAL SYNTHETIC PROCEDURE

Compound **<u>30</u>** (0.200 g, 0.737 mmol, 1.0 eq) and K_2CO_3 (0.255 g, 1.843 mmol, 2.5 eq) were diluted in acetonitrile (15 mL). To that various benzyl bromides/chlorides (0.885 mmol, 1.2 eq) were added and stirred overnight at 55 °C, equipped with chlorcalcium tube. Next day, after evaporating the solvent, 70 mL of DCM was added and extracted with 3 × 20 mL of distillated water. Organic phase was dried over sodium sulphate, filtered and evaporated under reduced pressure. The compounds were purified by recrystallization, using ethanol.

Table XVI: Derivatives of *N*-(piperidin-4yl)-6-(1*H*-pyrazol-yl)nicotinamide and their reagents.

R	Final compound	The reagent	Amount
R = Cl R_1 = H R_2 = Cl	 **31**	4-chlorobenzyl chloride	0.143 g
R = Br R_1 = Cl R_2 = Cl	 **32**	3,4-dichlorobenzyl bromide	0.212 g
R = Cl R_1 = H R_2 = H	 **33**	Benzyl chloride	0.10 mL
R = Cl R_1 = H R_2 = OMe	 **34**	4-methoxybenzyl chloride	0.12 mL

4.15.1 *N*-(1-(4-chlorobenzyl)piperidin-4-yl)-6-(1*H*-pyrazol-1-yl)nicotinamide (31)

Description: white solid

Reaction yield: η = 91.5% (0.267 g)

Rf = 0.42 (MF = DCM: methanol = 9:1)

Melting point = 235-139 °C

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.53-1.63 (m, 2H, NHCH(C$\underline{H_2}$CH$_2$)$_2$N), 1.79-1.84 (m, 2H, NHCH(C$\underline{H_2}$CH$_2$)$_2$N), 2.01-2.08 (m, 2H, NHCH(CH$_2$C$\underline{H_2}$)N), 2.75-2.86 (m, 2H, NHCH(CH$_2$C$\underline{H_2}$)N), 3.47 (s, 2H, Ar-C$\underline{H_2}$-N), 3.77-3.81 (m, 1H, NHC$\underline{H}$(CH$_2$CH$_2$)$_2$N), 6.62 (dd, *J* = 2.6, 1.6 Hz, 1H, Ar-$\underline{H}$), 7.30-7.42 (m, 4H, Ar-$\underline{H}$), 7.89 (d, *J* = 1.6 Hz, 1H, Ar-$\underline{H}$), 7.99 (dd, *J* = 8.6, 0.8 Hz, 1H, Ar-$\underline{H}$), 8.39 (dd, *J* = 8.6, 2.4 Hz, 1H, Ar-$\underline{H}$), 8.49 (d, *J* = 7.6 Hz, 1H, Ar-CO-N$\underline{H}$), 8.66-8.70 (m, 1H, Ar-$\underline{H}$), 8.88 (dd, *J* = 2.4, 0.8 Hz, 1H, Ar-$\underline{H}$).

^{13}C (100 MHz, DMSO-d6) δ(ppm) = 31.43 (NHCH(CH$_2$CH$_2$)$_2$N), 46.95 (NHCH(CH$_2$CH$_2$)$_2$N), 52.06 (NHCH(CH$_2$CH$_2$)$_2$N), 61.12 (Ar-CH$_2$), 108.78 (Ar-C), 111.18 (Ar-C), 127.51 (Ar-C), 128.02 (Ar-C), 128.10 (2×Ar-C), 130.44 (2×Ar-C), 131.30 (Ar-C), 137.71 (Ar-C), 138.56 (Ar-C), 142.90 (Ar-C), 147.79 (Ar-C), 152.04 (Ar-C), 163.33 (Ar-CO).

MS (ES$^+$) *m/z* = 396.2 (MH$^+$)

HRMS (ES$^+$) *m/z* for $C_{21}H_{22}ClN_5O$ calculated: 396.1591, found: 396.1602

The compound can be purchased [20].

4.15.2 *N*-(1-(3,4-dichlorobenzyl)piperidin-4-yl)-6-(1*H*-pyrazol-1-yl)nicotinamide (32)

Description: white solid

Reaction yield: η = 58.9% (0.187 g)

Rf = 0.42 (MF = DCM: methanol = 9:1)

Melting point = 245-247 °C

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.55-1.64 (m, 2H, NHCH(CH$_2$CH$_2$)$_2$N), 1.77-1.87 (m,2 H, NHCH(CH$_2$CH$_2$)$_2$N), 2.04-2.11 (m, 2H, NHCH(CH$_2$CH$_2$)$_2$N), 2.81 (d, *J* = 11.4 Hz, 2H, NHCH(CH$_2$CH$_2$)$_2$N) 3.49 (s, 2H, Ar-CH$_2$-N), 3.78-3.82 (m, 1H, NHCH(CH$_2$CH$_2$)$_2$N), 6.62 (dd, *J* = 2.7, 1.7 Hz, 1H, Ar-H), 7.32 (dd, *J* = 8.2, 2.0 Hz, 4H, Ar-H), 7.54-7.63 (m, 2H, Ar-H), 7.89 (d, *J* = 1.7 Hz, 1H, Ar-H), 8.00 (d, *J* = 8.6 Hz, 1H, Ar-H), 8.39 (dd, *J* = 8.6, 2.3 Hz, 1H, Ar-H), 8.49 (d, *J* = 7.6 Hz, 1H, Ar-CO-NH), 8.68 (d, *J* = 2.7 Hz, 1H, Ar-H), 8.88 (d, *J* = 2.3 Hz, 1H, Ar-H).

^{13}C (100 MHz, DMSO-d6) δ(ppm) = 31.44 (NHCH(CH$_2$CH$_2$)$_2$N), 46.84 (NHCH(CH$_2$CH$_2$)$_2$N), 52.01 (NHCH(CH$_2$CH$_2$)$_2$N), 60.41 (Ar-CH$_2$), 129.24 (Ar-C), 103.57 (Ar-C), 108.79 (Ar-C), 111.19 (Ar-C), 127.52 (Ar-C), 128.01 (Ar-C), 128.87 (Ar-C), 130.34 (3×Ar-C), 138.56 (Ar-C), 140.16 (Ar-C), 142.91 (Ar-C), 147.79 (Ar-C), 152.04 (Ar-C), 163.32 (Ar-CO).

MS (ES$^+$) *m/z* = 430.1 (MH$^+$)

HRMS (ES$^+$) *m/z* for $C_{21}H_{21}Cl_2N_5O$ calculated: 430.1201, found: 430.1209

4.15.3 *N*-(1-benzylpiperidin-4-yl)-6-(1*H*-pyrazol-1-yl)nicotinamide (33)

Description: white solid

Reaction yield: η = 60.4% (0.161 g)

Rf = 0.45 (MF = DCM: methanol = 9:1)

Melting point = 218-222 °C

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.57-1.64 (m, 2H, NHCH(C$\underline{H_2}$CH$_2$)$_2$N), 1.80-1.83 (m, 2H, NHCH(C$\underline{H_2}$CH$_2$)$_2$N), 1.99-2.09 (m, 2H, NHCH(CH$_2$C$\underline{H_2}$)$_2$N), 2.84 (d, *J* = 11.6 Hz, 2H, NHCH(CH$_2$C$\underline{H_2}$)$_2$N), 3.48 (s, 2H, Ar-C$\underline{H_2}$-N), 3.75-3.85 (m, 1H, NHC$\underline{H}$(CH$_2$CH$_2$)$_2$N), 6.63 (dd, *J* = 2.7, 1.7 Hz, 1H, Ar-$\underline{H}$), 7.23-7.28 (m, 1H, Ar-$\underline{H}$), 7.29-7.37 (m, 4H, Ar-$\underline{H}$), 7.89 (dd, *J* = 1.7, 0.8 Hz, 1H, Ar-$\underline{H}$), 8.00 (dd, *J* = 8.6, 0.8 Hz, 1H, Ar-$\underline{H}$), 8.39 (dd, *J* = 8.6, 2.4 Hz, 1H, Ar-$\underline{H}$), 8.50 (d, *J* = 7.6 Hz, 1H, Ar-CO-N$\underline{H}$), 8.68 (dd, *J* = 2.7, 0.8 Hz, 1H, Ar-$\underline{H}$), 8.88 (dd, *J* = 2.4, 0.8 Hz, 1H, Ar-$\underline{H}$).

^{13}C (100 MHz, DMSO-d6) δ(ppm) = 31.45 (NHCH(C$\underline{H_2}$CH$_2$)$_2$N), 47.01 (NHC$\underline{H}$(CH$_2$CH$_2$)$_2$N), 52.14 (NHCH(CH$_2$C$\underline{H_2}$)$_2$N), 62.09 (Ar-C$\underline{H_2}$), 108.78 (Ar-$\underline{C}$), 111.18 (Ar-$\underline{C}$), 126.82 (Ar-$\underline{C}$), 127.51 (Ar-$\underline{C}$), 128.03 (Ar-$\underline{C}$), 128.13 (2×Ar-$\underline{C}$), 128.69 (2×Ar-$\underline{C}$), 138.56 (Ar-$\underline{C}$), 138.61 (Ar-$\underline{C}$), 142.90 (Ar-$\underline{C}$), 147.79 (Ar-$\underline{C}$), 152.04 (Ar-$\underline{C}$), 163.32 (Ar-$\underline{C}$O).

