# SYNTHESIS OF PURPUREALIDIN E DERIVATIVES

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Tiivistelmä – Referat – Abstract

Seas are one of the most biodiverse and species-rich areas on the planet. Many of the underwater species are yet to be found and identified. The marine based drug discovery and the clinical pipeline of marine compounds have increased lately. Thus, there is a strong believe that the marine-derived compounds will provide new pharmaceutical lead compounds.

Marine sponges are one of the most studied marine species. Sponges can be found in shallow and deep waters all over the world. *Pseudoceratina purpurea* is a Verondiga order sponge that is known to be a source of bromotyramines. Bromotyramines are tyramine derivatives that have represented biological activity including cytotoxity, antivirality and antimicrobial effects.

Purpurealidin E is a bromotyramine that has been identified from *Pseudoceratina purpurea*. Purpurealidin E hasn't showed remarkable biological activity by itself, but it can be used as starting point for synthesis of novel bromotyramine derivatives. By forming an amide bond between carboxylic acid and primary amine of purpurealidin E, new bromotyramines can be synthesized.

In this master's thesis, purpurealidin E was successfully synthesized. Total amount of 11 novel bromotyramine derivatives were synthesized by amide coupling. Three of the new bromotyramine derivatives and purpurealidin E were purified and their biological activity against hepatitis C virus (HCV) was evaluated. Purpurealidin E did not show any antiviral activity, but all the three compounds showed potential biological activity against HCV.

This work can be considered to a continuum to the now ended MAREX project (Exploring Marine Resources for Bioactive Compounds: From Discovery to Sustainable Production and Industrial Applications).

Avainsanat – Nyckelord – Keywords

Purpurealidin E, tyramine, bromotyramine derivatives, *Pseudoceratina purpurea*

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Tiivistelmä – Referat – Abstract

Meret ovat biologisesti monimuotoisia ja lajistoltaan maailman rikkaimpia alueita. Monet vedenalaiset lajit ovat edelleen löytämättä ja tunnistamatta. Merieliöihin perustuva lääketutkimus ja kehitys ovat kasvaneet voimakkaasti viime aikoina. Näin ollen on oletettavaa, että merestä tullaan löytämään uusien lääkeaineiden kehittämiseen käytettäviä yhdisteitä.

Merisienet ovat yksi tutkituimmista meren lajeista. Merisienet elävät sekä matalissa että syvissä vesissä ympäri maailmaa. *Pseudoceratina purpurea* on Verondiga lahkoon kuuluva merisieni, joka on tunnettu bromityramiinien lähde. Bromityramiinit ovat tyramiinijohdannaisia, joilla on havaittu olevan runsaasti biologista aktiivisuutta kuten sytotoksisuutta, antiviraalisuutta ja antimikrobiologisuutta.

Purpurealidiini E on bromityramiinijohdannainen, joka on löydetty *Pseudoceratina purpurea* merisienestä. Purpurealidiini E:llä ei ole havaittu olevan merkittävää biologista aktiivisuutta, mutta sitä voidaan käyttää lähtöaineena uusien bromityramiinijohdannaisten synteesissä. Muodostamalla amidisidoksia purpurealidiini E:n primäärisen amiinin ja karboksyylihappojen välille, voidaan syntetisoida uusia bromityramiinijohdannaisia.

Tässä Pro gradu tutkielmassa purpurealidiini E:tä syntetisoitiin onnistuneesti. Käyttämällä purpurealidiini E:tä lähtöaineena, syntetisoitiin yhteensä 11 uutta bromityramiinijohdannaista muodostamalla amidisidos. Kolme bromityramiinijohdannaista ja purpurealidiini E puhdistettiin ja niiden biologinen aktiivisuus C-hepatiittia (HCV) vastaan testattiin. Purpurealidiini E:llä ei todettu olevan antiviraalista vaikutusta, mutta kaikilla kolmella uudella bromityramiinijohdannaisella havaittiin potentiaalista biologista aktiivisuutta HCV:tä vastaan.

Tätä työtä voidaan pitää osana viime vuonna päättynyttä MAREX projektia.



Avainsanat – Nyckelord – Keywords

Purpurealidiini E, tyramiini, bromityramiinijohdannaiset, *Pseudoceratina purpurea*

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ABBREVIATIONS

| | |
|---|---|
| AADC | Amino acid decarboxylase |
| AcOH | Acetic acid |
| AVG | Average |
| $Boc_2O$ | Di-*t*-butyl dicarbonate |
| BA | Biological activity |
| BTAC | Benzyltriethylammonium chloride |
| CDI | 1,1'-Carbonyldiimidazole |
| DBH | Dopamine hydroxylase |
| DCC | Dicyclohexylcarbodiimide |
| DCM | Dichloromethane |
| DIC | Diisopropylcarbodiimide |
| DIPEA | *N*,*N*-Diisopropylethylamine |
| DMAP | 4-Dimethylaminopyridine |
| DMC | 2-Chloro-1,3-dimethylimidazolinium chloride |
| DMF | Dimethylformamide |
| EDC·HCl | *N*-(3-Dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride |
| ESI | Electrospray ionization |
| EtOAc | Ethyl acetate |
| GPCR | G-protein-coupled receptors |
| HBTU | *N,N,N′,N′*-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate |
| HOBt | Hydroxybenzotriazole |
| HCV | Hepatitis C virus |
| Hex | Hexane |
| HRMS | High resolution mass spectrometry |
| IR | Infrared |
| MIC | Minimum inhibitory concentration |
| NMR | Nuclear magnetic resonance |
| PheOH | Phenylalanine hydroxylase |
| RSD | Relatively standard deviation |

