CHARLES UNIVERSITY IN PRAGUE
FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Departement of Inorganic and Organic Chemistry

Synthesis of 1-(3-Methoxyphenyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine and Study of its Physicochemical Properties

DIPLOMA THESIS

Supervisors: PharmDr. Jaroslav Roh, PhD, Carine Deleuze-Masquefa

Hradec Králové 2013 Lenka Valášková

DECLARATION

„Hereby I declare that this paper is my own work. All literature and sources of information I used are listed in the list of used literature and they are properly cited. This work has not been used to gain equal or different degree.

Lenka Valášková

Acknowledgement

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ABSTRAKT

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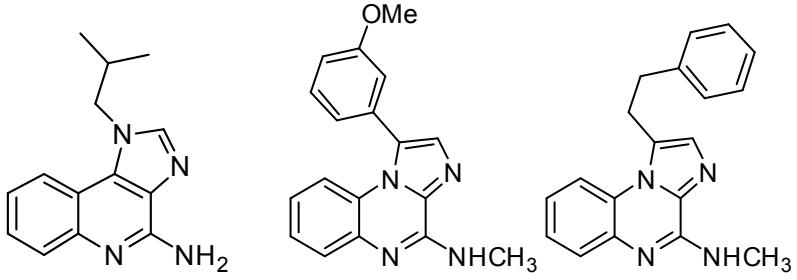
Název diplomové práce: Syntéza 1-(3-methoxyfenyl)-*N*-methylimidazo[1,2-*a*]chinoxalin-4-aminu a studium jeho fyzikálně-chemických vlastností.

Melanom je zhoubný nádor obvykle se vykytující v kůži, sliznicích nebo vzácně v jiných částech organismu. Výskyt tohoto nádoru v populaci stoupá každým rokem. Nádory objevené v počátečních stádiích lze účinně odstranit chirurgickým zásahem a úspěšnost této léčby je velká, ale pokud dojde k výskytu metastáz, je léčba tohoto typu rakoviny velmi složitá. Některé nádory, i když jsou diagnostikovány včas, nelze odstranit chirurgickým zákrokem (např. nádory na problematických místech jako je obličej apod.). V těchto případech mohou být použita topicky podávaná léčiva. Jedním z těchto léčiv je imiquimod (Aldara®). Tato látka má antivirové, imunostimulační a cytotoxické vlastnosti. Problémem je její celková toxicita, díky níž může být použita jen zevně. Skupina prof. Pierre-Antoine Bonneta se zabývá syntézou analogů imiquimodu. Syntetizované molekuly patří do tří skupin lišících se v orientaci imidazolového cyklu. Několik nově připravených látek prokázalo mnohonásobně lepší cytotoxickou aktivitu *in vitro* než imiquimod a fotemustin, který je momentálně v Evropě schválen k léčbě metastatického melanomu. Tyto nejúspěšnější látky jsou EAPB0203 a EAPB0503 (Obr.1). Hlavním problémem těchto látek je velmi malá rozpustnost ve vodě. Ve své práci jsem se zaměřila na syntézu EAPB0503 a tvorbu jeho ve vodě rozpustných solí. Bohužel žádný z postupů, které jsem zkoušela, nevedl k tvorbě voděrozpustných solí látky EAPB0503.



Obrázek 1. Struktury imiquimodu, EAPB0503 a EAPB0203

ABSTRACT

Charles University in Prague

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Title of diploma thesis: Synthesis of 1-(3-methoxyphenyl)-*N*-methylimidazo[1,2-*a*]quinoxalin-4-amine and study of its physicochemical properties

Melanoma is malign tumor usually located in the skin, mucous membranes or rarely in other parts of the organism. Every year the prevalence of this tumor is growing. Tumors which are detected in early stages can be successfully removed, but when metastasis appear treatment of this type of cancer is difficult. Some tumors (e.g. on problematic places such as on face) cannot be removed by surgery, even if they are soon detected. In these cases, topically administered anticancer drugs can be used. One of those substances is imiquimod (ALDARA®; Figure 1), possesses antiviral, immunostimulating and cytotoxic activity. Limiting factor of this substance is its toxicity- it can be used only topically. The research group of prof. Pierre-Antoine Bonnet deals with the synthesis of imiquimod analogues. Synthesized molecules belong to three chemical groups, which differ in the orientation of imidazole moiety. Their lead structures, providing higher *in vitro* cytotoxic activity against human melanoma cells than imiquimod and fotemustine, which is approved in Europe for metastatic melanoma treatment, are EAPB0203 and EAPB0503 (Figure 1).

The main problem of EAPB0503 is its poor solubility in water. In this work I focused on the synthesis of EAPB0503 and its water-soluble salts.

Unfortunately I did not succeed in formation of salts. Every attempt to dissolve EAPB0503 in water failed.

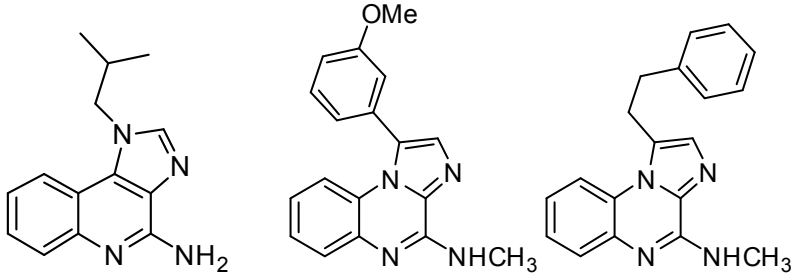


Figure 1. Structures of imiquimod, EAPB0503 and EAPB020

Content

1 Introduction

Cancer is a serious illness that affects more and more people all over the world. It is caused by abnormal proliferation of cells in normal tissues. Cancer cells can infinitely multiply, which incidentally is the main difference between normal cells and cancer cells (1).

There is no universal cure for cancer because every tumor has specific properties and tumor cells are able to develop resistance against treatment. That is why scientists are trying to discover new ways of treatment.

Melanoma is malign tumor caused by mutation of melanocytes which are located in the skin, mucous membranes or rarely in other part of the organism. It is a very aggressive form of cancer with capability of metastasis through migration of cancer cells in blood or lymphatic system. Primary tumor affects the most frequently skin, eye or mucous membranes. Metastases usually occur in lymphatic nodes, liver, lungs and brain. Tumors diagnosed in early stages, before metastasis appearance, can be successfully removed by surgery and the prognosis of therapy is very good. The prognosis of metastatic form of melanoma is unfavorable. Due to migration of tumor cells it is difficult to treat it by chemotherapy, radiotherapy or surgery(2).

Therapy of melanoma depends on stage of the tumor, location and type of metastasis. There are two types of metastasis: regional and distant. Tumors with regional metastasis have generally a better prognosis. Primary tumors (or metastases located in skin) can be eliminated by surgery or by topic administration of anticancer drugs, if surgery is not possible – especially when the tumor is located on exposed area on face. One of the drugs used for this purpose is the imiquimod (ALDARA®; Figure 1)(2). This molecule has antiviral, immunostimulant properties and also cytotoxic effect (3). The limiting factor of use of this substance is its toxicity. This is the reason why it is used only for topic administration. The aim of the research is to develop substances based on imiquimod with better cytotoxic activity against melanoma and with lower toxicity for the organism.

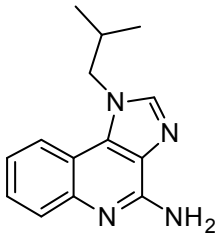


Fig. 1. Imiquimod

The research group of prof. Pierre-Antoine Bonnet synthesizes analogues of imiquimod and studies chemical properties and mechanism of action of their substances. Synthesized molecules belong to three chemical groups, which differ in five-membered fused heterocycle.

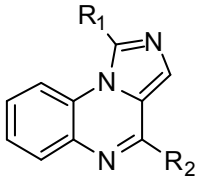


Fig. 2. Imidazo[1,5-a]quinoxaline

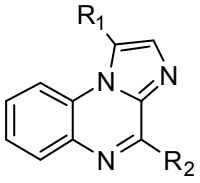


Fig. 3. Imidazo[1,2-a]quinoxaline

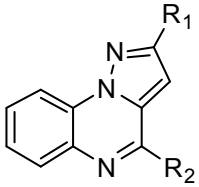


Fig. 4. Pyrazolo[1,5-a]quinoxaline

Among all obtained molecules there were substances providing much greater in vitro cytotoxic activity against human melanoma cells than imiquimod. Those leading molecules are EAPB0203 and EAPB0503.

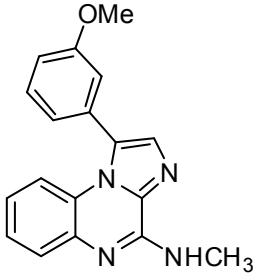


Fig. 5. EAPB0503

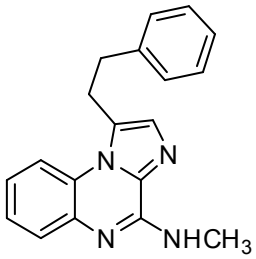


Fig. 6. EAPB0203

2 Theoretical part

2.1 Melanoma

2.1.1 Epidemiology, etiology and risk factors

Malign melanoma is increasing in the number of new cases every year, especially in white populations. Fair-skinned people receiving excessive UV irradiation are particularly at risk (2). Studies have proven that premenopausal women using oral contraception are at higher risk than women who do not use oral contraception (4). In white population, most of melanomas arise in skin, mostly on sun exposed parts of the body, while in black and dark skinned population the tumors appear on parts of the body not exposed to the sun such as various mucous membranes, uvea, palms etc. (5). Socio-economical status also plays a role in prevalency of melanomas. UV exposure is supposed to be one of the most important exogenous factors, but studies proved that the impact of this factor is more complicated. It seems that the most dangerous is UV exposure for untanned skin, especially great intensity of UV irradiation during short time (e.g. holidays). Workmen that spend a long time outside, exposed daily to UV irradiation, had smaller prevalence of melanoma than people working inside and exposing their skin to sun just during holidays(5).

It has been found that geographic locations are connected with melanoma prevalence. Fair-skinned population living closer to the equator are in higher risk, prevalence of melanoma in these populations is two or three times bigger than in northern populations(5).

One of potential precursors of melanomas are nevi. Those benign clusters of melanocytes are common in fair-skinned population and usually do not mean high risk. On the other hand, higher amount of nevi, atypical nevi and melanoma in family anamnesis mean increased potential of developing skin cancer(5).

Other endogenous factors include genetic predisposition and immunity condition. Immunodeficiency means danger of development of various tumors, melanomas including. Patients with immunosuppressive treatment or with diseases that affects immune system (AIDS, autoimmune diseases) should pay attention to prevention.