MS (ES$^+$) *m/z* = 362.2 (MH$^+$)

HRMS (ES$^+$) *m/z* for $C_{21}H_{23}N_5O$ calculated: 362.1981, found: 362.1986

4.15.4 *N*-(1-(4-methoxybenzyl)piperidin-4-yl)-6-(1*H*-pyrazol-1-yl)nicotinamide (34)

Description: white-silver solid

Reaction yield: η = 54.8% (0.158 g)

Rf = 0.18 (MF = DCM: methanol = 9:1)

Melting point = 207-211 °C

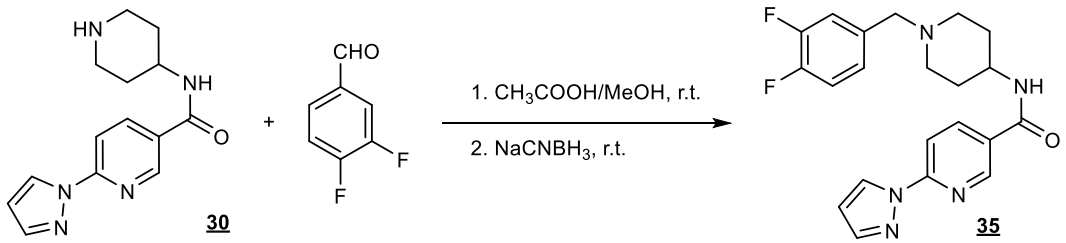
^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.42-2.20 (m, 6H, NHCH(C$\underline{H_2}$CH$_2$)$_2$N and NHCH(CH$_2$C$\underline{H_2}$)$_2$N), 2.93 (d, *J* = 84.5 Hz, 2H, NHCH(CH$_2$C$\underline{H_2}$)$_2$N), 3.77 (s, 5H, Ar-C$\underline{H_2}$-N and Ar-OC$\underline{H_3}$), 4.20 (m, 1H, NHC$\underline{H}$(CH$_2$CH$_2$)$_2$N), 6.63 (dd, *J* = 2.7, 1.6 Hz, 1H, Ar-$\underline{H}$), 6.81-7.60 (m, 4H, Ar-$\underline{H}$), 7.29-7.37 (m, 4H, Ar-$\underline{H}$), 7.89 (d, *J* = 1.6 Hz, 1H, Ar-$\underline{H}$), 8.00 (d, *J* = 8.6 Hz, 1H, Ar-$\underline{H}$), 8.35-8.44 (m, 1H, Ar-CO-N$\underline{H}$), 8.68 (d, *J* = 2.7 Hz, 1H, Ar-$\underline{H}$), 8.90 (s, 1H, Ar-$\underline{H}$).

^{13}C (100 MHz, DMSO-d6) δ(ppm) = 38.84-40.14 (NHCH(C$\underline{H_2}$CH$_2$)$_2$N together with DMSO-d6), 55.09 (NHC$\underline{H}$(CH$_2$CH$_2$)$_2$N, NHCH(CH$_2$C$\underline{H_2}$)$_2$N, Ar-C$\underline{H_2}$ and OC$\underline{H_3}$), 108.81(Ar-$\underline{C}$), 111.16 (Ar-$\underline{C}$), 113.89 (3×Ar-$\underline{C}$), 127.52 (4×Ar-$\underline{C}$), 138.66 (Ar-$\underline{C}$), 142.94 (Ar-$\underline{C}$), 147.93 (Ar-$\underline{C}$), 152.11 (Ar-$\underline{C}$).

MS (ES$^+$) *m/z* = 392.2 (MH$^+$)

HRMS (ES$^+$) *m/z* for $C_{22}H_{25}N_5O_2$ calculated: 392.2087, found: 392.2082

4.16 Synthesis of *N*-(1-(3,4-difluorobenzyl)piperidin-4-yl)-6-(1*H*-pyrazol-1-yl)nicotinamide (35)



Compound **30** (0.200 g, 0.737 mmol, 1.0 eq) was dissolved in 10 mL of anhydrous methanol, where acetic acid ≥ 99% (0.042 mL, 0.737 mmol, 1.0 eq) was added. After 15 minutes of stirring at room temperature under argon atmosphere, $NaCNBH_3$ (0.097 g, 1.548 mmol, 2.1 eq) was dissolved in 5 mL of methanol and added dropwise. After 10 minutes 3,4-difluorobenzaldehide (0.08 mL, 0.737 mmol, 1.0 eq) was added and the reaction was left stirring overnight. 20 mL of DCM and 10 mL of saturated $NaHCO_{3(aq)}$ was added in the reaction mixture, and the pH of the water phase was adjusted to 11 with 4M NaOH. Water phase was than extracted with 2 × 10 mL of DCM, combined organic phases were dried over sodium sulphate, filtered and evaporated under reduced pressure. The crude product was purified by column chromatography (MF = EtOAc: hexane = 2:1).

Description: white-silver solid

Reaction yield: η = 35.5% (0.104 g)

Rf = 0.36 (MF = EtOAc: hexane = 2:1)

Melting point = 225-229 °C

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.54-1.64 (m, 2H, NHCH(C$\underline{H_2}$CH$_2$)$_2$N), 1.80-1.84 (m, 2H, NHCH(C$\underline{H_2}$CH$_2$)$_2$N), 2.03-2.09 (m, 2H, NHCH(CH$_2$C$\underline{H_2}$)$_2$N), 2.79-2.84 (m, 2H, NHCH(CH$_2$C$\underline{H_2}$)$_2$N), 3.47 (s, 2H, Ar-C$\underline{H_2}$-N), 3.76-3.83 (m, 1H, NHC$\underline{H}$(CH$_2$CH$_2$)$_2$N), 6.62 (dd, *J* = 2.6, 1.7 Hz, 1H, Ar-$\underline{H}$), 7.14-7.18 (m, 1H, Ar-$\underline{H}$), 7.31-7.43 (m, 2H, Ar-$\underline{H}$), 7.89 (dd, *J* = 1.7, 0.7 Hz, 1H, Ar-$\underline{H}$), 8.00 (dd, *J* = 8.7, 0.8 Hz, 1H, Ar-$\underline{H}$), 8.39 (dd, *J* = 8.7, 2.3 Hz, 1H, Ar-$\underline{H}$), 8.50 (d, *J* = 7.6 Hz, 1H, Ar-CO-N$\underline{H}$), 8.68 (dd, *J* = 2.6, 0.7 Hz, 1H, Ar-$\underline{H}$), 8.88 (dd, *J* = 2.3, 0.8 Hz, 1H, Ar-$\underline{H}$).

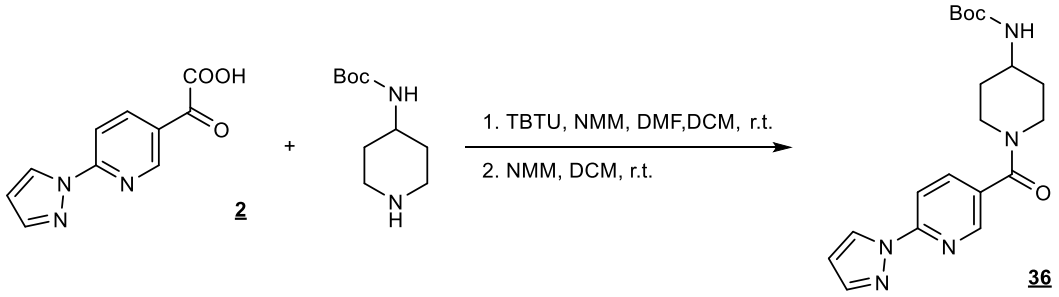
^{13}C (100 MHz, DMSO-d6) δ(ppm) = 31.42 (NHCH(C$\underline{H_2}$CH$_2$)$_2$N), 46.90 (NHC$\underline{H}$(CH$_2$CH$_2$)$_2$N), 51.98 (NHCH(CH$_2$C$\underline{H_2}$)$_2$N), 60.64 (Ar-C$\underline{H_2}$), 108.78 (Ar-$\underline{C}$), 111.18 (Ar-$\underline{C}$), 117.11 (3×Ar-$\underline{C}$), 127.51 (Ar-$\underline{C}$), 128.01 (Ar-$\underline{C}$), 137.59 (3×Ar-$\underline{C}$), 142.90 (2×Ar-$\underline{C}$), 147.79 (Ar-$\underline{C}$), 152.04 (Ar-$\underline{C}$), 163.33 (Ar-$\underline{C}$O).

MS (ES^+) ***m/z*** = 398.2 (MH^+)

HRMS (ES^+) ***m/z*** for $C_{21}H_{21}F_2N_5O$ calculated: 398.1792, found: 398.1782

The compound can be purchased. [20]

4.17 Synthesis of *tert*-butyl (1-(6-(1*H*-pyrazol-1-yl)nicotinoyl)piperidin-4-yl)carbamate (<u>36</u>)



Compound <u>**2**</u> and TBTU were dissolved in the mixture of DMF (10-15 mL) and DCM (10-15 mL), and stirred under the argon atmosphere at room temperature. After few minutes, NMM was added, leaving the reaction for 40 minutes. 4-(*N*-Boc-amino)piperidine was dissolved in DCM (10-15 mL) and added dropwise. In the end NMM was added and the reaction was left stirring overnight. On the next day the reaction mixture was evaporated and diluted in 50 mL of EtOAc and then extracted with 3 × 20 mL of distilled water, 3 × 20 mL of saturated $NaHCO_{3(aq)}$ and 1 × 20 mL of saturated $NaCl_{(aq)}$. The organic phase was dried over sodium sulphate, filtered and the solvent evaporated under reduced pressure. The compound was taken to the next step withouth further characterization. The used quantities are in the table XVII.

Table XVII: Reagents in synthesis of *tert*-butyl (1-(6-(1*H*-pyrazol-1-yl)nicotinoyl) piperidin-4-yl)carbamate.

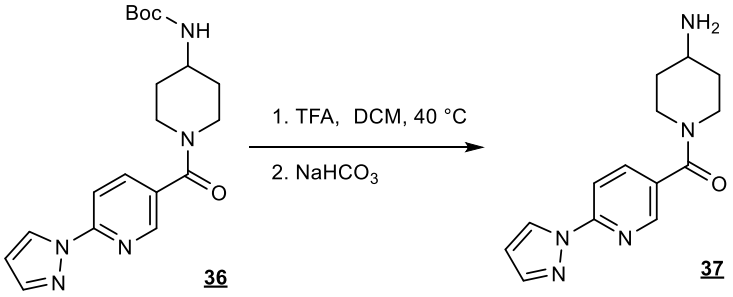
The reagent	Amount	n (mmol)	Eq
Compound <u>**2**</u>	1.417 g	7.49	1.0
	0.944 g	4.99	
TBTU	3.126 g	9.74	1.3
	2.083 g	6.49	
NMM	2.47 mL + 0.82 mL	22.47 + 7.49	3.0 + 1.0
	1.65 mL + 0.55 mL	14.98 + 4.99	
4-(*N*-Boc-amino)piperidine	1.500 g	7.49	1.0
	1.000 g	4.99	

Description: white solid

Reaction yield: η_1 = 83.4%, (2.319 g); η_2 = the compound was not complitely dry

Rf = 0.19 (MF = DCM: methanol = 20:1)

4.18 Synthesis of (6-(1*H*-pyrazol-1-yl)pyridin-3-yl)(4-aminopiperidin-1-yl)methanone (37)



Compound **36** was diluted in redistilled DCM (40 mL), trifluoroacetic acid was added, and the mixture stirred at 40 °C under the argon atmosphere overnight. 20 mL of DCM and 20 mL of saturated $NaHCO_{3(aq)}$ were added and stirred on magnetic stirrer to determine pH of the water phase. The pH was adjusted to 12 with 4M NaOH. Organic phase was than extracted with 2 × 20 mL of saturated $NaHCO_{3(aq)}$. Because the substance was still in aqueous phase, it was extracted with 2 × 30 mL of DCM and 3 × 30 mL of EtOAc. Organic phase was dried over sodium sulphate, filtered and evaporated under reduced pressure. The compound was not purified. The compound was taken to the next step withouth further characterization. The used quantities are in the table XVIII.