| | |
|---|---|
| SAR | Structure-activity relationship |
| SD | Standard deviation |
| SEM | Standard error of the mean |
| SIM | Selected-ion monitoring chromatogram |
| *p*-TsCl | 4-Toluenesulfonyl chloride |
| TA | Trace amine |
| TAAR | Trace amine-associated receptor |
| TBAT | Tetrabutylammonium tribromide |
| TEA | Triethyl amine |
| TFA | Trifluoroacetic acid |
| TLC | Thin layer chromatography |
| TOF | Time of flight |
| TyrOH | Tyrosine hydroxylase |

# 1 INTRODUCTION

Natural products have always been an important source of medicines (Molinski *et al.* 2009). In the last few decades every fourth drug approved to the market is a derivative from a nature product (Newman and Cragg 2012). Due to the limitations of underwater research the natural medicines used today are mostly from terrestrial plants and microbes, e.g. morphine, digitalis toxin (Molinski *et al.* 2009). However, due to the success stories in the marine discovery and enormous biodiversity of the sea, there is a growing interest for marine species exploration as novel lead compounds.

Sponges are one of the most studied marine species (Gerwick and Moore 2012). The research of these primitive metazoans has already offered approved drugs and they remain to stay in important role partly due to their interesting role to live with symbiosis with microorganisms (Taylor *et al* 2007). *Pseudoceratina purpurea* is a sponge that belongs to Verondiga order (Tilvi *et al* 2004). Sponges from this order are known to be source of many novel isolated bromotyramines. Bromotyramines have shown biological activity and recently there have been efforts to synthesize these isolated bromotyramines in order to have sufficient amounts of pure compounds (Kottakota *et al*. 2012). This is crucial for proper biological activity evaluation.

MAREX (2010-2014) was an EU funded international project which target was to explore the biodiversity of seas and offer novel marine-based lead compounds for European industries (MAREX 2015). Even though the actual MAREX project is now ended, this work can be considered as continuum of the project.

# 2 MARINE PHARMACEUTICALS

Unlike terrestrial products, marine organisms do not have a significant history of use in medicine (Cragg and Newman 2013). This might be surprising, since world's oceans covers more than 70% of the earth's surface and it represent an enormous resource for the discovery of potential medicines. One of the reasons for short history of marine

pharmaceuticals was limited and unreliable scuba diving techniques. Before their improvement about 45-50 years ago, the collection of marine organisms was limited to people able to skin-dive. When depths till approximately 40 m became routinely available, the discovery of marine compounds as novel bioactive compounds was able to begin. However, this was only a start. It is estimated, that over 95% of the sea area is deeper than 1 km and at the same time it is known that deep sea is one of the most biodiverse and species-rich areas on the planet, rivaling with coral reefs and rainforest (Skropeta and Wei 2014).

## 2.1 The clinical pipeline

Even though the marine exploration is far beyond ready, the clinical pipeline of marine derived compounds reveals some success already. Till the year 2010 there was only three marine based drugs approved by FDA and one EU registered drug (Mayer *et al.* 2010). Total 13 marine natural products or their variations were in clinical pipeline and large amount in the preclinical pipeline. Four years later, 2014, the amount of FDA approved marine-derived compounds was six while there was only one EU registered marine drug (Mayer 2014). In 2014, there were two marine-derived compounds in phase III trials, six in phase II, three between the phase I/II and 14 in phase I trials. The total amount of marine-derived compounds in the clinical pipeline was 25 in June 2014. This means that the amount of marine-derived compounds in clinical pipeline has doubled in four years. In the last 20 years there have been more than 1 000 marine chemicals in the preclinical pipeline that have a large amount of miscellaneous mechanism of action. At the moment, the pipeline mainly consist novel anticancer agents but there are also compounds that have activity against inflammation and mental illnesses. Thus, there is a strong believe that the marine-derived compounds in the preclinical pipeline will provide new lead compounds to the marine clinical pharmaceutical pipeline (Mayer *et al.* 2013).

## 2.2 FDA and EU approved marine drug(s)

The preclinical pipeline is strong and there are marine derived compounds in clinical pipeline that will most likely make their way to market (Gerwick and Moore 2012). However, there are already 6 marine compounds approved by FDA and one EU registered compound. The FDA approved marine drugs are cytarabine (Cytosar-U), vidarabine (Vira-A), ziconotide (Prialt), eribulin mesylate (Halaven), brentuximab vedontin (Adcetris) and omega-3-acid ethyl esters (Lovaza) (Figure 1). The EU registered drug is trabectedin (Yondelis).