Etiology of melanoma is complex and includes many factors. Exact mechanism of tumorogenesis has not been discovered, yet. All above mentioned factors together play role in potential cancer development.

2.1.2 Pathology

As I previously mentioned, melanoma is caused by malign mutation of melanocytes. These cells are located in basal membrane and their function is synthesis, storage and transport of melanin. These functions remain in primary melanoma cells and it is one of the main difference between other types of tumor (5). Primary lesions usually arise on skin. It can be pigmented or even amelanotic. One of the exogenous factors is UV irradiation exposition. UV irradiation causes mutation in DNA. It induces dimerization of thymine and can lead to GC→AT transition. Most of nonmelanoma skin tumors have this mutagenic fingerprint in tumor suppressing gene p53. This mutagenic fingerprint did not appear in any significant frequency in genes Ink4a or BRAF which are melanoma specimens. This could mean that UV irradiation do not play as important role in tumorogenesis as it was supposed but epidemiology studies in Caucasians proved the relation between UV-B exposure and increased melanoma incidence. Exact mechanism of action is object of current studies (5).

Cells have ability to repair these mutations, but if the reparation mechanism fails, mutated gene can express. If this happens, there are self-control pathways that can cause apoptosis of cell expressing damaged genes. The immune system also is able to detect and remove cells with damaged DNA. There are multiple ways how to prevent tumorogenesis, there must be failure of more mechanism.

Primary tumor grows horizontally first- it spreads superficially and radially. In this stage tumor is localized in epidermis without metastasis. As time goes by, tumor cells start to spread vertically – into other layers of dermis. In this phase usually metastases appear through migration of cancer cells in blood or lymphatic system. Metastases attack lymphatic nodes in the tumor area or even distant parts of body, as well as other organs (liver, brain etc.).

Tumor can be divided into a few subtypes according to their properties and molecular characteristic. Older classification is based on basic tumor properties.

Lentigo maligna melanoma is a slow developing tumor that appears on parts of body that are chronically sun exposed. It usually develops in older people from lentigo maligna- atypical melanocytes located at dermo-epidermal junctions.

Superficialy spreading melanoma is characteristic by its initial radial or superficial growth in epidermis. Typical are irregular borders and multiple, pale colors that may change. Nodular areas can secondary develop, but the main histological feature is lateral epidermal component pagetoid spread of clear melanocytes through epidermis(2).

Nodular melanoma is one of the most aggressive tumors. The radial growth is almost absent, while vertical growth is dominant. The color of the tumor is usually brown-black. This type of melanoma can develop metastasis really quickly.

Acral lentiginous melanoma is usually located on palms, fingers, sole of foot or underneath nails.

2.1.3 Diagnosis, staging and treatment

More than 85% of tumors are discovered in early stages. In this case surgery is indicated as the most effective. Early stage means primary tumor localized in skin without any evidence of metastasis. Prognosis in these cases is very good and tumor specific 10 years survival is 75-85%.

The most important histological factor for prognosis is vertical tumor thickness (Breslow's depth), presence of histological recognized ulcerations, mitotic activity of cancer cells and level of invasion (Clark's level). Thickness is measured with an optical micrometer. Ulcerations mean combination of histological features of local inflammation like full-thickness epidermal defect including absence of stratum corneum and basement membrane, fibrin depositions, thinning or reaction hyperplasia of surrounding epidermis. Level of invasion serves as prognostic factor only for thin tumors (>1 mm).

Staging uses TNM system. T is for primary tumors and is further divided into 4 stages by tumor thickness, presence of ulcerations and mitotic rate. N is for regional lymphatic metastases. This stage is divided into 3 substages by number of affected lymph nodes and presence of micrometastases (these metastases are not clinically recognizable, diagnosis is possible via sentinel lymph node biopsy), macrometastases (clinically recognizable metastases), satellite metastases (metastases up to 2 cm from primary lesion) and in-transit metastases (in the skin between 2 cm from the primary tumor and first draining lymph node). M is for distant metastases. The prognosis in patient with distant metastases is very poor, survival of untreated patients is usually 6-9 months. This stage is also divided into 3 substages by affection of organs and level of

lactate dehydrogenase (LDH) in serum. In the first two substages serum level of LDH is normal, while in the third stage the level is elevated. Following tables of staging were approved by AJCC and UICC. Staging is important in determination of appropriate treatment (2).

Table 1: Staging of primary tumor

Classification	Tumor thickness	Additional prognostic parameters
Tis		Melanoma in situ, no tumor invasion
T1	<1,0 mm	a) no ulceration, no mitosis b) ulceration or mitotic rate >1/mm^2
T2	1,01 - 2,00 mm	a) no ulceration b) ulceration
T3	2,01 - 4,00 mm	a) no ulceration b) ulceration
T4	>4,00 mm	a) no ulceration b) ulceration

Table 2: Staging of regional lymph nodes melanoma

Classification	Number of affected lymph nodes	Additional prognostic parameters
N1	1	a) micrometastasis b) macrometastasis
N2	2 - 3	a) micrometastasis b) macrometastasis c) satellite or in-transit metastasis
N3	>4	satellite or in-transit metastasis

Table 3: Staging of distant metastasis melanoma

Classification	Type of distant metastasis	Serum level of lactase dehydrogenase
M1a	Skin, subcutaneous tissue, lymph node	Normal
M1b	Lungs	Normal
M1c	All other distant metastasis	Normal or elevated

2.1.3.1 Surgery

Indication of surgery is early stages of primary lesions T1-T4. Screening of high risk patients is important for early diagnosis. Parameters of resection depend on tumor thickness and presence of ulceration. For thin tumors cca 1 mm thick, margins 1 cm are recommended, while for tumors thicker than 4 mm, margins 2 cm or bigger (if it is anatomically possible, if not, margins 1 cm are recommended). Clinical reviews did not prove improved reduction of relapses in 2 cm margins and 3 cm or bigger margins, but

some reports recommend 3 cm margins in tumors thicker than 4 mm. In these thick tumors biopsy of regional lymph nodes is indicated. T4 stage is the most likely to develop regional metastasis. Only biopsy of lymph nodes can discover micrometastases. Also to prevent relapses, in T4 stages adjuvant therapy is advised(6). If metastasis affect regional lymph nodes, guidelines recommend resection of these lymph nodes (7).

2.1.3.2 Adjuvant therapy

Surgical dissection of skin tumors is always indicated. As I mentioned above, in stage T4 and later stages adjuvant therapy is employed. Metastatic melanoma is one of the most difficult tumors to treat and there is not many approved possibilities in treatment. The main therapeutic approach is administration of interferon α 2b. The efficacy of this treatment has been studied and conclusions are full of contradictions. Effect of low dose interferon α 2b was studied in Europe in patients with high risk lesions and intermediate risk lesions. Results showed that recurrence-free survival was improved just in patients with intermediate risk melanoma which lead to approval of low dose interferon α 2b as adjuvant therapy for tumors of intermediate thickness. Limiting factor of interferon α 2b is its toxicity and side effects including nausea, headache, vomiting, weight loss, myelosuppresion and depression. Patients who are intolerant to this therapy can participate in clinical trials. In these trials usually combinations of interferon α 2b and new approaches are tested. Antitumor vaccination seems to be promising method since specific epitopes of tumor cells are better known. Compared with high dose interferon α 2b most of vaccines showed inferior relapse-free survival, so the studies were terminated early (6).

Treatment of metastatic disease is complicated and usually palliative, because prognosis is grim. Dacarbazine and fotemustine approved in USA and Europe, and high dose interleukin 2 used is approved only by FDA. Due to side effects it is indicated only in specific patients treated by experienced specialists.

Dacarbazine produce tumor response in 5-12% of patients and only 1-2% achieve long-term response. Dacarbazin has also peroral analogue, pro-drug temozolomide which is metabolized to active intermediate.

Fotemustine showed better effectivity and improved survival although not much significantly. Many clinical trials tested many therapy approaches including cisplatine, monoclonal antibodies and their combinations but none of these trials showed better survival than dacarbazine alone.

2.1.3.2.1 Aldara

Aldara is commercial name for imiquimod. It is produced as 5% cream and destined for external therapy of genital and perianal warts, basal cell carcinoma and actinic ceratosis. Randomized, double-blind, vehicle-controlled trials proved that it is effective and safe treatment for these indications. Off-label use is treatment of melanomas. It is alternative method to surgical excision of primary tumor, for example if the tumor is located on problematic places where cicatrix would be undesirable. Application of imiquimod leads to complete clearance of primary tumor. In melanomas it may clear malignant skin metastasis, but it cannot prevent lymphatic metastasis.

Aldara is usually well tolerated, adverse effects are mild. There are some skin reactions that can lead to interruption of treatment if the intensity is intolerable. These reactions are erythema, edema, indurations, vesicles, pustules, erosion, excoriation, ulceration, weeping, exudate, flaking, scaling, dryness, scabbing, crusting, itching, soreness, and burning. Although it is not frequent, imiquimod can exacerbate skin inflammatory conditions like psoriasis, pemphigus, aphthous ulcers or angioedema. Imiquimod systematic absorption is very small but it can cause systematic reactions including fatigue, fever, malaise, pain, myalgia, arthralgia, headache, nausea, diarrhea and influenza-like symptoms(8).

2.2 Imidazole derivatives

Imidazole derivatives were originally discovered as nucleoside analogues with antiviral activity. During *in vitro* test they did not possess any antiviral activity, while *in vivo* they proved good antiviral activity. Further studies came to conclusion that the mechanism of action is immune response modification via various stimulations of immune system. Imiquimod was the first imidazole derivative used in treatment. As I mentioned above, its systematic toxicity is too big to be administered parenterally for treatment of later stages of melanoma. There are already some derivatives synthesized , whose activities were evaluated on culture of melanoma cells, but further toxicity and activity tests *in vivo* depends on synthesis of water soluble salts of these derivatives which are practically insoluble in water.

2.2.1 Mechanism of action

Imiquimod (IM) possesses more actions than antiviral and antitumor effect. It is strong TLR (toll like receptors) agonist, especially TLR-7. These receptors are presented by professional antigen-presenting cells like macrophages, monocytes and plasmacytoids dendritic cells. When IM is bounded to TLR-7 receptor, its intracellular domain triggers and activate pathways that via activation of transcription leads ultimately to increased production of interferon α (INF α) which is the main cytokine induced by imiquimod. Imiquimod also induces tumor- necrosis- factor α (TNF α), interleukins IL-1 α, IL-1 β, IL-1 receptor agonist, IL-6, IL-8, IL-10, IL-12, granulocyte-macrophage- colony stimulating factor (CSF), granulocyte CSF and macrophage inflammatory protein-1 α. On the other hand, imiquimod suppress synthesis of IL-4 and IL-5 which leads to inhibition of development of T helper cells type 2 (Th2) of adaptive immune response.