Table XVIII: Reagents in synthesis of (6-(1*H*-pyrazol-1-yl)pyridin-3-yl)(4-aminopiperidin-1-yl)methanone.

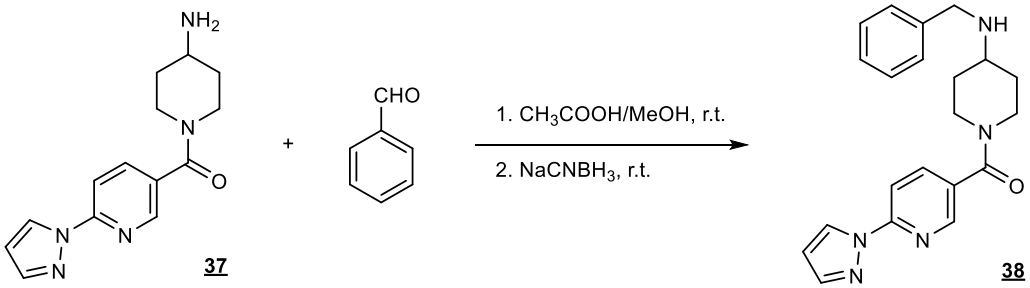
The reagent	Amount	n (mmol)	Eq
Compound **36**	2.319 g	6.243	1.0
	1.951 g	5.25	
Trifluoroacetic acid	4.64 mL	62.43	10.0
	3.90 mL	52.5	

Description: viscous colorless oil

Reaction yield: η_1 = 97.6% (1.654), η_2 = 100% (1.439 g)

Rf = 0.04 (MF = DCM: methanol = 9:1)

4.19 Synthesis of (6-(1*H*-pyrazol-1-yl)pyridin-3-yl)(4-(benzylamino) piperidin-1-yl)methanone (38)



Compound **37** (0.700g, 2.58 mmol, 1.0 eq), was dissolved in 35 mL of anhydrous methanol, acetic acid (≥ 99%, 0.147 mL, 2.58 mmol, 1.0 eq) was added to the solution, and after 30 minutes of stirring at room temperature under the argon atmosphere, $NaCNBH_3$ (0.341 g, 5.42 mmol, 2.1 eq) dissolved in 5 mL of MeOH was added dropewise. After 10 minutes benzaldehide (0.263 mL, 2.58 mmol, 1.0 eq) was added and the reaction was left stirring overnight. Into the reaction mixture 70 mL of DCM and 30 mL of saturated $NaHCO_{3\ (aq)}$ was added, and pH of the water phase was adjusted to 11 with 4M NaOH. Organic phase was than extracted with 2 × 20 mL of DCM, dried over sodium sulphate, filtered and evaporated under reduced pressure. The compound was cleaned by column chromatography (MF = EtOAc: methanol = 9:1).

Description: white solid

Reaction yield: η = 56.7% (0.529 g)

Rf = 0.27 (MF = EtOAc: methanol = 9:1)

Melting point = 100-101 °C

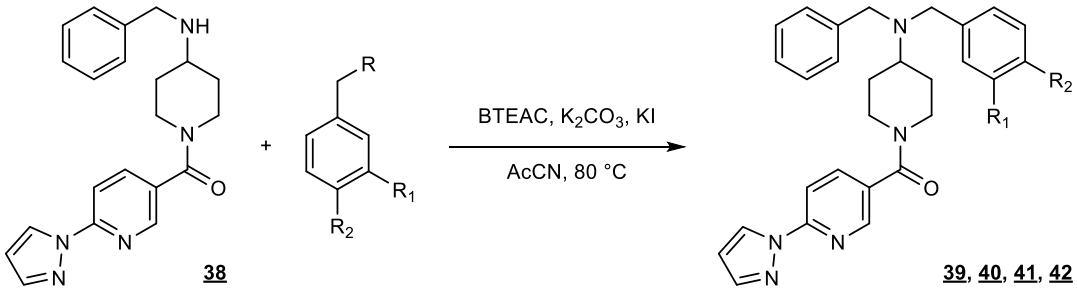
^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.21-1.39 (m, 2H, $N(CH_2C\underline{H_2})_2CH$), 1.74-1.90 (m, 2H, $N(CH_2C\underline{H_2})_2CH$), 2.16 (s, 1H, N<u>H</u>), 2.65-2.71 (m, 1H, $N(CH_2CH_2)_2C\underline{H}$), 3.03-3.12 (m, 2H, $N(C\underline{H_2}CH_2)_2CH$), 3.60 (s, 1H, $N(C\underline{H_2}CH_2)_2CH$) , 3.74 (s, 2H, NH-$C\underline{H_2}$-Ar), 4.24 (s, 1H, $N(C\underline{H_2}CH_2)_2CH$), 6.62 (dd, *J* = 2.6, 1.6 Hz, 1H, Ar-<u>H</u>), 7.18-7.24 (m, 1H, Ar-<u>H</u>), 7.27-7.37 (m, 4H, Ar-<u>H</u>), 7.88 (dd, *J* = 1.6, 0.8 Hz, 1H, Ar-<u>H</u>), 7.97 (dd, *J* = 8.4, 0.9 Hz, 1H, Ar-<u>H</u>), 8.02 (dd, *J* = 8.4, 2.2 Hz, 1H, Ar-<u>H</u>), 8.50 (dd, *J* = 2.2, 0.9 Hz, Ar-<u>H</u>), 8.65 (dd, *J* = 2.6, 0.8 Hz, 1H, Ar-<u>H</u>).

^{13}C (100 MHz, DMSO-d6) δ(ppm) = 45.79 ($N(CH_2\underline{C}H_2)_2CH$), 49.74 ($N(\underline{C}H_2CH_2)_2CH$), 52.85 ($N(CH_2CH_2)_2\underline{C}H$ and Ar-$\underline{C}H_2$), 108.63 (Ar-<u>C</u>), 111.50 (Ar-<u>C</u>), 126.40 (Ar-<u>C</u>), 127.28 (Ar-<u>C</u>), 127.83 (2×Ar-<u>C</u>), 128.03 (2×Ar-<u>C</u>), 130.01 (Ar-<u>C</u>), 138.28 (Ar-<u>C</u>), 141.20 (Ar-<u>C</u>), 142.72 (Ar-<u>C</u>), 146.59 (Ar-<u>C</u>), 150.96 (Ar-<u>C</u>), 165.98 (Ar-<u>C</u>O).

MS (ES^+) ***m/z*** = 362.2 (MH^+)

HRMS (ES^+) ***m/z*** for $C_{21}H_{23}N_5O$ calculated: 362.1981, found: 362.1986

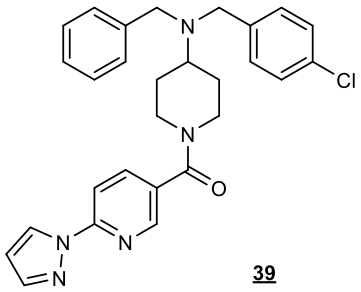
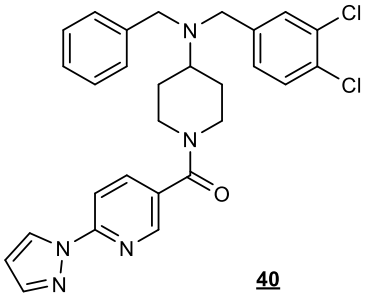
4.20 Derivatives of (6-(1*H*-pyrazol-1-yl)pyridin-3-yl)(4-(benzylamino) piperidin-1-yl)methanone

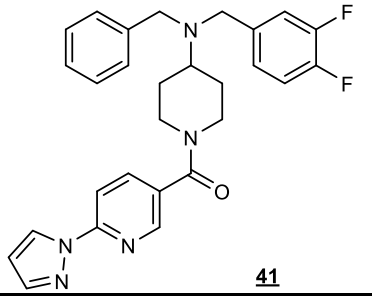
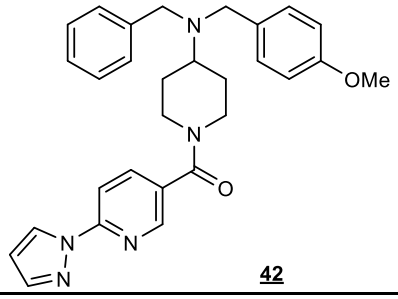


GENERAL SYNTHETIC PROCEDURE

Compound **38**, K_2CO_3 (4.0 eq), BTEAC (0.1 eq) and KI (0.1 eq) were diluted in acetonitrile (15 mL). To that various benzyl derivatives (2.0 eq) were added and stirred overnight at 80 °C, equipped with chlorcalcium tube. The K_2CO_3 was removed by filtration, the solvent was evaporated under reduced pressure and the compounds were cleaned by column chromatography.

Table XIX: Derivatives of (6-(1*H*-pyrazol-1-yl)pyridin-3-yl)(4-(benzylamino)piperidin-1-yl)methanone and their reagents.

R	Final compound	The reagent	Amount	n (mmol)
R = Cl R_1 = H R_2 = Cl	 **39**	Compound **38**	0.105 g	0.29
		4-chlorobenzyl chloride	0.056 g	0.35
		K_2CO_3	0.101 g	0.73
		BTEAC	0.007 g	0.03
R = Cl R_1 = Cl R_2 = Cl	 **40**	Compound **38**	0.150 g	0.42
		3,4-dichlorobenzyl bromide	0.199 g	0.83
		K_2CO_3	0.229 g	1.66
		BTEAC	0.009 g	0.04

R = Br R_1 = F R_2 = F	 **41**	Compound **38**	0.130 g	0.36
		3,4-difluorobenzyl bromide	0.09 mL	0.72
		K_2CO_3	0.199 g	1.44
		BTEAC	0.008 g	0.04
R = Cl R_1 = H R_2 = OMe	 **42**	Compound **38**	0.150 g	0.42
		3,4-methoxybenzyl chloride	0.11 mL	0.83
		K_2CO_3	0.229 g	1.66
		BTEAC	0.009 g	0.04

4.20.1 (6-(1*H*-pyrazol-1-yl)pyridin-3-yl)(4-(benzyl(4-chlorobenzyl)amino)piperidin-1-yl)methanone (39)

For the column chromatography the mobile phase EtOAc: hexane = 1:1 was used.