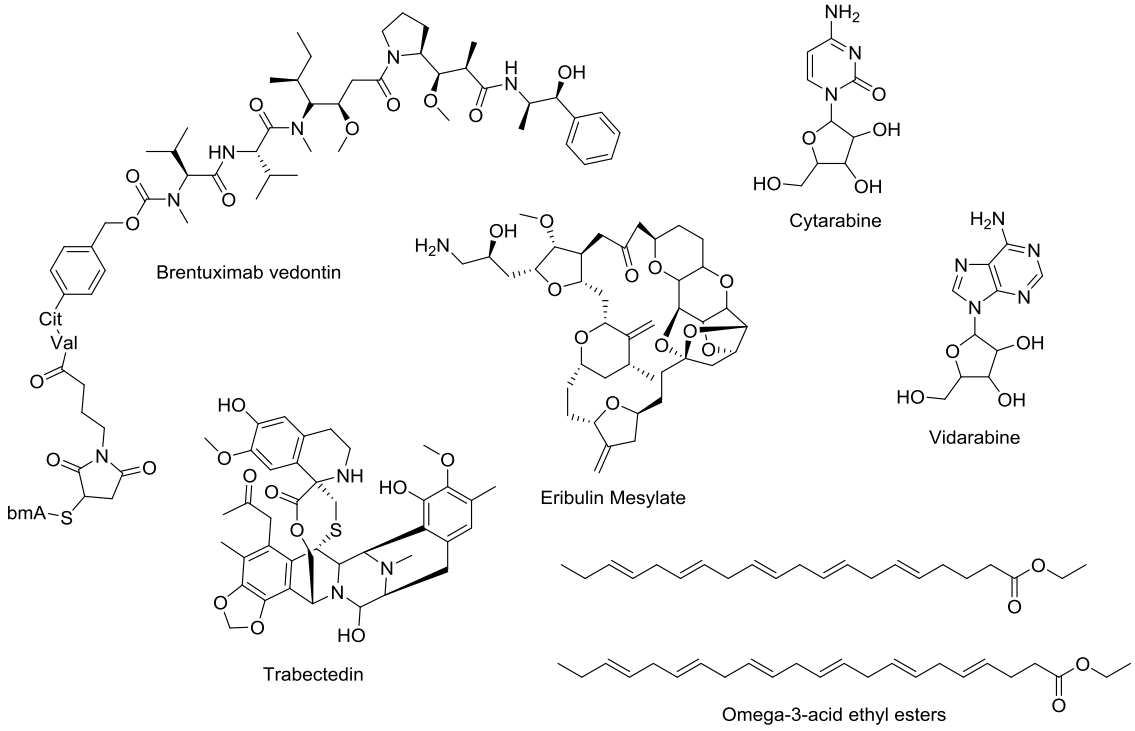



Figure 1. FDA approved and EU registered marine drug(s). Ziconotide is not included due to its complex structure.

Cytarabine (Cytosar-U) was the first marine derived product that was approved by FDA in 1969 (Mayer *et al.* 2010). Cytarabine was originally isolated from the Caribbean sponge *Tethya crypta* and it inhibits DNA polymerase and DNA synthesis which causes apoptosis. It is primarily used for to cure leukemia's. Five years later, the next marine derived medicine vidarabine (Vira-A) was approved by FDA in 1976 (Mayer *et al.* 2010). It is a synthetic purine nucleoside that was synthesized based on a molecule

found from the same Caribbean sponge *Tethya crypta*. In human, vidarabine inhibits DNA replication of viruses. Vidarabine's marketing status is currently discontinued, since better antiviral medicines have been developed. Both vidarabine and cytarabine have been remarkable medicines in the field of anticancer medication. After vidarabine was brought to the market, it took almost 30 years for the next approved marine based medicine.

Ziconotide (Prialt) was approved by FDA 2004 (Cragg and Newman 2013). Ziconotide is a non-narcotic analgesic (Atanassoff *et al.* 2000). The compound was originally isolated from venom of cone snail genus *Conus* that stuns their prey prior to capture (Cragg and Newman 2013). Ziconotide is mainly used as last option since it has to be delivered via intrathecal injection route. The same year new marine based product containing omega-3-acid ethyl esters (Lovaza) was approved by FDA (Koski 2008). Lovaza is a prescription drug that contains high amount of DHA and EPA. It is made of fish purified fish oil and it is used to treat hypertriglyceridemia.

Trabectedin (Yondelis) was approved in September, 2007, by EMEA for the treatment of soft tissue sarcomas (Cragg and Newman 2013). Trabectedin was originally isolated from a colonial tunicate *Ecteinascidia turbinata* and it as complex alkaloid. It is believed that complex structure of trabectedin allows it to bind to the guanines in the minor groove of the DNA double helix and thus cause apoptosis (Petek *et al.* 2015).