Another effect of imiquimod is stimulation of activation of Langerhans cells (LC)- bone marrow derived epidermal dendritic cells that are majority antigen-presenting cells in the skin. These cells take up viral and tumor antigens and migrate through skin into regional lymphatic nodes. Antigens presented by LC are processed to naive CD4 T lymphocytes. These lymphocytes differentiate into activated T cells that migrate back to epidermis where they express Th1 cytokines like INF α, INF γ and TNF α.

Imiquimod also enhance infiltration of treated area by lymphocytes and macrophages, especially cytotoxic T- lymphocytes (CD8+, peroforin +, granzyme B+). Specific mechanism of action for genital and common warts is decrease of HPV DNA and mRNA expression and stimulation of expression of 2‘5‘ oligoadenylate synthetase. Furthermore it was proved that anogenital warts unresponsive to imiquimod treatment, showed lower density of dermal dendritic cells and lack of LC activation. That could mean that compartment of antigen presenting cells can be affected in imiquimod resistant lesions.

Specific mechanism of action of imiquimod in basal cell carcinoma (BCC), actinic keratosis (AK), and melanoma has been studied. It seems that the main mechanism is the increase of inflammatory infiltrate containing CD4+ T-helper mixed with dendritic cells, CD8+ cytotoxic cells, CD68+ macrophages and CD20+ B lymphocytes. Expression of cytotoxic granules, T cell-restricted intracellular antigen

and granzyme B suggest that T-cells mediated immune response is probably mediated by CD8+ T lymphocytes.

Tumors with decreased presence of epidermal dendritic cells were resistant to imiquimod treatment which means that these cells are important in mechanism of action of imiquimod.

In vitro, imiquimod enhance apoptosis in melanoma, squamous cell carcinoma, human epithelial and human keratinocyte cell lines. *In vivo* it induces apoptosis in BCC and melanomas. Apoptosis is physiological process which helps to maintain tissue homeostasis, participates in morphogenesis and regulates cell cycle. Apoptosis is programmed cell death regulated by many pathways and alteration in genes can lead to cancerogenesis. The main pro-apoptotic pathway is mediated via death receptors p53, BAX and via activated cytotoxic cells. Main anti-apoptotic pathway is regulated via Bcl2 receptor which interferes with BAX in the mitochondria. During studies in vivo, imiqiomod proved downregulation of Bcl2 expression through this way increased apoptosis of BCC. It seems not to affect expression of p53 or cell proliferation of BCC cells.

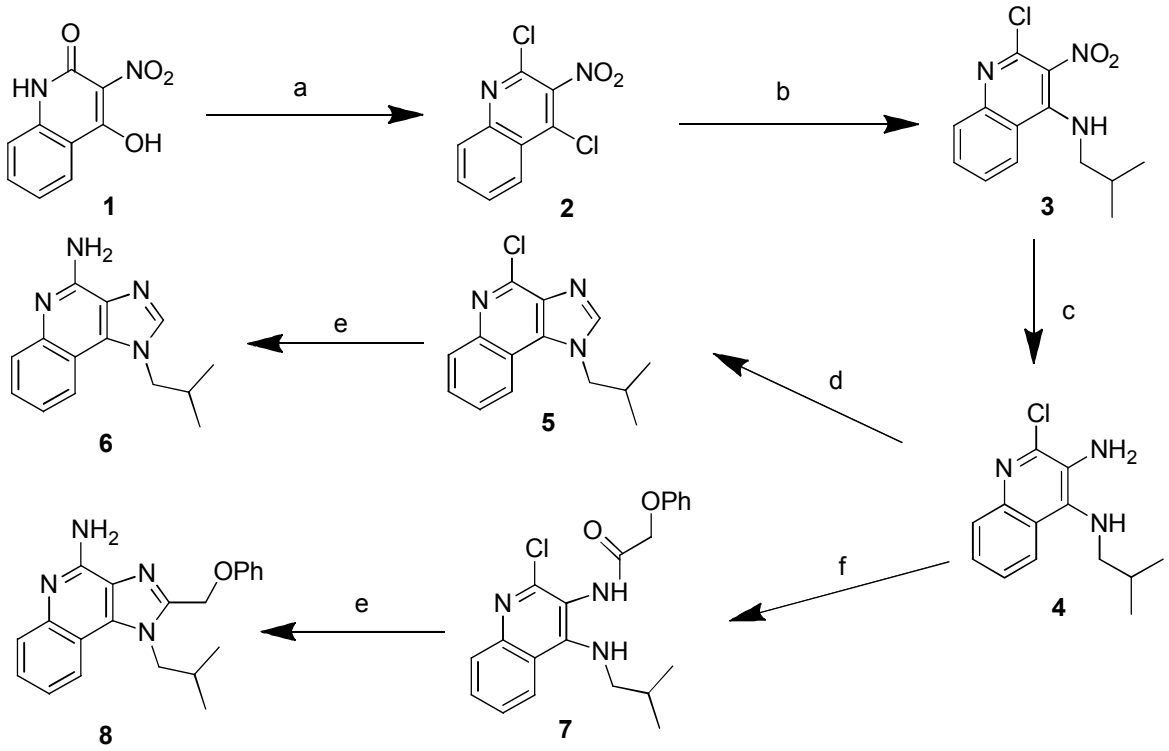
Another anticancerous effect of imiquimod is strong inhibition of tumor cell induced angiogenesis. This effect is mediated via IL-18 induction. IL-18 promote INF γ production and this interferon is strong and the most important inhibitor of angiogenesis(8).

Imiquimod analogues that I have been studying possess partly the same mechanism of action, but there are differences and exact mechanism of action is now object of research. It seems that the main effect is basically the same or very similar, but these analogues possess better *in vitro* cytotoxic activity against various tumors (not only melanoma, but also some lines of leukemia, colon and breast cancer cells).

2.2.2 Synthesis and SAR studies of imiquimod

2.2.2.1 Synthesis

Synthesis of imiquimod and its analogues, according to (9), was mostly realized via two synthetic routes (scheme 1 and scheme 2).

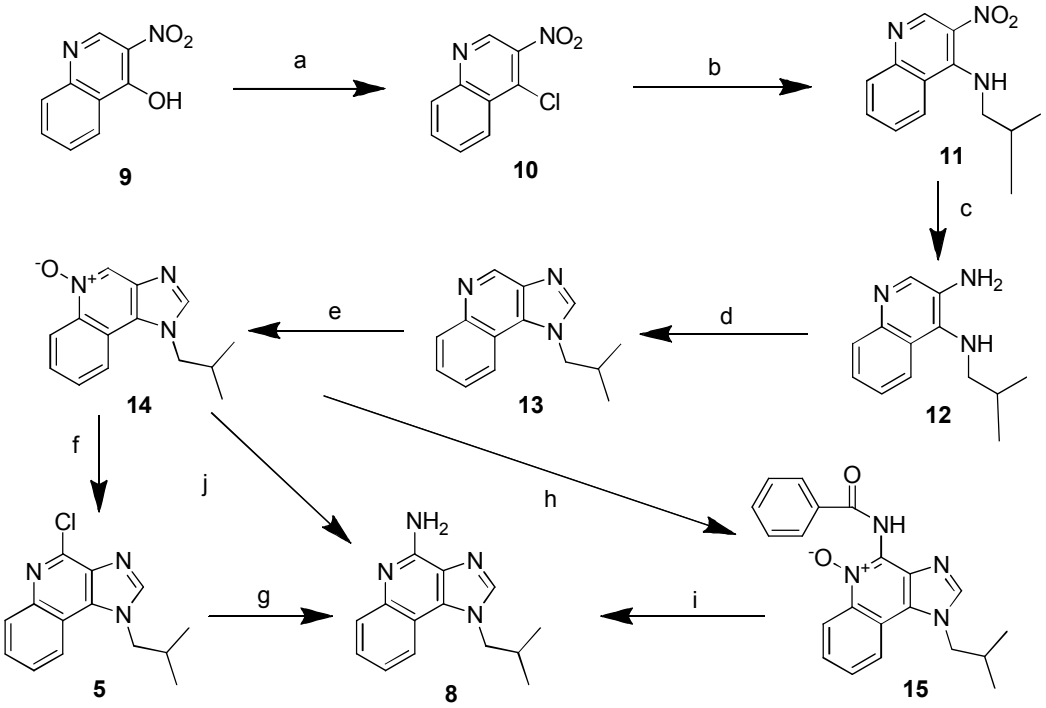


(a) $POCl_3$, toluene, Et_3N, heat or $PhPOCl_2$, heat; (b) isobutylamine, Et_3N, CH_2Cl_2, reflux; (c) 5% Pt/C, H_2, ethyl acetate; (d) $HC(OEt)_3$, toluene, reflux; (e) NH_3/MeOH, 150 °C; (f) $PhOCH_2COCl$, Et_3N, CH_3CN

Scheme 1. Synthesis of imiquimod

The first route, pictured on the Scheme 1, started with chlorination of 4-hydroxy-3-nitro-1*H*-quinolin-2-one. Reaction was based on method of Gabriel(10). This method employed phosphorus oxychloride in toluene and base represented by triethylamine. Alternative way of chlorination include phenylphosponic dichloride(11). Product of this reaction, 2,4-dichloro-3-nitroquinoline, with isobutylamine under careful regulation of reaction temperature provides substance **3**. Temperature need to be regulated to secure selective displacement of 4-chloro substituent. Catalytic hydrogenation of product **3** provided corresponding diamine **4**. Cyclization of diamine **4** was realized via reaction with triethyl orthoformate or diethoxymethyl acetate in refluxing toluene. Imidazole derivative **5** was treated with methanolic ammonia at 150°C to provide **6** (imiquimod). Change of primary amine in step 5 leads to synthesis of various analogues with different substituent in position **1** of imidazole moiety. Derivatives with substituent in position 2 of imidazole ring were prepared by change of step **d.** Instead of triethyl orthoformate, acyl chlorides or orthoesters were used. As it is demonstrated on Scheme 1, step **f** with phenoxyacetyl chloride, provided product **7**. This substance was treated with methanolic

ammonia under temperature 150°C to give cyclized imidazole derivative **8**. Imidazole cycle was closed and also displacement of 4-chloro substituent with amine occurred in one step. This manipulation brought variously substituted derivatives.