Description: white-yellow solid

Reaction yield: η = 64.6% (0.091 g)

Rf = 0.46 (MF = EtOAc: hexane = 1:1)

Melting point = 111-114 °C

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.65-1.98 (m, 4H, N($CH_2C\underline{H}_2$)$_2$CH), 2.63-2.77 (m, 2H, N($C\underline{H}_2CH_2$)$_2$CH), 2.99 (s, 1H, N(CH_2CH_2)$_2C\underline{H}$), 3.63 (s, 5H, 2×Ar-$C\underline{H}_2$-N and N($C\underline{H}_2CH_2$)$_2$CH), 4.56 (s, 1H, N($C\underline{H}_2CH_2$)$_2$CH), 6.62 (dd, *J* = 2.6, 1.6 Hz, 1H, Ar-H), 7.19-7.24 (m, 1H, Ar-H), 7.28-7.40 (m, 9H, Ar-H), 7.87 (dd, *J* = 1.8, 0.7 Hz, 1H, Ar-H), 7.96 (dd, *J* = 8.5, 0.8 Hz, 1H, Ar-H), 8.04 (dd, *J* = 8.5, 2.2 Hz, 1H, Ar-H), 8.53 (dd, *J* = 2.2, 0.9 Hz, 1H, Ar-H), 8.65 (dd, *J* = 2.6, 0.7 Hz, 1H, Ar-H).

^{13}C (100 MHz, DMSO-d6) δ(ppm) = 39.94 (N($CH_2\underline{C}H_2$)$_2$CH), 52.45 (N($\underline{C}H_2CH_2$)$_2$CH), 53.28 (2×Ar-$\underline{C}H_2$), 56.19 (N(CH_2CH_2)$_2\underline{C}$H), 108.63 (Ar-C), 111.43 (Ar-C), 126.69 (Ar-C), 127.27 (Ar-C), 128.07 (2×Ar-C), 128.10 (2×Ar-C), 128.18 (2×Ar-C), 129.38 (2×Ar-C), 129.97 (Ar-C), 131.03 (Ar-C), 138.30 (Ar-C), 139.64 (Ar-C), 140.30 (Ar-C), 142.71 (Ar-C), 146.65 (Ar-C), 150.96 (Ar-C), 165.93 (Ar-$\underline{C}$O).

MS (ES$^+$) *m/z* = 486.2 (MH^+)

HRMS (ES$^+$) *m/z* for $C_{28}H_{28}ClN_5O$ calculated: 486.2061, found: 486.2053

4.20.2 (6-(1*H*-pyrazol-1-yl)pyridin-3-yl)(4-(benzyl(3,4-dichlorobenzyl)amino) piperidin-1-yl)methanone (40)

For the column chromatography the mobile phase EtOAc: hexane = 1:1 was used.

Description: white solid

Reaction yield: η = 81.0% (0.175 g)

Rf = 0.5 (MF = EtOAc: hexane = 1:1)

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.64-1.92 (m, 4H, N($CH_2C\underline{H}_2)_2CH$), 2.58-2.79 (m, 2H, N($C\underline{H}_2CH_2)_2CH$ and N($CH_2CH_2)_2C\underline{H}$), 3.01 (s, 1H, N($C\underline{H}_2CH_2)_2CH$), 3.65 (s, 5H, 2×Ar-$C\underline{H}_2$-N and N($C\underline{H}_2CH_2)_2CH$), 4.54 (s, 1H, N($C\underline{H}_2CH_2)_2CH$), 6.62 (dd, *J* = 2.6, 1.7 Hz, 1H, Ar-H), 7.19-7.25 (m, 1H, Ar-H), 7.28-7.38 (m, 5H, Ar-H), 7.54-7.58 (m, 1H, Ar-H), 7.88 (dd, *J* = 1.7, 0.8 Hz, 1H, Ar-H), 7.97 (dd, *J* = 8.4, 0.8 Hz, 1H, Ar-H), 8.05 (dd, *J* = 8.4, 2.2 Hz, 1H, Ar-H), 8.53 (dd, *J* = 2.2, 0.8 Hz, 1H, Ar-H), 8.65 (dd, *J* = 2.6, 0.8 Hz, 1H, Ar-H).

^{13}C (100 MHz, DMSO-d6) δ(ppm) = 40.14 (N($CH_2\underline{C}H_2)_2CH$), 53.57 (N($\underline{C}H_2CH_2)_2CH$ and Ar-$\underline{C}H_2$), 56.55 (Ar-$\underline{C}H_2$ and N($CH_2CH_2)_2\underline{C}H$), 108.64 (Ar-C), 111.43 (Ar-C), 126.75 (Ar-C), 127.27 (Ar-C), 128.13 (3×Ar-C), 128.19 (2×Ar-C), 128.24 (Ar-C), 128.90 (Ar-C), 129.79 (Ar-C), 129.96 (Ar-C), 130.30 (Ar-C), 130.71 (Ar-C), 138.30 (Ar-C), 140.10 (Ar-C), 142.22 (Ar-C), 142.72 (Ar-C), 146.66 (Ar-C), 165.94 (Ar-CO).

MS (ES$^+$) *m/z* = 520.2 (MH^+)

HRMS (ES$^+$) *m/z* for $C_{28}H_{27}Cl_2N_5O$ calculated: 520.1671, found: 520.1676

4.20.3 (6-(1*H*-pyrazol-1-yl)pyridin-3-yl)(4-(benzyl(3,4-difluorobenzyl)amino) piperidin-1-yl)methanone (41)

For the column chromatography the mobile phase EtOAc: hexane = 1:1 was used.

Description: yellow oil

Reaction yield: η = 84.3% (0.148 g)

Rf = 0.57 (MF = EtOAc: hexane = 1:1)

Melting point = 48-51 °C

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.50-2.00 (m, 4H, N($CH_2C\underline{H}_2)_2CH$), 2.57-2.77 (m, 2H, N($C\underline{H}_2CH_2)_2CH$), 3.00 (s, 1H, N($CH_2CH_2)_2C\underline{H}$) 3.63 (d, *J* = 3.7 Hz, 5H, 2×Ar-$C\underline{H}_2$-N and N($C\underline{H}_2CH_2)_2CH$), 4.57 (s, 1H, N($C\underline{H}_2CH_2)_2CH$), 6.62 (dd, *J* = 2.6, 1.7 Hz, 1H, Ar-H), 7.17-7.23 (m, 2H, Ar-H), 7.27-7.41 (m, 6H, Ar-H), 7.88 (d, *J* = 1.6 Hz,

1H, Ar-H), 7.94-8.00 (m, 1H, Ar-H), 8.04 (dd, *J* = 8.4, 2.2 Hz, 1H, Ar-H), 8.53 (dd, *J* = 2.2, 0.8 Hz, 1H, Ar-H), 8.65 (d, *J* = 2.6 Hz, 1H, Ar-H).

^{13}C (100 MHz, DMSO-d6) δ(ppm) = 39.35 ($N(CH_2\underline{C}H_2)_2CH$), 52.17 ($N(\underline{C}H_2CH_2)_2CH$), 53.43 (2×Ar-$\underline{C}H_2$), 56.42 ($N(CH_2CH_2)_2\underline{C}H$), 99.48 (Ar-C), 108.63 (Ar-C), 111.43 (Ar-C), 116.54 (Ar-C), 117.04 (Ar-C), 126.71 (Ar-C), 127.26 (Ar-C), 128.15 (5×Ar-C), 129.97 (Ar-C), 139.96 (5×Ar-C), 146.66 (Ar-C), 150.97 (Ar-C), 165.94 (Ar-CO).

MS (ES$^+$) *m/z* = 488.2 (MH^+)

HRMS (ES$^+$) *m/z* for $C_{28}H_{27}F_2N_5O$ calculated: 488.2262, found: 488.2267

4.20.4 (6-(1*H*-pyrazol-1-yl)pyridin-3-yl)(4-(benzyl(4-methoxybenzyl)amino)piperidin-1-yl)methanone (42)

For the column chromatography mobile phases EtOAc: hexane = 1:1 and EtOAc: methanol = 9:1 were consecutively used.

Description: orange oil

Reaction yield: η = 83.1% (0.166 g)

Rf = 0.33 (MF = EtOAc: hexane = 1:1)

Melting point = 60-62 °C

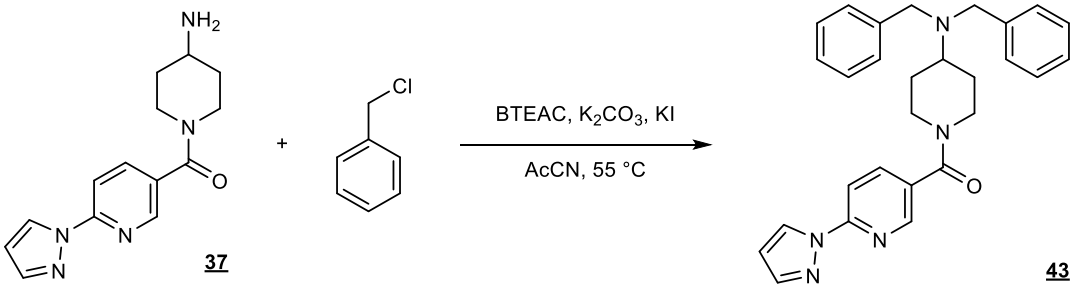
^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.53-1.95 (m, 4H, $N(CH_2\underline{C}H_2)_2CH$), 2.55-2.76 (m, 2H, $N(\underline{C}H_2CH_2)_2CH$), 2.97 (s, 1H, $N(CH_2CH_2)_2C\underline{H}$), 3.58 (d, *J* = 25.2 Hz, 5H, 2×Ar-$C\underline{H}_2$-N and $N(C\underline{H}_2CH_2)_2CH$), 3.72 (s, 3H, Ar-$OC\underline{H}_3$), 4.56 (s, 1H, $N(C\underline{H}_2CH_2)_2CH$), 6.61 (dd, *J* = 2.6, 1.7 Hz, 1H, Ar-H), 6.84-6.90 (m, 2H, Ar-H), 7.17-7.38 (m, 7H, Ar-H), 7.87 (dd, *J* = 1.7, 0.8 Hz, 1H, Ar-H), 7.96 (dd, *J* = 8.4, 0.9 Hz, 1H, Ar-H), 8.04 (dd, *J* = 8.4, 2.2 Hz, 1H, Ar-H), 8.52 (dd, *J* = 2.2, 0.9 Hz, 1H, Ar-H), 8.65 (dd, *J* = 2.6, 0.8 Hz, 1H, Ar-H).