Eribulin mesylate (Halaven) was approved 2010 by FDA (Cragg and Newman 2013). The compound is derived from halichondrin that can be found from different marine species including sponge *Halichondria okadai* and bryzoa *Bugula neritina*. Due to the complexity of the molecule the total synthesis was impractical for drug development (Doherty and Morris 2015). Fortunately, it was found that only a half of the molecule was demanded for the pharmacological effect. Brentuximab vedotin is an antibody drug conjugate that was approved by FDA 2011 (Cragg and Newman 2013). It was derived originally from marine mollusca (Mayer 2014). It consist three different components: a chimeric immunoglobulin specific for human CD30-antibody, microtubule disrupting agent MMAE and protease-cleavable covalent linker (Younes *et al.* 2012). The part

chimeric immunoglobulin is produced is Chinese hamster ovary cells and the small-molecule parts are synthesized. Brentuximab vedotin is used e.g. against Hodgkin's lymphoma.

## 2.3 Marine pharmaceuticals future and challenges

The approved drugs are important success stories in marine based drug discovery that brings confidence to the field of study. The efforts have not been useless: the rate of discovering a new medicine from all the marine compounds studied is approximately 1.7- to 3.3-fold better than the industry average (Gerwick and Moore 2012). The promising results of marine field have inspired more researchers to find new interesting compounds from the sea.

However, there are ethical issues that need to be considered (Cragg *et al.* 2012). The most accessible marine biodiversity and the majority of species are inside economic zones of countries. Thus, there is an organization United Nations Convention on Biological Diversity (CBD) that is establishing international manners in marine drug discovery. The objects in the CBD are to conserve the marine biodiversity, use components sustainably and to share the benefits arising out of the utilization of genetic resources. This serves the field of marine drug discovery and the collaboration between biodiversity-rich and scientifically-rich countries. The agreements made helps the researchers to explore the marine species.

While the ethical issues and rules have been partly solved, there has also been development in the analytical methods (Gerwick and Moore 2012). Due to this, the needed amounts of the samples have become smaller. This helps to destroy marine biodiversity as little as possible. Also, it allows the exploration of marine species existing in very low biomass in nature, such as thinly encrusting invertebrates or micro algal slimes. Unfortunately, the small sample sizes have also downsides. Often, the amounts collected from the seas are enough for the identification but not for the biological activity evaluation. Thus, it is important to find synthesis routes to study the possible biomedical activity of novel compounds.

There are also cases were new interesting biological activity have been reported, but the research have not been continued. For instance, between the years 2009 and 2011 there were 230 reported compounds that showed biological activity (Mayer *et al.* 2013). There is a great need to proceed the research of these new compounds. One approach are projects where academic investigators are partnered with pharmaceutical companies and institutes such as National Cancer Institute's (NCI) (Gerwick and Moore 2012). The programs have been successful providing new compounds that have gone to clinical trials.

Even though marine species are still relatively poorly known, a large number of marine compounds in shallow water have been characterised (Gerwick and Moore 2012). Large marine species or the ones that are easy to access are becoming more and more familiar while the technology for exploring new compounds from deeper parts of the sea still needs to develop (Skropeta and Wei 2014). Thus, there has been a growing interest to explore small creatures such as marine bacteria and fungi that have been left out of attention (Gerwick and Moore 2012). It is now understood that a large number of new nature compounds that are isolated from macroorganisms, such as sponges, are actually metabolic products of microbes. Due to the estimation that only a percent of all bacteria presented in sea water is cultured, it is clear that there is a great potential for new lead compound discoveries from the sea.

## 3 MARINE SPONGES

Sponges (Porifera) are considered to the most primitive of metazoans (Taylor *et al.* 2007). They arise at least from the Precambrian time 600 million years ago. The divergence of sponges from the other species may have happened even 1.3 billion years ago. Today, sponges are still well presented in the marine ecosystem covering up to 80% of some shallow- and deep-water communities (Taylor *et al.* 2007; Skropeta and Wei 2014). There are over 8000 sponge species known to live in the shallow and the deepest parts of the seas. Sponges are the largest source of new marine natural products reported annually. The diagram visualizes the growing interest towards sponges and the

number of publications retrieved from SciFinder-database until the end of year 2014 (Figure 2).