(a) $SOCl_2$, DMF, CH_2Cl_2, reflux or $POCl_3$, DMF, heat; (b) isobutylamine, CH_2Cl_2, reflux; (c) 5% Pt/C, H_2, ethyl acetate; (d) diethoxymethyl acetate, heat or $HC(OEt)_3$, toluene, reflux; (e) CH_3CO_3H, EtOH, heat; (f) $POCl_3$, CH_2Cl_2, reflux; (g) NH_3/MeOH, 150 °C; (h) benzoyl isocyanate, CH_2Cl_2, heat; (i) $NaOCH_3$, CH_3OH; (j) concentrated NH_4OH, CH_2Cl_2, tosyl chloride.

Scheme 2. Alternative synthesis of imiquimod

The second route (Scheme 2) begun with chlorination of 3-nitro-4-quinolinol via thionyl chloride and *N,N*-dimethylformamide or phosphorus oxychloride in *N,N*-dimethylformamide. 4-chloro substituent of product **10** was then displaced by isobutylamine to provide product **11**. Product **11** was catalytically reduced to give diamine **12**. Cyclization of diamine **12** to imidazoquinoline **13** was realized via heating the solution of diamine with dimethoxymethyl acetate. Imidazoquinoline **13** was oxidated with peracetic acid or *m*-chloroperbenzoic acid to provide *N*-oxide **14**. In this step the synthetic route offers three possibilities to obtain final product **8**. One of them was chlorination of *N*-oxide **14** and following substitution of chlorine with amine by methanolic solution of ammonia. This manipulation gave substance **8** (imiquimod). The second alternative was the reaction of *N*-oxide with benzoyl isocyanate in dichloromethane. Corresponding derivative provided imiquimod after saponification

with sodium methoxide. Direct way to obtain imiquimod from *N*- oxide is modification of Hamana and Hoshino procedure(12). This manipulation involves dichloromethane (DCM) as solvent, ammonium hydroxide and tosyl chloride(9)

Various analogues were prepared via these synthetic routes and their modification to provide good basis for SAR studies.

2.2.2.2 Structure-activity relationship study

All synthesized imidazoquinolines were tested *in vitro* for their ability to increase interferon production. Human peripheral blood mononuclear cells were cultured overnight with each test compound in RPMI medium containing 10% autologous serum at 37°C. Supernatants were collected and analyzed for interferon α by a virus cytopathic effect assay. Human lung carcinoma cells and encephalomyelitis virus(9)(13). Determination of minimum effective concentration (MEC) at which compounds showed induction of interferon production was made.

Most compounds with different alkyl substituents at N-1 position showed interferon induction at similar concentration (0.5 μg/ml). Longer chain alkyl analogues (C9 and more) failed to induce interferon production, as well as the bulkier substituent like *tert*-butyl. Analogues with phenyl attached directly to N-1 position did not induced interferon production also. Phenyl group at the terminus of alkyl chain provided analogues that induce interferon production. Exception is 3-phenylpropyl derivative which proved to be inactive in repeated experiments. An unsubstituted analogue is also active interferon inducer. Hydroxy group attached to the N-1 substituent did not prove any significant affection of activity, however dihydroxypropyl derivative showed smaller potential to induce interferon (MEC= 5,0 μg/ml).

SAR studies were primarily focused on imiquimod which MEC was similar as many other compounds, but in animal models it proved to be more effective and progressed to further studies as potential candidate. *In vivo* imiquimod is metabolized to the 1-(2-hydroxy-2-methylpropyl) which is also active interferon inducer(14).

To investigate what impact C2 substituent has on interferon induction, series of coumpounds with various C2 substituents was prepared. Imiquimod was used as reference compound. Straight chain alkyl C1-C5 increased effect on interferon production 10-50 fold (MEC= 0,01-0,05 μg/ml) while longer chains had decreasing effect on activity. Electronacceptor substituents like trifluoromethyl seem to withdraw

interferon induction potential, as well as phenyl core attached directly on C2. Terminal phenyl moiety is tolerated, phenoxymethyl and benzyl derivatives showed 5-10 fold better interferon induction. Oxygen directly attached to the C2 eliminates activity (2-methoxy and 2-phenoxy derivatives failed to induce interferon production)

C4 position substituent is the most important for ability to induce interferon production. SAR study came to conclusion that only primary amino substituent leads to active compounds. Methylation or hydroxylation completely eliminated activity.

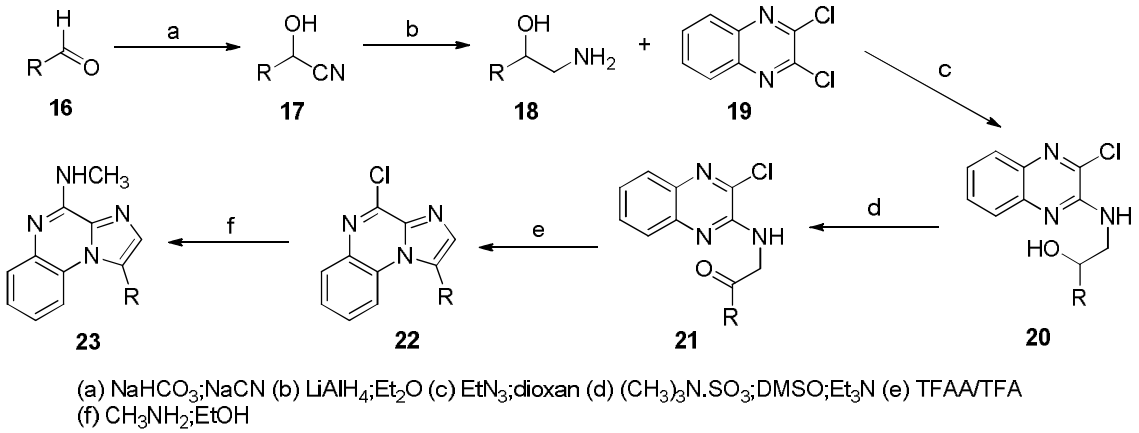
Substitution on benzene ring brought various results. C6, C8 and C9 substitution withdraw activity. The only exception is C6 hydroxyl-substituted analogue which exhibits 2 fold less activity than imiquimod. C7 substitution was tolerated, MEC were comparable to imiquimod.

Although imiquimod did not show the best interferon- induction potential, it was chosen for further examination because it is small molecule with balanced activity and tolerability.

2.2.3 Synthesis and SAR studies of imidazo[1,2-a]quinoxaline derivatives

2.2.3.1 Synthesis

There are two synthetic routes to imidazo[1,2-a]quinoxaline derivatives. The older one which brought the first successful derivative EAPB0203 is pictured on scheme 3

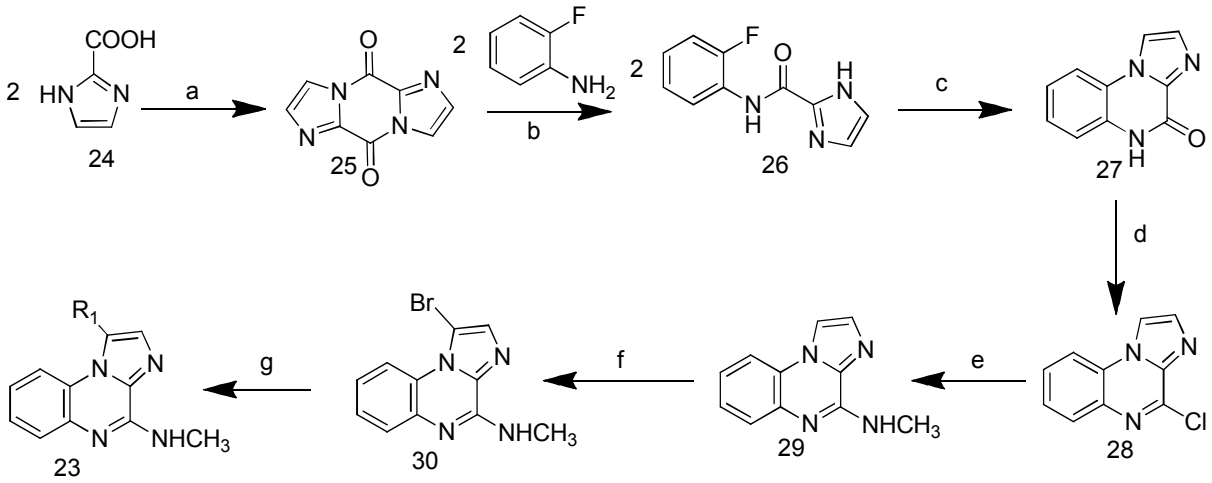


Scheme 3 Synthesis of imidazo[1,2-a]quinoxaline derivatives

The first synthetic route begins with synthesis of corresponding aminoalcohole. In the case of synthesis of EAPB0203 it was 3-phenylpropionaldehyde. Aldehyde was

treated with sodium cyanide and provided compound **17** which was reduced by lithium aluminum hydride to obtain aminoalcohole **18**. Condensation of **18** with 2,3-dichloroquinoxaline **19** in the presence of triethylamine in dioxan gave product **20**. Oxidation of hydroxyl moiety by sulphur trioxide trimethylamine complex provided compound **21**. Subsequent cyclisation was realized by dehydration in trifloroacetic acid or trifluoroacetic anhydride gave exclusively cyclized **22**. The last step of synthesis was nucleophylic substitution of chlorine with methylamine in ethanol which provided final product **23** in good yields. Disadvantage of this synthetic route is number of purification. Due to this fact it is time demanding synthesis (15).

The second route (Scheme 4) led to EAPB0503. The difference is that substituent in the position 1 is attached to the core via Suzuki coupling at the end of the synthesis. The tricyclic system remained ususbstituted until the last step of the synthesis, the Suzuki coupling. This new synthetic route reduced number of purifications.



(a) $SOCl_2$ reflux, 18 h; (b) NaHMDS, THF, 5 h; (c) NaH, DMA, reflux, 10 h; (d) $POCl_3$, reflux, 6 h; (e) EtOH, $NHCH_3$, 20 h, rt; (f) NBS, $CHCl_3$, reflux, 2 h; (g) arylboronic acid (R–$B(OH)_2$), $Pd(PPh_3)_4$, Na_2CO_3, DME, MW (140°C, 20 min).

Scheme 4. Synthesis of imidazo[1,2-a]quinoxaline derivatives using Suzuki coupling

Synthesis started with dimerization of imidazole acid **24** in presence of thionyl chloride. Following step was coupling of 2-fluoroaniline with compound **25** that brought derivative **26**. Intramolecular cyclization in presence of strong base (NaH) was the third step of synthesis. It provided product **27** that was chlorinated via phosphorus oxychloride. Chlorinated derivative **28** with methylamine gave compound **29.** *N*-bromosuccinimide was used as bromination agent in following step which led to

product **30.** Final step of synthesis was Suzuki coupling that provided variously substituted derivatives (16)

2.2.3.2 Structure-activity relationship study

EAPB0503 proved the best carcinoma cell inhibition among all derivatives. IC_{50} was determined to compare activity of synthesized derivatives with imiquimod and fotemustine. Substances were tested on A375 melanoma cells. Cultivation lasted 96 hours and detection was realized via colorimetric assay by addition of MTT which is reduced by living tumor cells to blue colored product.