^{13}C (100 MHz, DMSO-d6) δ(ppm) = 39.94 ($N(CH_2\underline{C}H_2)_2CH$), 52.52 ($O\underline{C}H3$), 52.96 ($N(\underline{C}H_2CH_2)_2CH$), 54.91 (2×Ar-$\underline{C}H_2$), 55.85 ($N(CH_2CH_2)_2\underline{C}H$), 108.63 (Ar-C), 113.55 (Ar-C), 126.59 (2×Ar-C), 127.27 (2×Ar-C), 128.01 (2×Ar-C), 128.14 (2×Ar-C), 129.19 (2×Ar-C), 129.98 (Ar-C), 132.09 (Ar-C), 138.30 (2×Ar-C), 140.63 (Ar-C), 142.71 (Ar-C), 146.66 (Ar-C), 150.96 (Ar-C), 158.03 (Ar-C), 165.93 (Ar-CO).

MS (ES$^+$) *m/z* = 482.2 (MH^+)

HRMS (ES$^+$) *m/z* for $C_{29}H_{31}N_5O_2$ calculated: 482.2556, found: 482.2553

4.21 Synthesis of (6-(1*H*-pyrazol-1-yl)pyridin-3-yl)(4-(dibenzylamino) piperidin-1-yl)methanone (43)



Compound **37**, K_2CO_3, BTEAC and KI were dissolved/dispersed in acetonitrile (15 mL). To that benzyl chloride was added and stirred overnight at 50 °C under argon atmosphere. The K_2CO_3 was removed by filtration, the solvent was evaporated under reduced pressure and the compound was purified by column chromatography, using mobile phase EtOAc: hexane = 2:1. The used quantities are in the table XX.

Table XX: Reagents in synthesis of (6-(1*H*-pyrazol-1-yl)pyridin-3-yl)(4-(dibenzylamino)piperidin-1-yl)methanone.

The reagent	Amount	n (mmol)	Eq
Compound **37**	0.200 g	0.74	1.0
	0.990 g	3.65	
Benzyl chloride	0.13 mL	1.10	1.5
	4.62 mL	4.01	1.1
BTEAC	0.017 g	0.07	0.1
	0.083 g	0.37	
K_2CO_3	0.612 g	4.43	6.0
	3.025 g	0.37	
KI	0.012 g	0.07	0.1
	0.061 g	0.37	

Description: brown oil

Reaction yield: η_1 = 56.0% (0.186 g), η_2 = 88.8% (1.463 g)

Rf = 0.80 (MF = EtOAc: hexane = 2:1)

^{1}H NMR (400 MHz, DMSO-d6) δ(ppm) = 1.65-2.00 (m, 4H, N(CH_2CH_2)$_2$CH), 2.61-2.75 (m, 2H, N(CH_2CH_2)$_2$CH), 2.98 (s, 1H, N(CH_2CH_2)$_2$CH), 3.64 (s, 5H, 2×Ar-CH_2-N and N(CH_2CH_2)$_2$CH), 4.58 (s, 1H, N(CH_2CH_2)$_2$CH), 6.62 (dd, *J* = 2.6, 1.7 Hz, 1H, Ar-H), 7.17-7.26 (m, 2H, Ar-H), 7.28-7.43 (m, 8H, Ar-H), 7.88 (dd, *J* = 1.7, 0.7 Hz, 1H, Ar-H),

7.97 (dd, *J* = 8.4, 0.8 Hz, 1H, Ar-H), 8.05 (dd, *J* = 8.4, 2.2 Hz, 1H, Ar-H), 8.53 (dd, *J* = 2.2, 0.8 Hz, 1H, Ar-H), 8.65 (dd, *J* = 2.6, 0.7 Hz, 1H, Ar-H).

^{13}C (100 MHz, DMSO-d6) δ(ppm) = 39.15 ($N(CH_2\underline{C}H_2)_2CH$), 39.94 ($N(\underline{C}H_2CH_2)_2CH$), 53.18 (2×Ar-$\underline{C}H_2$), 56.06 ($N(CH_2CH_2)_2\underline{C}H$), 108.63 (Ar-C), 111.43 (Ar-C), 126.63 (2×Ar-C), 127.27 (Ar-C), 128.04 (4×Ar-C), 128.15 (4×Ar-C), 129.98 (Ar-C), 138.30 (2×Ar-C), 140.48 (Ar-C), 142.71 (Ar-C), 146.66 (Ar-C), 150.96 (Ar-C), 158.03 (Ar-C), 165.93 (Ar-CO).

MS (ES$^+$) *m/z* = 452.2 (MH^+)

HRMS (ES$^+$) *m/z* for $C_{28}H_{29}N_5O$ calculated: 452.2450, found: 452.2443

5 BIOLOGICAL ASSAYS

5.1 Cell culture and transfection

The human embryonic kidney (HEK) 293T cells were cultured in a petri dish in DMEM/F-12 medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% penicillin-streptomycin and incubated at 37 °C in a humid atmosphere with 5% CO_2. The cells for cAMP accumulation assay were cultured in 10 cm petri dish and transfected with 2 μg of the wild type CXCR4/CXCR3 and 2 μg of biosensor, after they reached 50-70% of the maximum occupancy of the petri dish. Cells for β-arrestin 2 assay were transfected with 1μg of CXCR3-PK1 at the same occupancy in 4 cm petri dish. Transfection was performed using TransIT-293 transfection reagent (Mirus Corporation) according manufacturers' instructions. A day after transfection, the cell were seeded in a microtiter plate as described below.

5.2 Signaling assays

5.2.1 β-Arrestin 2 recruitment assay

To estimate the ability of novel allosteric modulators to inhibit chemokine mediated β-arrestin recruitment, we used the PathHunter assay (DiscoverX) as described previously in [5]. Briefly, HEK293T cells stably expressing β-arrestin 2/incomplete β-gal enzyme chimera were transfected with the CXCR3-PK1 receptor. PK1 annotates the second half of the β-gal enzyme. 24 hours after transfection, the cells were seeded in the 384-well plate at the density of 10.000 cells per well and in the volume of 20 μL. After incubation overnight at 37 °C and 5% CO_2, various concentrations of the chemokine CXCL11 and test compounds, dissolved in phosphate buffered saline (PBS) containing 0.2% bovine serum albumin (BSA), were added and the cells incubated for additional 4 hours. Afterwards the detection mix was added and the plate incubated for additional 60 minutes at room temperature. The activation or inhibition of the β-arrestin 2 recruitment was determined by measuring the chemiluminescence using BMG Clariostar. An increase in luminescence is proportional to the β-arrestin 2 recruitment, because the recruitment of β-arrestin 2 to the receptor results in the complementation of β-galactosidase, which is now able to hydrolyse the substrate and to generate chemiluminescent signal [16].

The same approach unfortunately did not work for the CXCR4-PK1 construct, so the measurement of chemokine mediated β-arrestin 2 recruitment to CXCR4 was not possible. We have not observed any increase in signal upon the stimulation with CXCL12.

5.2.2 BRET based cAMP assay

The bioluminescence resonance energy transfer (BRET) is a phenomenon at which the energy transfers from a donor enzyme to acceptor after substrate oxidation. The donor is *Renilla luciferase* (Rluc), which uses coelenterazine as substrate, and the acceptor is yellow fluorescent protein (YFP). In the case of BRET based cAMP assay, Epac biosensor named CAYMEL is used. Epac protein drastically changes its conformation upon cAMP binding. In CAMYEL, Epac is fused to RLuc and YFP. In the absence of cAMP the donor and acceptor are separated by less than 100 Å and resonance energy transfer can occur. The oxidation of substrat caused by donor excites the acceptor which emites fluorescence. When the biosensor binds intracellular cAMP the donor and acceptor are too far away and the energy transfer is not possible, which inhibits fluorescence [17, 18].

To preform this assay, we transfected HEK293T cells with wild type CXCR4 or CXCR3 receptor and biosensor CAMYEL (purchased at ATCC). 24 hours after transfection, the cells were seeded in the 96-well plate at the density of 20.000 cells per well and in the volume of 100 μL. After incubation overnight at 37 °C and 5% CO_2 the medium was replaced with 30 μL of Dulbecco's phosphate-buffered saline (dPBS) and incubated for 1 hour. After substrate 10 μL of 25 μM Coelenterazin h was added to a final concentration 5 μM and incubated for additional 5 minutes. Concomitantly various concentrations of chemokine CXCL12 or CXCL11 and test compounds, dissolved in phosphate buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) and forskoline (final concentration 10 μM), were added and incubated for 20 minutes. Forskoline is a labdane diterpene that activates adenylyl cyclases and caused the raise of cAMP concentration in the cells. The emission from Renilla luciferase (RL) and YFP were simultaneously measured at 475-30 nm/535-30 nm with BMG Clariostar. The netBRET signal calculated as the ratio between the light emitted by YFP (505 to 555 nm) and the light emitted by RLuc (465 to 505 nm).

BRET based cAMP assay is a new assay for CXCR3 and CXCR4 receptors and it has not been performed before on these two receptors. It is a very useful tool for receptor

characterization, because it measures the activity of receptors via activation or inhibition of adenylate cyclase, which is the main target of CXCR3 and CXCR4 G proteins. In comparison to β-Arrestin 2 recruitment assay, which was the previously used test, BRET based cAMP is faster and it also enables the measurments, which has not been possible with β-Arrestin 2 recruitment assay. For example, in the compounds characterization on CXCR4 receptor with β-Arrestin 2 recruitment assay we have not observed any increase in signal upon the stimulation with CXCL12, but when we used BRET based cAMP assay it worked withouth any problem.

6 RESULTS

6.1 Results of assays on CXCR4

Synthesized compounds were tested on the CXCR4 receptor in the BRET based cAMP assay. To validate the assay, we performed dose-reponse experiments with CXCL12 and known antagonist IT1t. To determine the optimal concentration at which the inhibition would be tested, we first stimulated CXCR4 with various concentration of endogenous ligand CXCL12 to determine 80% of maximal effective concentration (EC_{80}) for this chemokine. Next, we inhibited activated receptor (at EC_{80} of CXCL12) with IT1t, which is known antagonist of CXCR4 (Thoma G. et al (2008): *Orally bioavailable isothioureas block function of the chemokine receptor CXCR4 in vitro and in vivo*).