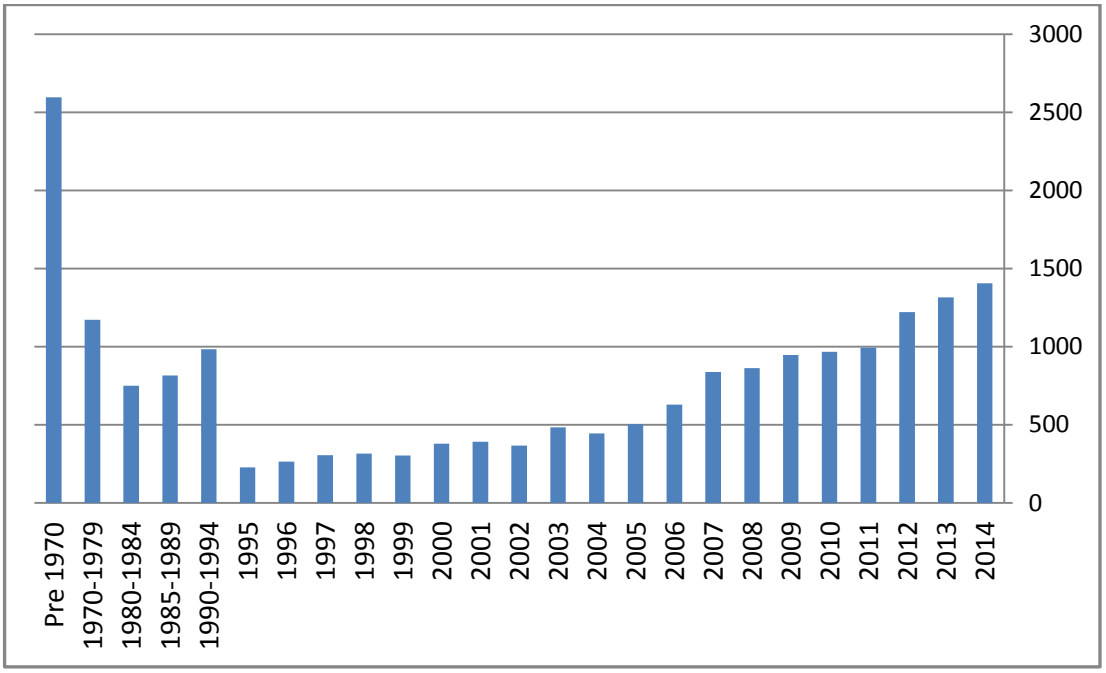



Figure 2. Amount of publications found from SciFinder-database when the used search terms are *sponge* or *porifera*.

## 3.1 Sponge morphology

The environmental atmosphere for marine species is enormously different (Skropeta and Wei 2014). For instance, there are large variations in the pressure and temperature under water. In the deep parts of the sea, species needs to adjust their metabolism that they can function at depressed temperatures and high pressure. Needless to say, the organisms and sponges under sea level have to be adapted to their environment.

Sponges can be divided in to three main classes by paraphyletic grouping: Hexactinellida (glass sponges), Calcarea (calcareous sponges) and Demospongiae (demosponges) (Borchiellini *et al.* 2001). Demospongiae contains the majority of extant sponges while Calcarea is a small group. The sponges of Hexactinellida group are mostly deep-water sponges. The discrepancy in sponges structure and tissue coordination is relatively small considering that they are one of the oldest multicellular

animals (Taylor *et al.* 2007). However, the morphology is otherwise highly diverse. There are sponges with different colors, shapes and sizes. The diameter of small sponges can be only few millimeters and the big sponges can be up to one meter.

The morphology of sponges depends often on the surrounding area (Taylor *et al.* 2007). Sponges are sessile and they are efficient at obtaining food by filtering the nearby water. Thus, they need to adapt their function depending on the environment. Sponges living in the areas with short amount of food need to filter more water in order to get enough nutrients (Leys and Hill 2012). On the other hand, sponges living in the areas of high nutrient levels or in symbiosis with microbes need to process less water. It is estimated, that up to 24 000 liters of water can be pumped trough 1 kg sponge in a day (Taylor *et al.* 2007).

With the large amount of water running through sponges, it is obvious that there are large amount of microorganisms and other particles going through sponges. Sponges have a capability to recognize different bacteria and microorganisms and discriminate the food they consume (Wilkinson *et al.* 1984). This may be due to protective capsular material of the symbiotic bacteria. The incapability to consume some type of bacteria can lead to high density of microorganism inside the sponge that can occupy up to 40% of the total sponge tissue volume (Taylor *et al.* 2007). The microorganisms that stay inside the sponge and can't be consumed as food can live in symbiosis with sponge. Some of the microorganisms can protect the sponge by expressing metabolic products that are not harmful for the sponge itself, but that protect it from other animals. On the other hand, microorganisms that only live inside the sponge but are not useful for the sponge are parasites. The realization of the importance of microorganisms in sponges have awakened the researchers that majority of the identified compounds may be metabolic products. In mature of fact, all three FDA approved medicines from sponges (cytarabine, vidarabine, eribulin mesylate) are predicted to be biosynthetic product of bacteria (Gerwick and Moore 2012).

## 3.2 *Pseudoceratina purpurea*

*Pseudoceratina purpurea* is a widely dispersed sponge (World porifera database 2015). It can be found for instance from Indian Ocean and Pacific Ocean. *Pseudoceratina purpurea* can live in different depths and it have been collected from shallow water (-1 m) and deeper parts of seas (-40 m) (Jurek *et al.* 1993; Gotsbacher and Karuso 2015). Even though -40 m was the deepest collection depth that was found from the literature, it is possible that the sponge can live even in deeper parts of seas.