Generally substitution of position 4 of the imidazo[1,2-a]quinoxaline core with amine or methylamine brought better results than bulkier substituents. Methylamine proved the best influence on cytotoxic activity, so in following studies only impact of substituent on position 1 was observed. The best results were achieved with variously 3-substitued phenyl derivatives. One of them is EAPB0503 that is 3-methoxyphenyl derivative. Interesting fact is that 3-methoxyphenyl and 4-methoxyphenyl derivatives possess great cytotoxic activity while 2-methoxyphenyl derivative activity was more than 1000 times lower. 3-ethoxyphenyl derivative showed good cytotoxic activity as well as other studied 3-substituted phenyl derivatives. Exception were 3-fluoro, cyano and nitro derivatives but still their activity was better than imiquimod and fotemustine(16,17)

2.2.4 Physico-chemical properties

All obtained molecule proves similar or close physico-chemical properties. Values of logP are high, ranging between 2,99 – 5,80, which means these compounds are lipophilic and insoluble in water. Exceptions are imidazo[1,5-a]quinoxalines which proved less lipophilic properties- logP 0,93 – 1,19. Main reason of this is substitution of position 1 just with hydrogen or methyl (16,17). All substances showed pK_a of conjuged acid between 4.50 – 5.60. That means that they possess basic properties. Only a few exceptions had pK_a lower- these exceptions had chlorine or methoxy group attached to position 4(16).

3 Aim

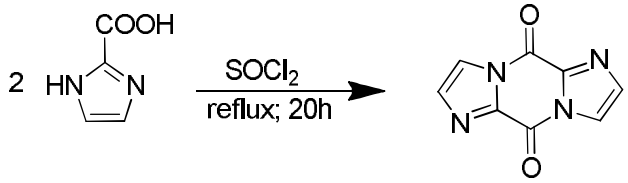
The aim of this study was to synthesize new anticancer compounds and modify their structure and chemical properties in order to improve solubility in water. EAPB0503, the lead molecule, proved advanced antiproliferative activity on tumor cell cultures in previous studies, but due to its poor solubility it is difficult to reach therapeutic concentration in solution for administration to animals, which is essential for further research. I focused on synthesis of this substance and then on formation of salts with various acids and under various conditions.

4 Experimental section

4.1 Synthesis of EAPB0503

4.1.1 Step 1 – Synthesis of diimidazo[1,2-a]piperazine-5,10-dione (25)

Reaction



24 **25**

Reactants

1*H*-imidazole-2-carboxylic acid (**24**)

ME= 1.0

$SOCl_2$

ME= excess

Manipulation

$SOCl_2$ is very reactive with H_2O, for the reason of security it is recommended to wear 2 pairs of gloves. I prepared saturated solution of $NaHCO_3$ for neutralization of $SOCl_2$ in case of an accident.

1*H*-imidazole-2-carboxylic acid (**24**) and $SOCl_2$ were placed to the three-neck round-bottom flask with water cooled condenser with drying tube. Reaction mixture was brought to reflux (bp $SOCl_2$= 79°C) with probe set for 105°C. After 20 h of reflux the reaction mixture was cooled down to laboratory temperature.

Treatment

The reaction mixture was filtered on frit n°4. The product on the frit was rinsed with toluene in order to get rid of impurities. The filtrate from the reaction was neutralized by saturated solution of $NaHCO_3$. $NaHCO_3$ was poured into Erlenmeyer flask 1 l and then the filtrate was added slowly by pasteur pipette under constant stirring.

The product was placed into desiccator for 24 hours.

Diimidazo[1,2-a]piperazine-5,10-dione (**25**)

M.p.= 164°C

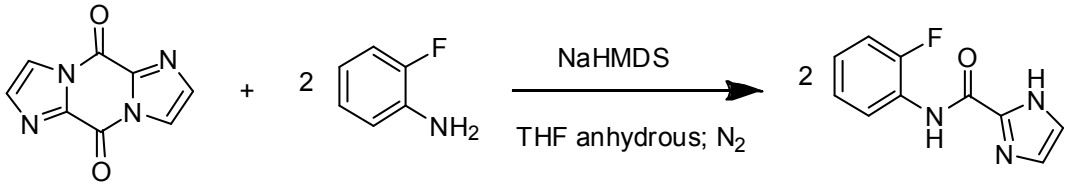
^{1}H NMR (300 MHz, DMSO-d6) δ: 8.85 (s, 2H); 8.2 (s, 2H)

^{13}C NMR (300 MHz, DMSO-d6) δ: 145.85, 144.95, 125.20, 120.15

entry	amount of **24**	amount of $SOCl_2$	**Yield of 25**
1	10.54 g; 0.094 mol	193 ml; 2.65 mol	**60%** (5.3 g)
2	10.0 g; 0.089 mol	183 ml; 2.65 mol	**66%** (5.57 g)
3	9.95 g; 0.089 mol	180 ml; 2.49 mol	**75%** (6.23 g)

4.1.2 Step 2 – Synthesis of (2-fluoroaniline)-*1H*-imidazole-4-carboxamide (26)

Reaction



Reactants

2-fluoroaniline (**31**)

ME= 2.1

Sodium bis(trimethylsilyl)amide (NaHMDS) 1,0 M THF

ME= 2.7

Diimidazo[1,2-a]piperazine-5,10-dione (**25**)

ME= 1.0

Manipulation

2-fluoroaniline (**31**) was placed to the three-necks-round-bottom flask with NaHMDS solution in THF and reaction mixture was stirred for 1 hour under N_2 atmosphere and temperature -10°C (cooled by ice + NaCl). The color of reaction mixture changed into violet.

After 1 hour of stirring under above described conditions, compound **25** in THF (21,4 ml for 0,01 mole of substrate **25**) was added into the reaction mixture and it was stirred under N_2 atmosphere and laboratory temperature for 3 hours.

Treatment

Reaction mixture was placed into 1 l Erlenmeyer flask, which was cooled by ice, and then acetic acid was added slowly- drop by drop- by pasteur pipette in order to neutralize the solution. The neutralization was stopped when pH=7 had been reached. Neutralization was very exothermic and produced gases. A formation of brick/brown colored clot was observed. Solvent was evaporated on vacuum rotavapor.

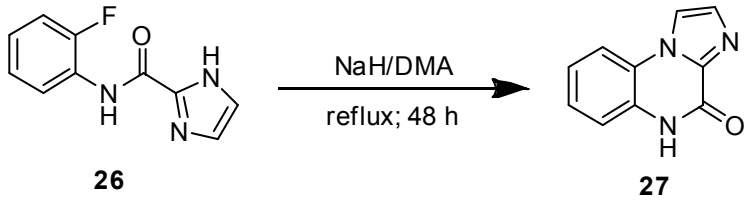
When the solvent had been evaporated, H_2O (17.9 ml for 0.01 mol of substrate **25**), saturated solution of Na_2CO_3 and cyclohexane (each in the same amount as H_2O) was added and the flask was placed to the ultrasound for cca 10 minutes. The impurities are red, the product is brown. The reaction mixture was then filtered on the frit n°4. The product **26** had been rinsed with cyclohexane.
Brown solid product was placed to the desiccator for 48 hours.

(2-Fluoroaniline)-*1H*-imidazole-4-carboxamide (**26)**
M.p.= 250°C
^{1}H NMR (300 MHz, DMSO-d6) δ: 8.12 (dd, 1H), 7.92 (dd, 1H), 6.92 (t, 1H), 7.13 (t, 1H), 7.07 (s, 2H).
^{13}C NMR (300 MHz, DMSO-d6) δ: 151.69, 150.92, 150.52, 130.14, 128.79, 126.96, 123.97, 121.03, 119.53, 115.03

entry	substrate **25**	NaHMDS 1M THF	substrate **31**	**Yield of 26**
1	5.30 g; 0.028 mol	75.6 ml; 0.076 mol	5.7 ml; 0.059 mol	**74%** (8.49 g)
2	5.57 g; 0.030 mol	79.5 ml; 0.079 mol	6.0 ml; 0.062 mol	**56%** (6.78 g)
3	6.23g; 0.033 mol	90.5 ml; 0.090 mol	6.7 ml; 0.069 mol	**92%** (12.52 g)

4.1.3 Step 3 – Synthesis of imidazo[1,2-a]quinoxaline-6(*5H*)-one (27)

Reaction



26 **27**

Reactants

(2-Fluoroaniline)-*1H*-imidazole-4-carboxamide (**26**)

ME= 1

NaH

ME= 1.5 (x 2)

DMA

V= 81.8 ml for 0,01 mol of compound **26**

Manipulation

Substance **26** was mixed with DMA in the three-necks-round-bottom flask with water cooled condenser with drying tube. Then 1.5 ME of NaH was added to the reaction mixture by glass scoop. Reaction mixture was brought to reflux (bp DMA= 165°C; sond 185°C). The reflux had not been reached under these conditions, so the temperature of sond was elevated to 205°C and the flask was covered by aluminum foil.

The reaction mixture was cooled down to laboratory temperature after 24 hours of reflux and another 1.5 ME of NaH was added. The mixture was brought to reflux again under the same conditions for another 24 hours.

Treatment

The reaction mixture was cooled down to laboratory temperature. The mixture was brown. The excess of NaH was neutralized by saturated solution of NH_4Cl. Then the mixture was evaporated. The evaporation was slow and careful. It started with temperature 72°C, then the temperature and vacuum were step by step ameliorated to t= 80°C. When the DMA was evaporated, it was dried long time under t=80°C and deep vacuum. Content of the flask was dissolved in water and filtred. Product was dried in dessicator.

Imidazo[1,2-a]quinoxaline-6(*5H*)-one (**27**)

M.p.= 322°C

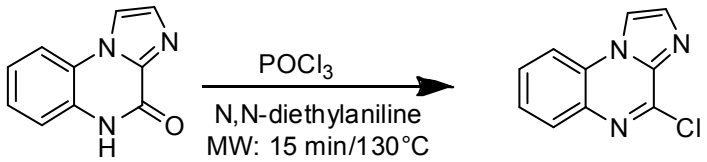
^{1}H NMR (300 MHz, DMSO-d6) δ: 9.4 (s, 1H), 8.2 (d, 1H), 7.8 (d, 1H), 7.31 (d, 1H), 7.1–7.3 (m, 3H).