In order to determine if novel compounds acts as allosteric agonists, we performed the screening at the concentration 10 μM and compared it to the effect of 20 nM of CXCL12.

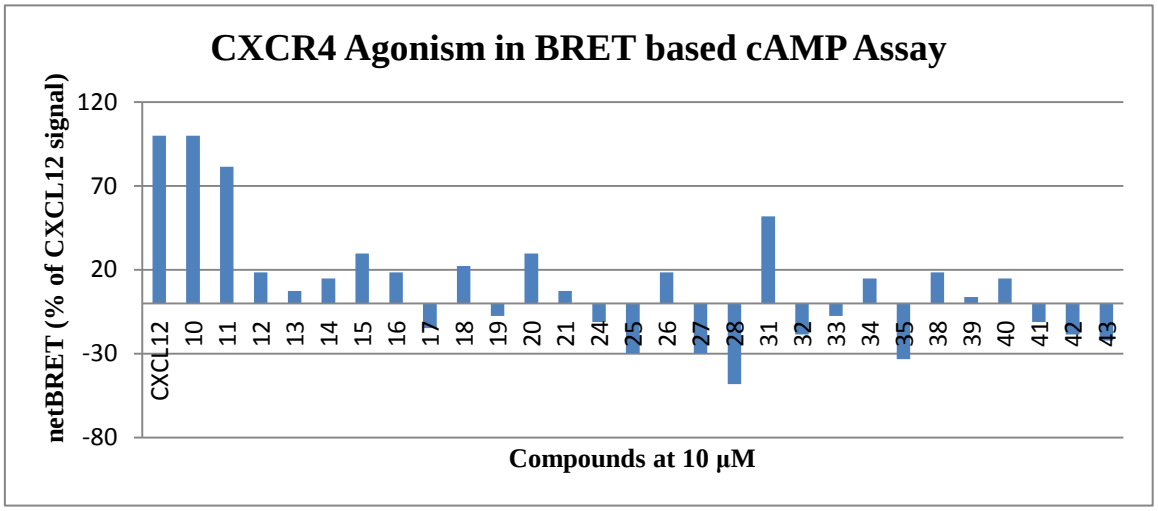


Figure 13: The results of agonism screening. The data of three experiments were normalized to the CXCL12 responsed and pooled.

Compounds **10**, **11** and **31** acted as allosteric agonists, meaning they activated the receptor in the absence of the chemokine (Figure 13).

Next we perfomed an antagonist screening to determine if novel compounds behave as negative allosteric modulators. In this experiment we tried to inhibit the activation of CXCR4 by 20 nM CXCL12 with novel allosteric ligands and the reference compound 1T1t. The concentration of the compounds was set to 10 μM.

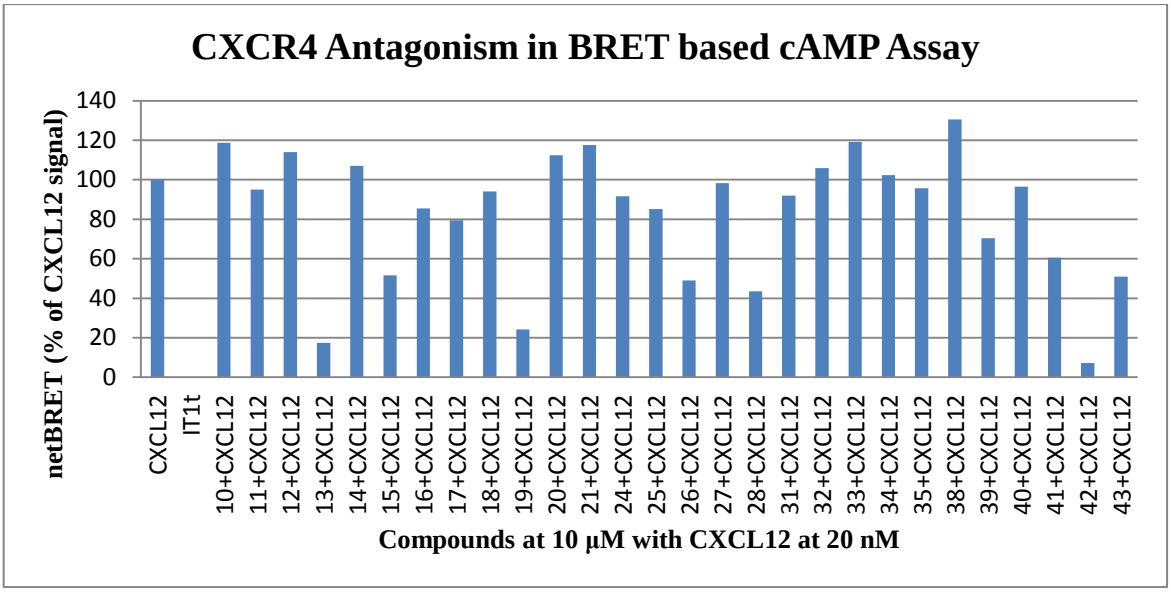


Figure 14: The chart of the tested compounds for antagonism in BRET based cAMP assay, representing the percents of netBRET signal in comparison with CXCL12 (100%) and IT1t (full inhibition, receptor activation reduced to 0%).

The compounds **13**, **19**, **26**, **28** and **42** acted as potent allosteric antagonists and thus inhibited the activation of the receptor by CXCL12. None of novel ligands was able to completely abolish the inhibiton of receptor as observed for the reference compound IT1t (Figuire 14). The compound **38** enhanced the efficacy of CXCL12 at CXCR4, which means that this compound acts as positive allosteric modulator that enhances the activity of the chemokine.

For the further characterization of allosteric profile for these promising compounds we performed dose-respone assays and analyzed the data with a ternary complex model of allosterism as described in [5]. The data from functional studies were fitted to the following equations using Prism 5.0:

$$K_{\text{app}} = \frac{K_{\text{A}}\left(1 + \frac{[\text{B}]}{K_{\text{B}}}\right)}{\left(1 + \frac{\alpha[\text{B}]}{K_{\text{B}}}\right)}$$

$$Y = \frac{Y_0(1 + K_{\text{A}})}{([c] + K_{\text{app}})}$$

K_{app} describes the occupancy of the orthosteric site; K_A is the EC_{50} value of CXCL11 or CXCL12. Y_0 is the basal value of luminescence in the absence of modulator, Y is the value of luminescence, [c] is the concentration (EC_{80}) of CXCL11/CXCL12 used, [B] is the

concentration of test compounds, K_B is the equilibrium dissociation constant of modulator binding and α is the ternary complex constant, which denotes the cooperativity factor of allosteric and orthosteric ligand binding and it is characterizing the affinity of orthosteric ligand. Values of α > 1 denote positive cooperativity (increase orthosteric ligand binding), whereas α < 1 denotes negative cooperativity (reduce orthosteric ligand binding). α = 0 can not be distinguishable from competitive antagonism. If α = 1, the result is unaltered ligand affinity [5, 19]. The K_A value for CXCL12 was set to 3 nM, concentration of the CXCL12 was set to 20 nM and compounds were tested in concentrations 10^{-9}-10^{-5} M.

The results are presented in the table XXI.

Table XXI: Characterization of allosteric modulators in the cAMP assay using CXCR4

Compound	**pK_b ±SEM**	**α ±SEM**	**pEC_{50} ±SEM**
10	/	/	5.02
11	4.64 ± 1.67	10.38 ± 0.05	/
13	6.04 ± 0.04	0.0	/
19	6.13 ± 0.17	0.04 ± 0.38	/
26	6.50 ± 0.48	0.45 ± 0.85	/
28	6.09 ± 0.06	0.0	/
31	5.60 ± 0.59	4.66 ± 0.54	/
38	/	/	5.23
42	5.83 ± 0.03	0.0	/
IT1t	8.54 ± 0.06	0.01 ± 0.77	/

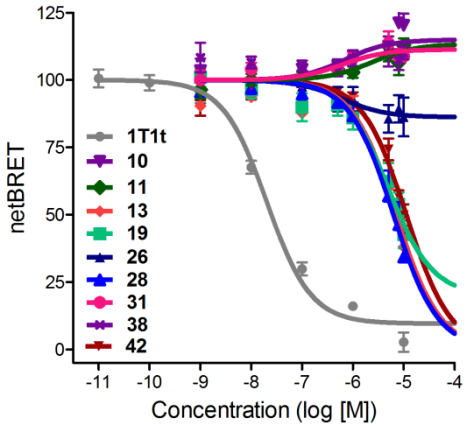


Figure 15: Representative dose-effect curves from BRET based cAMP assay. The dose-dependent receptor activity is plotted for known antagonist as a reference.

For compound **10** and **38** we were not able to estimate Kb and α. The data reported were fitted by non-linear regression curve fixing the Hills slope at 1 to obtain approximate EC_{50} values. Further experiments are needed to enable the estimate Kb and α.

6.2 Results of assays on CXCR3

For the evaluation of the synthesized compounds on the CXCR3 receptor the β-arrestin 2 recruitment and BRET based cAMP assay were used. To validate the assays, initially we obtained the dose-dependent activation of by endogenous ligand CXCL11 to determine EC_{80}. Next the CXCR3 antagonists (±)-NBI 74330 (Heise et al (2005): *Pharmacological characterization of CXC chemokine receptor 3 ligands and a small molecule antagonist*) and cRAMX3 [4] were used as reference negative allosteric modulators to determine the maximal inhibition of CXCR3.

For the β-arrestin 2 assay we were only able to perfom the screening of compounds. Because of the small assay window we were not able to determine 50% of maximal effective/inhibitory concentration (EC_{50}/IC_{50}). The compounds were tested in concentration 10 μM in the presence of CXCL11 at the concentration of 50 nM (EC_{80}).