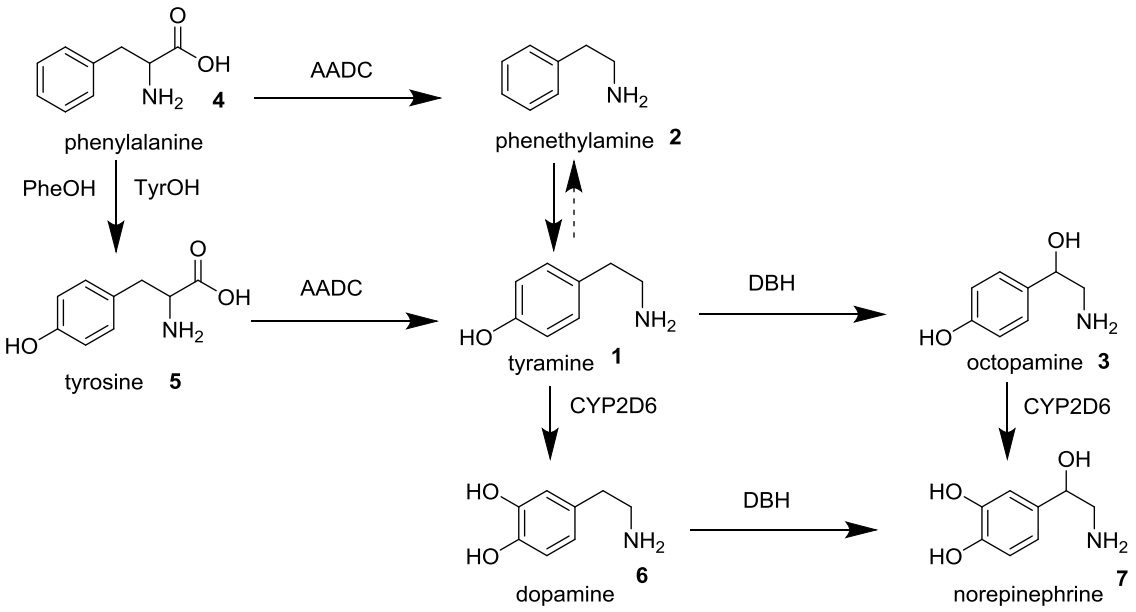
*Pseudoceratina purpurea* has often a dense collagen aggregations and sparse irregular dendritic fibers (Bergquist and Cook 2002). The reason why *Pseudoceratina purpurea* is an interesting sponge is that it is a major source of bromotyramines. Bromotyramines are a large group of molecules with various biological activity and new compounds are published constantly (Gotsbacher and Karuso 2015). Additionally, epibiotic microorganisms have been identified to live in symbiosis with *Pseudoceratina purpurea* that protects the sponge from pathogenic bacteria (Kanagasabhapathy and Nagata 2008).

The naming culture of sponges such as *Pseudoceratina purpurea* is diverse. This is understandable since the richness and diversity of marine species is fairly new subject. The naming of *Pseudoceratina purpurea* is covered more in other master's thesis work from University of Helsinki (Flemmich 2015). *Pseudoceratina purpurea* that belongs to Verondiga order of sponges was brought to general public in the late 19th century known as *Pseudoceratina* Carter (Bergquist 1995). During the years the same *Pseudoceratina* Carter-sponge was re-identified multiple times as a novel sponge specie and multiple names was given to the same sponge. An often used synonym for *Pseudoceratina purpurea* is *Psammaplysilla purpurea* but there are multiple other synonyms too (World porifera database 2015). Many of the bromotyramines covered in this thesis are originally isolated from *Pseudoceratina purpurea.*

# 4 BROMOTYRAMINES

Tyramine **1** is a small amine compound that can be found in many organism e.g. plants, bacteria, insects and other invertebrates to mammals, including human (Scheme 1) (Branchek and Blackburn 2003). It is a trace amine (TA) that is chemically related to biogenic amines. Some of the TA's in mammals are tyramine **1**, phenetylamine **2** and octopamine **3** that are metabolized from phenylalanine **4** (Branchek and Blackburn 2003). TA's are believed to act as neurotransmitters or neuromodulators.

Tyramine **1** is produced in the human body, but it is also ingested from food such as dairy products and wine (Ladero *et al.* 2009). In food supplements, **1** is produced by lactic acid bacteria which are crucial micro-organisms due to their food fermentation and their contribution of healthy homeostasis. In human body, **1** is metabolized from tyrosine **5** with amino acid decarboxylase (AADC) and through hydroxylation from **2** (Scheme 1) (Hiroi *et al.* 1998). Tyramine **1** is metabolized to dopamine **6** by CYP2D6, but **1** can be metabolized through other routes too. For instance, **1** is a substrate for both monoamineoxidase A (MAO-A) and B (MAO-B) which catalyze the oxidation of monoamines (Hauptmann *et al.* 1996).

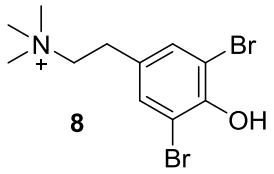



Scheme 1. Biosynthesis of tyramine **1** *in vivo*

Tyramine **1** and other TAs are linked to psychiatric disorders like depression and schizophrenia (Lindeman *et al.* 2005). Their concentration in central nervous system is generally low but they are known to have pharmacological activity. Nowadays, they are also known to have specialized G-protein-coupled receptors (GPCR), trace amine-associated receptor (TAARS) (Lindeman *et al.* 2005). Tyramine **1** displaces norepinephrine **7** from neuronal storage vehicles causing vasoconstriction and increased blood pressure through positive chronotropic effects (Shulman *et al.* 2013).