^{13}C NMR (300 MHz, DMSO-d6) δ: 149.26, 139.11, 137.89, 132.43, 126.43, 123.94, 123.02, 119.40, 118.17, 115.42

entry	product 2	NaH	DMA	**Yield of 28**
1	8.49 g; 0.041 mol	2.98 g; 0.124 mol	338.5 ml	**16%** (1.22 g)
2	6.78 g; 0.033 mol	2.38 g; 0.098 mol	270.3 ml	**0**%
3	4.17 g; 0.020 mol	1.46 g; 0.061 mol	176 ml	**13%** (1.49 g)
4	4.17 g; 0.020 mol	1.46 g; 0.061 mol	176 ml	
5	4.17 g; 0.020 mol	1.46 g; 0.061 mol	176 ml	

4.1.4 Step 4 – Synthesis of 6-chloroimidazo[1,2-a]quinoxaline (28)

Reaction



27 **28**

Reactants

Imidazo[1,2-a]quinoxaline-6(*5H*)-one (**27**)

ME= 1.0

$POCl_3$

ME= 20.0

N,N- diethylaniline

ME= 2.0

Manipulation

Product **27**, *N,N*-diethylamine and $POCl_3$ were added to the vial 10-20 ml. The vial was placed to the MW for 15 min under temperature 130°C, previously stirred for 1 min.

After this period, one drop of reaction mixture was shaken in test tube with mixture of dichloromethane/water in order to obtain sample for control TLC.

Treatment

$POCl_3$ was neutralized by saturated solution of Na_2CO_3. The neutralization was very exothermic and produced gases. About 100 ml of saturated solution of Na_2CO_3 was put into 1 l Erlenmeyer flask cooled by ice and reaction mixture was added drop by drop under constant stirring. When the all reaction mixture was added, another solution would have been poured into until the effervescence stopped.

Neutralized reaction was then extracted 2x with 250 ml of DCM. Organic phase from extractions was collected, dried with Na_2SO_4 and evaporated on vacuum rotavapor. The product was placed to the desiccator for a night.

Column chromatography

Product was purified on silica gel column chromatography. DCM was used as initial mobile phase. When the first impurity was out of the colon, a mobile phase was changed to DCM : MeOH 99 : 1.

6-Chloroimidazo[1,2-a]quinoxaline (**28**)

M.p.= 188°C

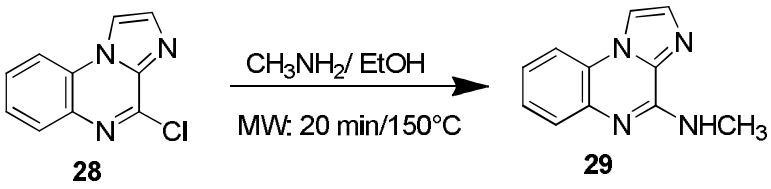
^{1}H NMR (300 MHz, DMSO-d6) d: 8.5 (s, 1H), 8.06 (d, 1H), 7.56 (s, 1H), 7.35 (m, 1H), 7.25 (m, 2H).

^{13}C NMR (300 MHz, DMSO-d6) d: 135.82, 132.19, 131.75, 130.4, 129.95, 127.12,125.03, 115.67, 113.4

entry	substrate **28**	$POCl_3$	N,N-diethylaniline	**Yield of 29**
1	1.32 g; 0.007 mol	13.1 ml; 0.143 mol	2.3 ml; 0.014 mol	**44%** (0.64 g)
2	1.00 g; 0.005 mol	9.9 ml; 0.11 mol	1.7 ml; 0.01 mol	**56%** (1.93 g)
3	0.91 g; 0.005 mol	8.9 ml; 0.10 mol	1.6 ml; 0.01 mol	
4	1.22 g; 0.007 mol	12.1 ml; 0.132 mol	2.1 ml; 0.013 mol	
5	1.49 g; 0.008 mol	14.8 ml; 0.161 mol	2.6 ml; 0.016 mol	**62%** (1.02 g)

4.1.5 Step 5 – Synthesis of *N*-Methylimidazo[1,2-a]quinoxalin-4-amine (29)

Reaction



Reactants

6-Chloroimidazo[1,2-a]quinoxaline (**28**)

ME= 1

CH_3NH_2

ME= 6.6

EtOH absolute

solvent

Manipulation

Chlorinated derivative **28** was placed into vial 20 ml with CH_3NH_2 (33% solution in EtOH). The reaction was carried out in microwave under conditions 150°C/ 20 min. Maximum amount of substrate that can be used in this size of vial is 200 mg. That's why the reaction was made several times. Then the content of all vials was collected and evaporated.

Treatment

Product was dissolved in DCM (cca 60 ml for every 1 g of compound **28**), extracted two times with saturated solution of $NaHCO_3$ (30 ml for every 1 g of compound **28**) and then washed with H_2O (the same amount as DCM). Organic phase was dried with Na_2SO_4 and evaporated under vacuum.

N-Methylimidazo[1,2-a]quinoxalin-4-amine (**29**)

M.p.= 180°C

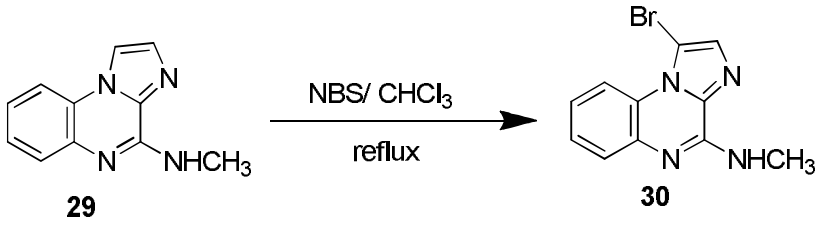
^{1}H NMR (300 MHz, DMSO-d6) d: 7.92 (s, 1H), 7.75 (dd, 1H), 7,65 (dd, 1H), 7.52 (s, 1H), 7.4 (t,1H), 7.25 (t, 1H), 6.15 (s, 1H), 3.25 (d, 3H)

^{13}C NMR (300 MHz, DMSO-d6) d: 142.25, 139.17, 131.99, 129.4, 128.17, 127.2, 125.14, 124.34, 114.14, 113.89, 29.20

entry	substrate **28**	CH_3NH_2 33% sol. in EtOH	EtOH	**Yield of 29**
1	1.93 g; 0.010 mol	7.72 ml; 0.064 mol	173.7 ml	**98%** (1.83 g)
2	1.02 g; 0.005 mol	4.00 ml; 0.034 mol	90 ml	**78%** (0.76 g)

4.1.6 Step 6 – Synthesis of 1-Bromo-*N*-methylimidazo[1,2-a]quinoxalin-4-amine (30)

Reaction



Reactants

N-Methylimidazo[1,2-a]quinoxalin-4-amine (**29**)

ME= 1

N-Bromosuccinimide (NBS)

ME= 1,1

$CHCl_3$

Solvent

Manipulation

Substance **29** was dissolved in sufficient amount of $CHCl_3$ and transferred into three-neck-round-bottom flask. Then NBS was added. Reaction mixture was brought to mild reflux under constant stirring (bp $CHCl_3$= 61°C) for 90 min.

Treatment

Reaction mixture was extracted two times with saturated solution $NaHCO_3$. Organic phase was dried with Na_2SO_4 and evaporated under vacuum.

1-Bromo-*N*-methylimidazo[1,2-a]quinoxalin-4-amine (**30**)

M.p.= 202°C

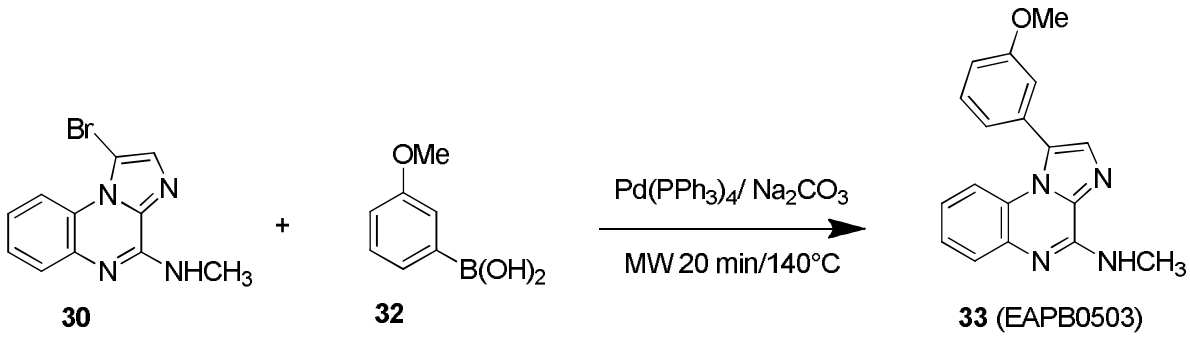
^{1}H NMR (300 MHz, DMSO-d6) d: 9.10 (s, 1H), 7.75 (dd, 1H), 7,65 (dd, 1H), 7.4 (t,1H), 7.25 (t, 1H), 6.15 (s, 1H), 3.25 (d, 3H)

^{13}C NMR (300 MHz, DMSO-d6) d: 141.97, 140.54, 138.85, 128.96, 127.78, 127.09, 126.03, 125.13, 113.86, 101.4, 29.20

entry	substrate **29**	NBS	$CHCl_3$	**Yield of 30**
1	1.83 g; 0.009 mol	1.8 g; 0.010 mol	177 ml	**98%** (2.50 g)
2	0.76 g; 0.004 mol	0.75 g; 0.004 mol	74 ml	**98%** (1.07 g)

4.1.7 Step 7- Suzuki coupling – Synthesis of 1-(3-Methoxyphenyl)-*N*-methylimidazo[1,2-a]quinoxaline4-amine (33)

Reaction



Reactants

1-Bromo-*N*-methylimidazo[1,2-a]quinoxalin-4-amine (**30**)

ME= 1

3-methoxyphenylboronic acid (**32**)

ME= 1.1

$Pd(PPh_3)_4$

ME= 0.05

Na_2CO_3

ME= 2.0

Manipulation

All the reactants were added to the vial 20 ml with DME : H_2O 2:1. Reaction was carried out in the microwave under conditions 140°C/ 20 min. Maximum amount of substrate **30** that could be used in one vial is 250 mg, so the reaction was carried out several times.

Treatment

12,4 ml H_2O for every 250 mg of **30** (content of 1 vial) was added and the mixture was extracted three times with the same amount of DCM. Organic phases were collected, dried with Na_2SO_4 and evaporated under vacuum.

Column chromatography

Product was purified by silica gel column chromatography. DCM was used as a mobile phase.