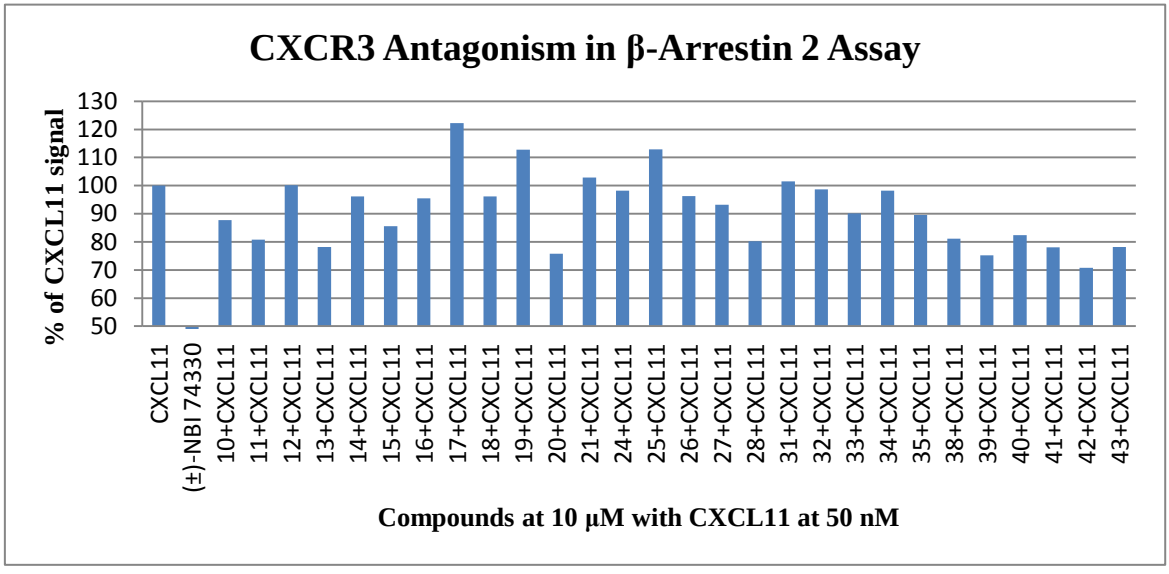


Figure 16: The chart of the tested compounds for antagonism in the β-arrestin 2 recruitment assay, representing the percents of chemiluminescence in comparison with CXCL11 (100% activation) and (±)-NBI 74330 (full inhibition).

As evident from the Figure 15 compounds **13**, **20**, **28**, **39**, **41**, **42** and **43** inhibit the receptor for more than 20%.

For further characterization compounds the BRET based cAMP assay was used. Initially, we performed the screening in agonist and antagonists mode as described for CXCR4. The most promising compounds were further characterized in dose-resposnse assay and the data analyzed with the ternary complex model allosterism [5]. The screening in antagonist mode was done in the presence of 50 nM CXCL11 and the compounds (including cRAMX3 [4]) were added at the concentration of 10 μM (Figure 17).

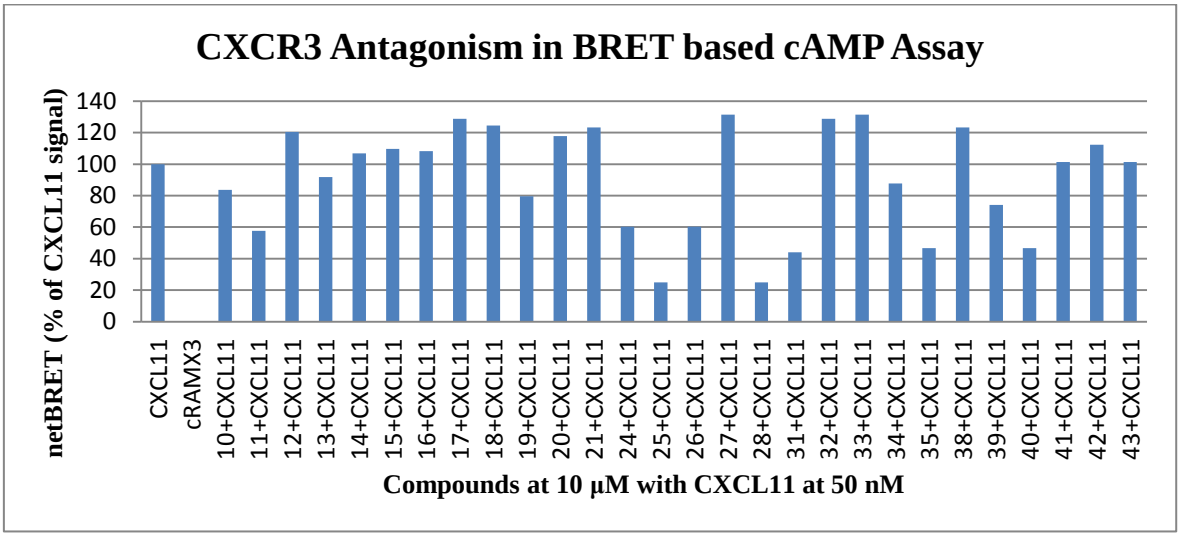


Figure 17: The screening in an antagonist mode on CXCR3. NetBRET signal in comparison with CXCL11 and cRAMX3.

The compounds **11**, **24**, **25**, **26**, **28**, **31**, **35** and **40** inhibited the receptor-mediated cAMP production for more than 40%. The compounds **25** and **28** showed the best efficacy and were further tested in dose-response assays. The K_A value for CXCL11 was set to 0.9 nM, the concentration of CXCL11 was set to 5 nM and compounds were tested in concentrations 10^{-12}– 10^{-5} M. The results are presented in the table XXII.

Table XXII: Resoults of BRET based cAMP assay for CXCR3

Compound	**$pK_b \pm$ SEM**	**α**
25	6.35 ± 0.10	0.0
28	6.15 ± 0.15	0.0
cRAMX3	7.33 ± 0.11	0.0

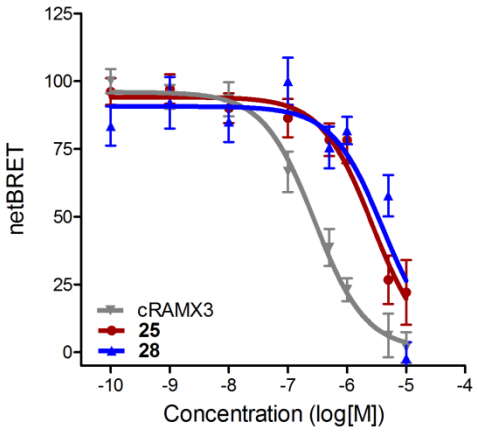


Figure 18: Representative dose-effect curves from BRET based cAMP assay. The dose-dependent receptor activity is plotted for known antagonist as a reference.

In comparison with the β-arrestin 2 recruitment assay only **28** acted as a negative allosteric modualtor in both assays. Intrigungly the compound **25** acted as a negative allosteric modulator in the cAMP assay, but showed no activity in the β-arrestin 2 recruitment assay. The compound **25** is thus highly interesting, because it shows ligand-biased signaling.

In order to determine, if some of compounds act as allosteric agonist, we performed the screening of the compounds at the concentration 10 μM and in the absence of CXCL11. As a comparison CXCL11 was used at the concentration of 50 nM.

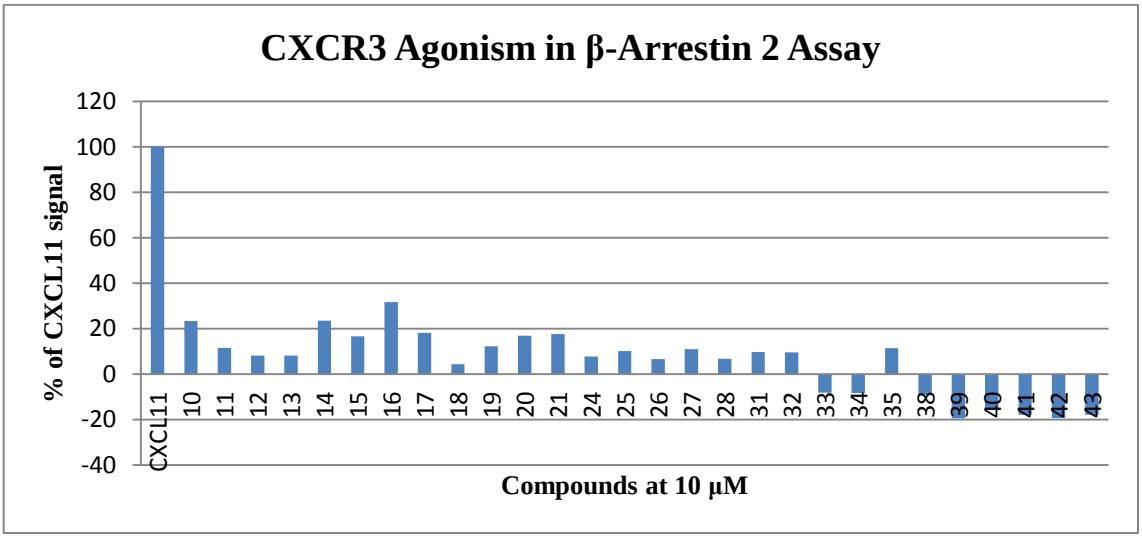


Figure 19: The chart of the tested compounds for agonism in β-arrestin 2 assay, representing the percents of chemiluminescence in comparison with CXCL11.

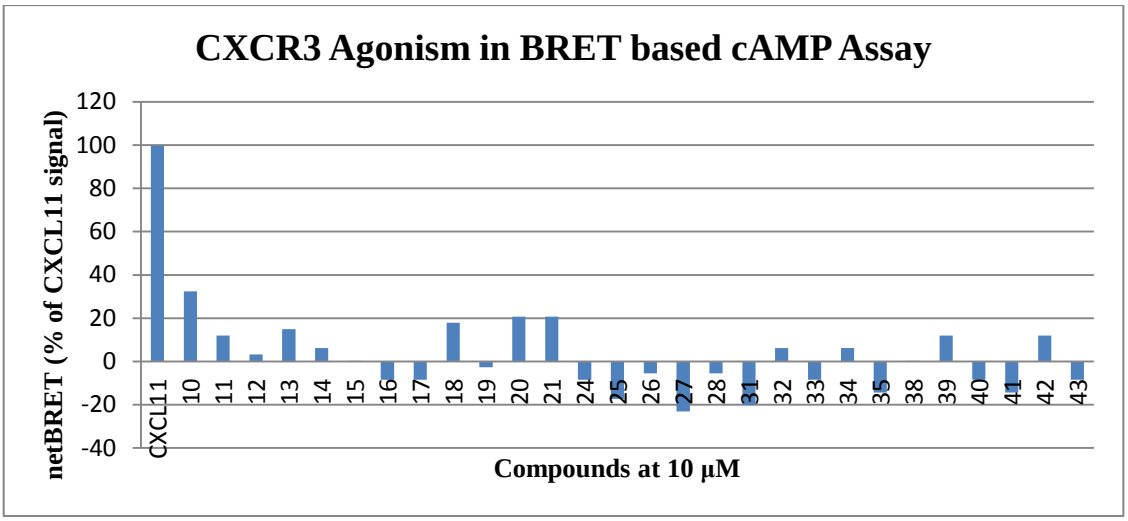


Figure 20: The chart of the tested compounds for agonism in BRET based cAMP assay, representing the percents of netBRET signal in comparison with CXCL11.