## 4.1 Bromotyramines and their biological activity

Bromotyramines are tyramine **1** derivatives where one or two hydrogens of the aromatic ring have been replaced by bromine. They are a large group of molecules that are mostly isolated from sponge *Pseudoceratina purpurea*. The molecule weight is more than doubled due to the atomic mass of bromine. The first marine bromotyramine derivative *N*,*N*,*N*-trimethyldibromotyramine **8** was indentified in 1978 from a sponge *Verongia fistularis* (Figure 3) (Peng *et al.* 2005).




**8**

Figure 3. *N*,*N*,*N*-trimethyldibromotyramine **8**

The naming of bromotyramines in literature is confusing. Compounds that are identified from marine species, have some of the TA's (**1**, **5** etc.) in the molecule structure and contain bromine are generally called as bromotyrosines (Peng *et al.* 2005). Tyrosine **5** is only one metabolic step away from **1** (Scheme 1). From this perspective, it is understandable to use the common name bromotyrosines. After all, bromotyramines could be metabolized from bromotyrosines in sponges either by the sponge itself or by the microorganisms living in symbiosis with sponge. However, there are a large amount of publications from bromotyrosines and bromotyramines both identified from sponges. It is reasonable to keep these two kind of brominated TA's in separate classes and use the correct terminology. In this master's thesis, the focus will be in bromotyramines that

are halogenated from the tyramine **1** aromatic ring. Additionally, all the brominated TA's that have substituents in the ethyl chain between the amine and the **1** aromatic ring were dropped out. There is one exception in the end of the chapter. In the end of this chapter there is also a table that summarizes the biological activity of the compounds (Table 1).

### 4.1.1 Bromotyramines isolated from *Pseudoceratina purpurea*

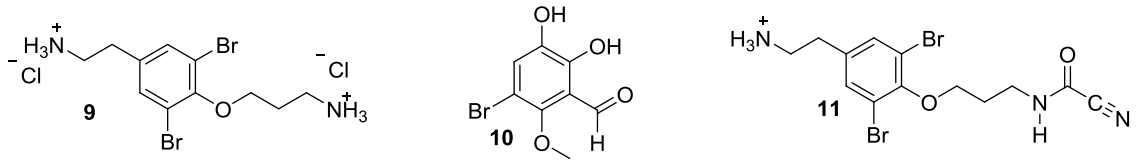



Figure 4. Moloka'iamine **9**, 3-bromo-5,6-dihydroxy-2-methoxybenzaldehyde **10** and ceratinamine **11**

Moloka'iamine **9** is often represented as a substructure for bromotyramines (Figure 4) (Hamann and Scheuer 1993). It was represented first time in 1993 from sponge that was most likely *Pseudoceratina purpurea*. Biological activity tests made in 1993 did not show potential antiviral, cytotoxic, immunomodulator or antifungal activity for **9**. In the same tests, non-bromotyramine derivative aromatic aldehyde 3-bromo-5,6-dihydroxy-2-methoxybenzaldehyde **10** isolated from the same sponge had cytotoxity with lower minimum inhibitory concentration (MIC). Additionally, **10** had antiviral activity with concentration 2 µg/ml while the needed concentration of **9** against the same cell line was >80 µg/ml. These results did not encourage to investigate the biological activity of **9** more.

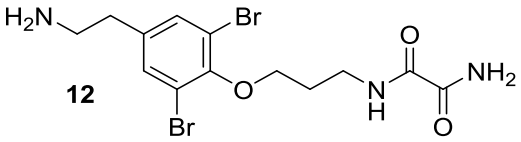



Figure 5. Moloka'iakitamide **12**

Couple years later after **9** was identified from *Pseudoceratina purpurea*, it was isolated again with its derivate ceratinamine **11** (Figure 4) (Tsukamoto *et al.* 1996). Tsukamoto's team (1996) made biological activity tests where **9** and **11** had both antifouling activity against *Balanus amphitrite* cyprid and cytotoxic against P388 murine leukemia cells. However, they did not show significant antifungal or antibacterial activity. Years later, **9** was isolated from Red Sea sponge *Pseudoceratina arabica* with other bromotyramine related compounds (Badr *et al.* 2008). Moloka'iamine **9** with the new isolated bromotyramine, moloka'iakitamide **12**, showed significant parasympatholytic effects on isolated rabbit heart and jejunum (Figure 5). The biological activity tests made by Badr's team (2008) showed weak antifungal activity for **12**.

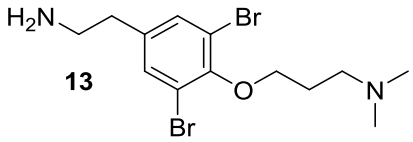



Figure 6. Purpurealidin E **13**

Purpurealidin E **13** was originally identified in 1998 from *Pseudoceratina purpurea* collected from Mandapam coast, India as an unknown and acetylated salt (Figure 6) (Venkateswarlu *et al.* 1998). The counter ion of the identified compound was not determined. One year later, in 1999, **13** was characterized as its *N*-acetyl derivative (Rama Rao *et al.* 1999). Purpurealidin E **13** was identified again from the same sponge five years later (Tilvi *et al.* 2004). However, later it was discovered that the identified **13** by Tilvi's team (2004) was probably a salt (Kottakota *et al.* 2008). Purpurealidin E **13** was tested against multiple Gram-negative and Gram-positive bacteria, and two pathogenic yeasts (Kottakota *et al.* 2012). It didn't show any biological activity against any of the tested compounds. This correlates the poor biological activity seen with the similar compound **9** (Hamann and Scheuer 1993; Tsukamoto *et al* 1996).