1-(3-Methoxyphenyl)-*N*-methylimidazo[1,2-a]quinoxalin-4-amine (**33**)
M.p.= 168°C
^{1}H NMR (300 MHz, CDCl3) d: 9.04 (s, 1H), 8 (dd, 1H), 7.82 (m, 2H), 7.4 (m, 3H), 6.99 (d, 1H), 6.86 (s, 1H), 5.7 (s, 1H), 3.83 (s, 3 H), 2.88 (d, 3H).

^{13}C NMR (300 MHz, CDCl3) d: 160.98, 142.86, 139.57, 131.13, 130.56, 129.5, 129.02, 128.4, 127.39, 124.9, 124.40, 122.10, 118.02, 114.96, 111.87, 108.08, 55.30, 29.20.

entry	substrate **30**	3-methoxy phenylboronic acid **32**	$Pd(PPh_3)_4$	Na_2CO_3	**Yield of 33**
1	10 x 250 mg; 0.90 mmol	10 x 150 mg; 0.99 mmol	10 x 52 mg; 0.05 mmol	10 x 191 mg; 1.80 mol	**58%** (1.76 g)
2	4 x 251 mg; 0.90 mmol	4 x 151 mg; 0.99 mmol	4 x 53 mg; 0.05 mmol	4 x 192 mg; 1.80 mol	**69%** (0.82 g)

4.2 Improving solubility of EAPB0503

4.2.1 Formation of salt

4.2.1.1 Sulfate

Manipulation

50,3 mg (1,65.10^{-4} mole) of EAPB0503 was placed to the vial and 1 ml of H_2O and 10 µl of H_2SO_4 was added under constant stirring. Every hour another 1 ml of H_2O and 10 µl of H_2SO_4 was added. When the volume of liquid in the vial reached 10 ml, the interval was shortened to 15 min. When the concentration of 2 mg/ml was reached (volume in the vial was 15 ml), the mixture was stirred for 24 hours.

Treatment

5 ml of solution were filtered on microfilter 0.22 µm. 4 ml of this filtrate was lyophilized.

4.2.1.2 Phosphate

Manipulation

10 mg of EAPB0503 (3.29.10^{-5} mol) was placed to the vial with 1 ml of H_2O and 4 µl of H_3PO_4 85%. The mixture was stirred for 15 min and another ml of H_2O and 4 µl of H_3PO_4 85% were added. This continued until the volume of mixture in vial reached 5 ml (the concentration of EAPB0503 was 2 mg.ml^{-1}). The mixture was stirred for 24 hours.

Treatment

5 ml of solution were filtered on microfilter 0.22 µm. 4 ml of this filtrate was lyophilized.

4.2.1.3 Mesylate

4.2.1.3.1 Approach 1

Manipulation

10 mg ($3.29.10^{-5}$ mol) of EAPB0503 was placed to the vial with 1 ml of H_2O and 4 µl of methanesulphonic acid. The mixture was stirred for 15 min and another 1 ml of H_2O and 4 µl of methanesulfonic acid were added. This continued until the volume of mixture in vial reached 5 ml (the concentration of EAPB0503 was 2 $mg.ml^{-1}$). The mixture was stirring for 24 hours.

Treatment

5 ml of solution was filtered on microfilter 0.22 µm. 4 ml of this filtrate was lyophilized.

4.2.1.3.2 Approach 2

Manipulation

80 mg ($2.63.10^{-4}$ mol) of EAPB0503 was placed into round-bottom flask and then acetone has been added step by step until all the EAPB0503 was dissolved. I used 13 ml of acetone. Then 35 µl of methanesulfonic acid (two molar equivalents of EAPB0503) were added.

After 2 hours of stirring another 350 µl was added. After another 24 hours 175 µl was added and the mixture was stirred 48 hours.
Control TLC was made (mobile phase DCM : MeOH 95:5).

Treatment

The mixture was diluted with acetone, dried with Na_2SO_4 and evaporated under vacuum. DCM was added to the flask with the rest from evaporation (methanesulfonic acid phase). The content of flask was extracted with distilled water. Aqueous phase was lyophilized, organic phase was dried with Na_2SO_4 and evaporated under vacuum. Before evaporation control TLC was made (mobile phase DCM : MeOH 95 : 5). Residual liquid in the flask after lyophilisation was drop by drop added to 30 ml of

acetone. It was stirring for 4 hours and there the mixture was filtered on frit nr. 4. Obtained solid was dried and analyzed by NMR.

4.2.1.3.3 Approach 3

Manipulation

77 mg ($2.53.10^{-4}$ mol) of EAPB0503 was placed into round-bottom flask and then dichloromethane have been added step by step until all the EAPB0503 was dissolved. I used 2 ml of dichloromethane. Then 35 µl of methanesulfonic acid (cca two molar equivalents of EAPB0503) were added. After 2 hours of stirring another 210 µl was added.

After 24 hours 175 µl was added and the mixture was stirred for 48 hours. Control TLC was made (mobile phase DCM : MeOH 95 : 5).

Treatment

The mixture was diluted with DCM, dried with Na_2SO_4 and evaporated under vacuum. Control TLC was made (mobile phase DCM : MeOH 95 : 5). No liquid was left in flask after evaporation, just white/yellow powder.

4.2.1.3.4 Approach 4

Manipulation

100 mg of EAPB0503 were added into round-bottom flask with 1 ml of solution methanol-water 1:1. After formation of white suspension, 27 µl of methanesulfonic acid (1,2 molar equivalents) was added. The mixture was stirred for 3 hours and then 5 ml of acetone was employed. Solvents were evaporated. The residue after evaporation was dissolved in acetone, excess of methanesulfonic acid was added and mixture was stirred for 48 hours.

Treatment

The mixture was diluted with acetone, dried with Na_2SO_4 and evaporated under vacuum. DCM was added to the flask with the rest from evaporation (methanesulfonic acid phase). The content of flask was extracted with distilled water. Aqueous phase was lyophilized, organic phase was dried with Na_2SO_4 and evaporated under vacuum. Before evaporation control TLC was made (mobile phase DCM : MeOH 95 : 5)

4.2.1.3.5 Approach 5

Manipulation

75 mg of EAPB0503 was added into round-bottom flask with 1 ml of solution methanol-water 1:1. After formation of white suspense, excess of methanesulfonic acid was added (1 ml). The mixture was stirred for 48 hours.

Treatment

30 ml of acetone were added and then solvents were evaporated. The rest after evaporatin was extracted with DCM and distilled water. Aqueous phase was lyophilized, organic phase was evaporated.

4.2.1.4 Citrate

Manipulation

10 mg ($3.29.10^{-5}$ mol) of EAPB0503 was placed to the vial and every 15 min 1 ml of citric acid solution (concentration 0.013 $g.ml^{-1}$.) was added. This continued until the volume of mixture in vial reached 5 ml. The mixture was then stirred for 24 hours.

Treatment

5 ml of solution were filtered on microfilter 0.22 μm. 4 ml of this filtrate was lyophilized.

4.2.1.5 Succinate

Manipulation

10 mg ($3,29.10^{-5}$ mol) of EAPB0503 was placed to the vial and every 15 min 1 ml of succinic acid solution (concentration 0.013 $g.ml^{-1}$.) was added. This continued until the volume of mixture in vial reached 5 ml. The mixture was then stirred for 24 hours.

Treatment

5 ml of solution were filtered on microfilter 0.22 μm. 4 ml of this filtrate was lyophilized.

5 Results and discussion

5.1 Synthesis

Whole synthesis was repeated two times. The first three steps were repeated three times, but in one case synthesis could not continue due to inconvenient quality of product **27**. The initial reaction provided good yields and purity of product was sufficient. Yields mentioned in the article that described the syntesis(16) were around 90-95%. My yields were not that high, but the tendencies were ameliorating with every attempt.

Step 2 was coupling of **26** with 2-fluoroaniline (**31**). Yields were generally good, but in one case (the second attempt) there were more impurities and less product than in other attempts. That was maybe one of the reasons of following failure of step 3. Yields were better than in the referenced article(16).

Intramolecular cyclization was the most problematic step of whole synthesis. It provided low yields. Previous experiments proved that the smaller amount of starting product is used, the better yields are. But even if I used small amount of **26**, yields were not better than 16%. One of possible reasons was impurity of **26** or maybe NaH was partially degraded. Exact reason of the failure was not discovered.

Step 4 was made two times with my own starting product and one time with starting product previously synthesized by my predecessor (as a part of synthesis attempt nr.1, so after purification it was mixed with my product). Yields were between 44-62%. My yields are incomparable with the referenced article, because the manipulation was different. I carried out the reaction in the microwave reactor, while boiling under reflux for 2 hour was previously employed.

Step 5 did not follow manipulation described in the referenced article. I made this reaction in microwave reactor several times, because maximum amount of starting product that could be placed into vial was 200 mg. Content of all vials was collected, treated and purified together. Yields were good.

Yields of **30** in step 6 were better than it was mentioned in referenced article, but my product was not purified on silica gel column chromatography, it was just extracted into organic solvent.

Yields of the final step of synthesis were not corresponding to the referenced article, they were smaller, but the mass of product that I obtained was sufficient for further experiments. In both cases I obtained product of high purity.

5.2 Improving of solubility of EAPB0503

5.2.1 Formation of salts

5.2.1.1 Sulphate

During manipulation I observed changes of appearance of the reaction mixture. EAPB0503 was hydrophobic when the first water was added, but the addition of acid caused that it became less hydrophobic. Later the mixture turned into white suspension. I weighted the content of the flask after lyofilisation- the theory was that if there was the salt of EAPB0503, I could determine its quantity gravimetrically. Unfortunately, due to imprecision of scales and very small mass of content of the flask I could not determine the weight. There was almost nothing so we came to conclusion that this way of synthesis is not suitable.

5.2.1.2 Phosphate

I did not observed any changes of appearance of the reaction mixture. EAPB0503 stayed hydrophobic even after addition of acid. I weighted the content of the flask after lyofilization, but as I mentioned above, it was not possible to determine the mass of the content. During observing the changes of the appearance this acid brought the least significant changes.

5.2.1.3 Mesylate

5.2.1.3.1 Approach 1

The changes of appearance were the same like in the case of sulfate. The suspension was a little clearer then the one mentioned in 5.2.1.1. Problems with determination of the mass in the flask after lyofilization were the same as above, but the changes of physicochemical properties were promising. Methanesulfonic acid seemed to be the best of examined acids. That is why we chose methanesulfonic acid for further experiments to try if different workup will bring better results.

5.2.1.3.2 Approach 2

Patent "Shimizu Hideaki, Uchida Miuki, Sawada Seigo, Kaneda Norimasa, Matsumoto Tsuneo. Acid addition salt of irinotecan. p. C07D491/22" describing formation of mesylate of irinotecan brought us to idea if workup mentioned in the patent would be useful for EAPB0503. Irinotecan is multicyclic heterocyclic compound that is very badly water-soluble. Some parts of the molecule can be compared with parts of molecule EAPB0503. This approach did not follow the patent, but it was inspired with it. I intended to dissolve EAPB0503 in acetone, then to add acid and let the mixture stir for at least 24 hours.