As seen in the Figures 19 and 20, none of the compounds acted as an allosteric agonist.

7 DISCUSSION

7.1 Synthetic procedures

During the comparison of various chemokine antagonists, we have found that they consist of two aromatic groups joined with an appropriate spacer. Among all aromatics, the most appropriate were the ones with halogen substitution. The idea of substitution pattern was taken on the basis of Topliss scheme, which is a tree-like graph of decision in search for suitable substitution pattern of aromatic compounds, with better biological effect compared to unsubstituted compound.

The synthetic plan was to synthesise the compounds with pyrazolopyridine scaffold as first biaromatic group, connected with the linker to second aromatic area. The prediction was, that by modification of the linker a selective and strong action at individual receptors will be achived. Four different linkers have been utilized to prove our hypothesis. The second aromatic area served as a tool to see, how the size and substitution pattern of aromatic groups affect selectivity and effectiveness. Compounds with piperazine and 4-aminopiperidine linker carried only one substituted aromatic group, while 1,3-diaminopropane and the second set of 4-aminopiperidine linker were more expanded by two aromatic groups.

The amide bond was formed with various carbodiimide coupling reagents. In the case of compounds **10**, **11** and **12** the amide bond was synthesised with coupling reagent EDC×HCl, which acts via the activation of carboxylic acid to form activated ester. The main advantage of this reagent is the urea byproduct, which is soluble in water and it is easy removable by aqueous extraction in acidic environment. Coupling reagent in amide bond synthesis of compounds **22**, **29** and **36** was TBTU. It causes the formation of another activated ester with similar leaving group like in the case of EDC×HCl. The advantage in use of coupling ragents is the selective activation of carboxylic acid.

Benzyl group of compounds **35** and **38** was attached by reductive amination of imine (formed between amine and aldehyde), performed using sodium cyanoborohydride, a reducent which converts imine to amine. Acetic acid was added to provide appropriate acidity of the reaction media. In synthesis of compound **35** aldehyde was used because of the lack of benzyl halogenide. As for compound **38**, reductive amination was used to

achive selective monosubstitution, as in the case of reaction with suitable benzyl bromide some degree of disubstitution is usually observed.

The most used reaction for N-alkylation was the alkylation of amines with benzyl halides. The mechanism of reaction is nucleophilic substitution in wich halogenides act as a leaving group. Compared to reductive amination, the problem of this reaction is selectivity. This problem has been encountered in the synthesis of compound **38**, where we first tried to achived monosubstitution with alkylation, but the major final product was always disubstituted one independent of equivalents of aryl halide used in the reaction. With the use of reductive amination selective monosubstitution was achived.

7.2 Biological caracterisation

Based on the given substance in [12] we developed and characterized several new compounds or compounds with proven novel mechanism of action with the aim to make step forward towards potential therapeutic agents. In particular, we have developed completely new assay for CXCR4 and CXCR3 receptors, namely the BRET based cAMP assay, which has not been performed before on these two receptors. We predicted that the most of the compounds act as allosteric antagonists, which was successfully proved. The significant step was also made for structure related activity, where we managed to determine which structural components are more important for activity and selectivity.

The most potent negative allosteric modulators of CXCR4 are the compounds **13** and **28** with Kb values of 900 nM and 800 nM, respectively, and the α values of 0.0. **13** consists of 1,3-diaminopropane linker, unsubstituted benzylamino and aniline group. **28** consist of piperazine linker and 4-metoxyanilino group. Compound **26** has the lowest Kb (300 nM) value, but shows only weak negative cooperativity (the α value 0.45).

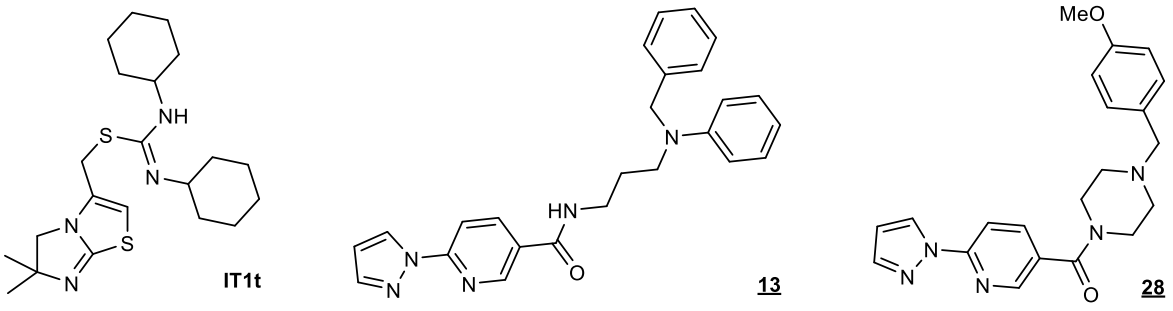


Figure 21: IT1t in comparison with compounds **13** and **28**.

In the Figure 21 we can see some similarities between known antagonist IT1t and the most potent negative allosteric modulators of CXCR4. They all consist of hydrophobic scaffold, with linker connected to two hydrophobic groups.

Compounds **25** and **28** are the most potent negative allosteric modulators of CXCR3; theirs Kb values are of 400 nM and 700 nM, respectively, and the α values of 0.0. The Kb values are only one order smaller than Kb value of cRAMX3, which is a great accomplishment when turning a virtual hit to real hit by structural modification of the virtual hit. Both compounds contain piperazine linker connected with mono-substituted benzyl moiety.

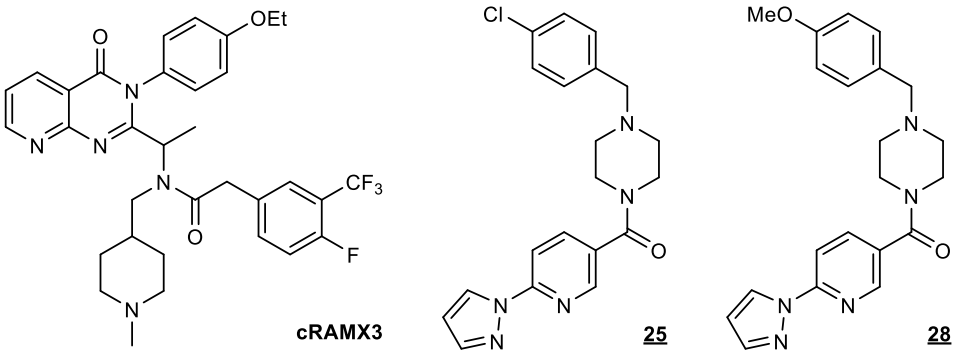


Figure 22: cRAMX3 in comparison with compounds **25** and **28**.

As for CXCR4 receptor ther are also similarities between known CXCR3 antagonist cRAMX3 and the most potent allosteric modulators of CXCR3 (Figure 21). They comprise a hydrophobic moiety, a linker and substituted hydrophobic groups.

In the set of these compounds we detected also a dual negative allosteric modulator, which inhibits the activation of CXCR3 and CXCR4. This is compound **28** and has similar Kb and α values for both receptors, 800 nm for CXCR4 and 700 nm for CXCR3.

In general we can say that the compounds with piperazine linker yielded the most potent negative allosteric modulators at both receptors (**25**, **26** and **28**). The compounds with 4-aminopiperidine linker were the least efficient, while 1,3-diaminopropane derivatives were more efficient at CXCR4 receptor. The substitution pattern is important for negative allosteric modualtion in both receptors. The exeption are compounds with aminopropane linker; unsubstituted compounds (**13** and **19**) demostrated stronger negative allosteric modulation than substituted. From these findings we conclude that compounds containing piperazine linker and substituted aromatic ring (mostly para substituted) are the most

promising negative allosteric modulators of CXCR3 and CXCR4, and represent the perspective starting point for further development.

In terms of agonism it is interesting that none of the compounds act as allosteric agonist on CXCR3, but only on CXCR4. **10**, **11** and **31** are able to partially activate CXCR4 and are so called partial allosteric agonists. Compounds **10** and **11** have aminopropane linker connected with aniline group, which is 4-chloro substituted in the structure of compound **11**, but unsubstituted in **10**. The relation between the structure and function is not clear in this case.

Surprisingly, compound **20**, which was our starting "hit" showed only weak negative allosteric effect on CXCR3 in both assays, but did not have any effect on CXCR4 in the same assays. This observation might be explained by different experimental conditions used for the initial characterization of compounds from the virtual screening [12]. In mentioned work, the compounds were characterized in the [^{35}S] GTPγS assay using membrane preparations of HEK cells expressing correponding receptor and not living cells, which is a further improvement of the screening assay methodology used in our laboratories.

8 CONCLUSION

This work is a remarkable achievement in the field of chemokine receptors, where we managed to synthesize totally new compounds that have proved successful as negative allosteric modulators. At the same time, we managed to introduced BRET based cAMP assay as a new method for testing the activity of receptors. It is a good closure of hard work and represents a large part in understanding of chemokine receptors.

In this thesis we synthesized 28 potentional allosteric modulators targeting the chemokine receptors CXCR3 and CXCR4. Novel ligands are characterized by pyrazolopiridine scaffold, connected through carbonyl group with 1,3-diaminopropane, 4-aminopiperidine and piperazine linkers to anilino and/or benzylamino group. Some of them were mono- or di- substituted.

All novel ligands were tested in the β-arrestin 2 recruitment and newly established BRET based cAMP assay.

Compounds **13**, **19**, **26**, **28** and **42** were identified as the most potent negative allosteric modulators on CXCR4 and compounds **25** and **28** on CXCR3.

Agonists were found only for CXCR4 and these compounds are **10**, **11** and **31**.

For the structure activity relationship we can postulate that the compounds with piperazine linker and benzylamino moiety are the most potent negative allosteric modulators, but the optimal substitution pattern cannot be determined based on the available library of the ligands. We also came to the conclusion, evidenced in Kb value, that there is not such a significant difference in the antagonism potency of both receptors.

Our study enabled initial insigt in the SAR of these ligands and provided valuable information for the future development of allosteric modulators targeting CXCR3 and CXCR4.

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