I did not observe any appearance change of solution. I expected precipitation of crystals of salt, but no matter how much acid was added, I had not noticed any significant effect on appearance of mixture. But after 48 hours of stirring, solution changed color from light yellow to red/brown- brick like color. TLC proved that there was still some EAPB0503 left, but there was also spot at the start which meant that there was some really polar substance. Another spot appeared higher than EAPB0503. Later it turned out that the spot at the start was methanesulfonic acid, not salt of EAPB0503 as I originally supposed.

After evaporation I obtained brown liquid. As the boiling point of methanesulfonic acid is 167°C/ 10 mmHg (conditions in vacuum rotavapor), it was clear that obtained liquid is methanesulfonic acid. I added DCM to this liquid. DCM phase turned yellow. TLC from this phase showed that EAPB0503 and the high impurity was extracted from methanesulfonic acid phase into DCM. We decided to extract this mixture with distilled water. Aqueous phase changed color into yellowish. Lyofilisation of aqueous phase was not successful in getting rid of methanesulfonic acid, so we decided to add the liquid into excess of acetone and try if precipitation would occure. Finally it worked. With first drops of the methanesulfonic acid liquid I observed formation of fine white clot. Unfortunatelly NMR later proved that this precipitate was not desired salt.

5.2.1.3.3 Approach 3

DCM and methanesulfonic acid cannot be mixed, so I observed formation of two phase system. With addition of methanesulfonic acid I expected precipitation, but it has not occurred no matter how much acid had been added. The color of DCM phase was light yellow, acidic phase was darker yellow and those colors remained the same during

whole experiment. According to TLC there was EAPB0503 and some polar substance. Later I made TLC of methanesulfonic acid and found out that the polar substance was probably just methanesulfonic acid.

Drying the mixture with Na_2SO_4 brought loss of acidic phase, because it was captured by Na_2SO_4.

This approach did not work because methanesulfonic acid and DCM are not miscible, so it created two phase system. After treatment I evaporated DCM phase to obtain EAPB0503 back.

5.2.1.3.4 Approach 4

When solution methanol-water was added to the flask with EAPB0503, I observed formation of white suspension. When the suspension was well homogenized, I added methanesulfonic acid. With first drop of acid mixture changed from suspension into solution with just a few precipitates. After 3 hours of stirring I obtained clear, transparent solution without any precipitations. Then acetone was added in order to crystallize the salt of EAPB0503. As in previous cases, this workup failed. One of possible reasons could be insufficient amount of acid, so I evaporated solvents, dissolved the residue in acetone and added excess of acid. The mixture was stirred for 24 hours and then the treatment followed the one described in approach 2. I obtained white precipitate, but NMR proved that the precipitate was not desired salt. Unfortunately NMR analysis of this clot was carried out at the same time as NMR analysis of clot from approach 2, so we found that this is an impasse too late.

5.2.1.3.5 Approach 5

This approach was mostly the same as approach 4, but the excess of acid was employed at the beginning. We supposed that excess of acid could improve condition for formation of salt, White suspension of EAPB0503 in water/methanol solution turned into clear solution after addition of acid as in previous case. Dilution of the mixture with acetone after 48 hours of stirring did not bring precipitation. The residue after evaporation of solvents was directly analyzed by NMR. As it turned out that the precipitate is not desired salt, we wanted to know if the salt is enclosed in methanesulfonic acid or if there is no salt at all. NMR proved that there was no sign of EAPB0503 salt.

5.2.1.4 Citrate

From the beginning of the experiment I have not observed any remarkable changes of appearance- just that EAPB0503 was not hydrophobic. But when the third dose of citric acid solution was added, the mixture, which was clear before this moment, turned into white suspension. As I mentioned in point 5.2.2.1, lyofilisation and gravimetric determination of content failed due to imprecision of scales. There were just crystals of citric acid and according to my calculations the approximate weight of the content after lyophilisation was almost the same as theoretical weight of used citric acid. Citric acid is less acidic than previously tested acids, and nitrogens of EAPB0503 are not much basic, so that is maybe the reason of the failure.

5.2.1.5 Succinate

As in the previous case, at the beginning I did not observed any remarkable changes, but after the last dose of succinic acid was added and the mixture was stirred for 2 hours, it turned into white suspension, too. Gravimetric determination of the residue after lyophilisation failed due to above mentioned facts. Succinic acid turned out to be not suitable for synthesis of EAPB0503 salt.

6 Conclusion

I synthesized EAPB0503 in sufficient yields for further experiments and good purity. Overall yield were 0.03-0.05 %. Synthetic route maybe need some further optimalisation, especially step 3- intramolecular cyclization. Attempts to formation of salts with chosen mineral and organic acids brought less success. Methods that I used proved to be unsuitable. It seems that nitrogens bounded in the molecule are not sufficiently basic to provide salts under common conditions. After all the failures I would suggest to try to treat EAPB0503 with sodium salicylate. This manipulation improved solubility of certain benzodiazepines(18) which has similar physicochemical properties - low basicity of nitrogen and therefore low potential of formation of salts under common conditions.

7 List of abbreviations

LBCE – Laboratoire de chimie de Biomolécules et l'Environnement

GC – guanine-cytosine pair

AT – adenosin-thymine pair

AJCC – American Joint Committee for Cancer

UICC – Union for International Cancer Control

LDH – lactate dehydrogenase

TLR- toll like receptors

LC – Langhanser cells

INF – interferon

TNF – tumor necrosis factor

IL – interleukin

BCC – Basal cell carcinoma

AK – actinic ceratosis

DMF – *N*,*N*-dimethylformamid

MEC – minimum effective concentration

SAR – structure-activity relationship

MTT – [3-(4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromid

THF – tetrahydrofurane

DMSO – dimethylsulfoxide

NaHMDS – sodium bis(trimethylsilyl)amide

DMA – *N*,*N*-dimethylacetamide

ME – molar equivalent

MW – microwave reactor

DCM – dichloromethane

Bp – boiling point

M.p – melting point

TLC – thin layer chromatography

NBS – *N*-bromosuccinimide

MeOH – methanol

EtOH – ethanol

NMR – nuclear magnetic resonance

DME – dimethoxyethan

8 Literature

1. Clarke MF, Fuller M. Stem Cells and Cancer: Two Faces of Eve. Cell. **2006**;*124(6)*:1111 – 1115.

2. Garbe C, Peris K, Hauschild A, Saiag P, Middleton M, Spatz A, et al. Diagnosis and treatment of melanoma. European consensus-based interdisciplinary guideline - Update 2012. Eur. J. Cancer. **2012**;*48(15)*:2375-2390

3. Schön M., Schön M. Imiquimod: mode of action. British Journal of Dermatology. **2007**;*157*:8–13.

4. Feskanich D, Hunter DJ, Willett WC, Spiegelman D, Stampfer MJ, Speizer FE, et al. Oral contraceptive use and risk of melanoma in premenopausal women. Br. J. Cancer. **1999**;*81(5)*:918–23.

5. Houghton AN, Polsky D. Focus on melanoma. Cancer Cell. **2002**;*2(4)*:275–8.

6. Tsao H, Atkins MB, Sober AJ. Management of cutaneous melanoma. N. Engl. J. Med. **2004**;*351(10)*:998–1012.

7. Garbe C, Eigentler TK, Keilholz U, Hauschild A, Kirkwood JM. Systematic review of medical treatment in melanoma: current status and future prospects. Oncologist. **2011**;*16(1)*:5–24.

8. Vidal D. Topical imiquimod: mechanism of action and clinical applications. Mini Rev Med Chem. **2006**;*6(5)*:499–503.

9. Gerster JF, Lindstrom KJ, Miller RL, Tomai MA, Birmachu W, Bomersine SN, et al. Synthesis and structure-activity-relationships of 1H-imidazo[4,5-c]quinolines that induce interferon production. J. Med. Chem. **2005**;*48(10)*:3481–91.

10. Gabriel, S. Zur Kenntnis einiger Chinolin-Abkommlinge (Information about Some Quinoline Derivatives). Chem. Ber. **1918**;*(51)*:1506–15.

11. Izumi T, Sakaguchi J, Takeshita M, Tawara H, Kato K, Dose H, et al. 1H-Imidazo[4,5-c]quinoline derivatives as novel potent TNF-alpha suppressors: synthesis and structure-activity relationship of 1-, 2-and 4-substituted 1H-imidazo[4,5-c]quinolines or 1H-imidazo[4,5-c]pyridines. Bioorg. Med. Chem. **2003**;*11(12)*:2541–50.

12. Hamana M, Hoshino O. Studies on tertiary amine oxides. XXII. reactions of quinoline 1-oxide with amines in tghe presence of acylating agents. Yakugaku Zasshi. **1964**;*84*:35–41.

13. Weeks CE, Gibson SJ. Induction of interferon and other cytokines by imiquimod and its hydroxylated metabolite R-842 in human blood cells in vitro. J. Interferon Res. **1994**;*14(2)*:81–5.

14. Chen L-Z, Liu X, Ridgewell RE. Identification of R-837 Metabolites in the Presence of Human Liver Microsomes. New Orleans, LA; **1999**.

15. Deleuze-Masquefa C, Gereptzoff G, Subra G, Febrequettes J, Ovens A, Carraz M, Strub M, Bompart J, George P, Bonnet P. Design and synthesis of novel imidazo[1,2-a]quinoxalines as PDE4 inhibitors. Bioorg. Med. Chem. **2004;***39(12)*:1129-1139.

16. Moarbess G, Deleuze-Masquefa C, Bonnard V, Gayraud-Paniagua S, Vidal J-R, Bressolle F, et al. In vitro and in vivo anti-tumoral activities of imidazo[1,2-a]quinoxaline, imidazo[1,5-a]quinoxaline, and pyrazolo[1,5-a]quinoxaline derivatives. Bioorg. Med. Chem. **2008**;*16(13)*:6601–10.

17. Deleuze-Masquefa C, Moarbess G, Khier S, David N, Gayraud-Paniagua S, Bressolle F, Pinguet F, Bonnet P. New imidazo[1,2-a]quinoxaline derivatives: synthesis and in vitro activity against human melanoma. Eur J Med Chem. **2009**;*44(9)*:3406–11.

18. Badwan AA, EI‐Khordagui LK, Saleh AM, Khalil SA. The solubility of benzodiazepines in sodium salicylate solution and a proposed mechanism for hydrotropic solubilization. Int J Pharm. **1983**;*13(1)*:67–74.