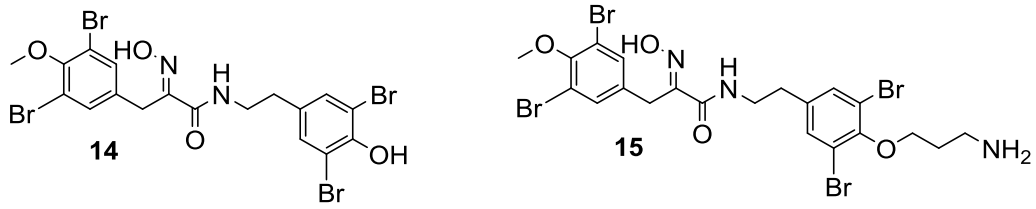



Figure 7. JBIR-44 **14** and aplysamine 4 **15**

JBIR-44 **14** and aplysamine 4 **15** were both extracted from *Pseudoceratina purpurea* collected from Kinwan bay, Japan (Figure 7) (Fujiwara *et al.* 2009). Both of the isolated compounds were tested against human cervical carcinoma HeLa cells and their cytotoxity was screened. Approximately equally strong cytotoxic effect was noticed and there was no difference between the longer or the shorter alkyl chain attached to the phenolic oxygen. This suggests that the alkyl chain does not play role in the cytotoxity. On the other hand, this could suggest that the non-tyramine aromatic ring is more crucial for the cytotoxity. This idea is supported by the tests made by Hamann and Scheuer (1993), since the small aromatic aldehyde **10** had more cytotoxic than **9**.

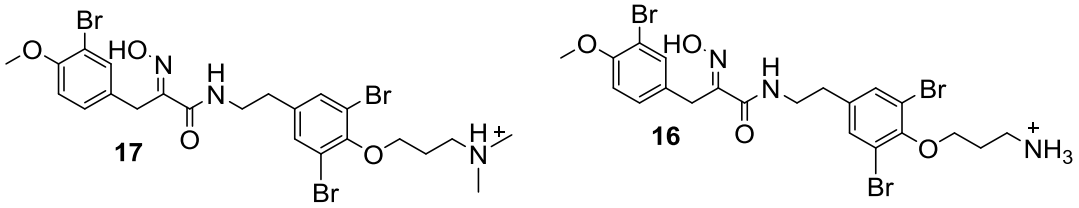



Figure 8. Aplysamine 2 **17** and aplysamine 3 **16**

Aplysamine 4 **15** was originally isolated in 1993 from *Pseudoceratina purpurea* (Jurek *et al.* 1993). It was collected by scuba on the south shore of Maui, Hawaii, at the depth of 40 m. It was identified with a bromotyramine salt that had three bromines instead of four. The compound is also known as aplysamine 3 **16** (Figure 8). Jurek's team (1993) did not try to determine the counter ion of the salt. Aplysamine 2 **17** was already found earlier and it was reported inactive in several tests against Gram-positive and Gram-negative bacteria. However, test made by Jurek's team (1993) showed mild activity against *Staphylococcus aureus* for **16** and **15**. No activity against *Escherichia coli* was observed. Compounds **17**, **16** and **15** were found to be cytotoxic in mouse lymphoid neoplasm, human lung carcinoma, human colon adenocarcinoma and human oral

epidermoid carcinoma assays. They were also tested against HIV-1 assay but they didn't have significant antiviral activity.

Purealidin Q **18**, 16-debromoaplysamine-4 **19**, Purpuramine I **20** and a novel bromotyramine compound Purealidin B **21** were extracted from *Pseudoceratina purpurea* in 2004 (Figure 9) (Tilvi *et al.* 2004). The sponge was collected by scuba diving at a depth of 8–10 m from Mandapam, India. Purealidin Q **18**, **21** and earlier identified purealidin P **22** have a heterocyclopentane formed from oxime, which makes the molecule more rigid (Kobayashi *et al* 1995). The antimicrobial and antifungal activity of the compounds identified by Tilvi's team (2004) was evaluated. The compounds (**18**, **19**, **20**, **21**) did not show any activity against *Klebsiella* sp. *Pseudomonas aeruginosa* strains and the fungal strains. Purealidin Q **18** and **19** showed activity against *Salmonella typhi*. Purealidin B **21** showed good activity and **19** minor activity against *Escherichia coli*. Moderate activity against *Staphylococcus aureus* was confirmed for **20**. It also showed moderate activity against *Escherichia coli* and *Vibrio cholerae*. Overall, the compounds were noticed to have from minor to good activity against several bacteria. Purealidin Q **18** and **22** were earlier noticed to have cytotoxity against murine lymphoma cells and human epidermoid carcinoma cells (Kobayashi *et al* 1995). They have also showed inhibitory activity against epidermal growth factor (EGF) receptor kinase.

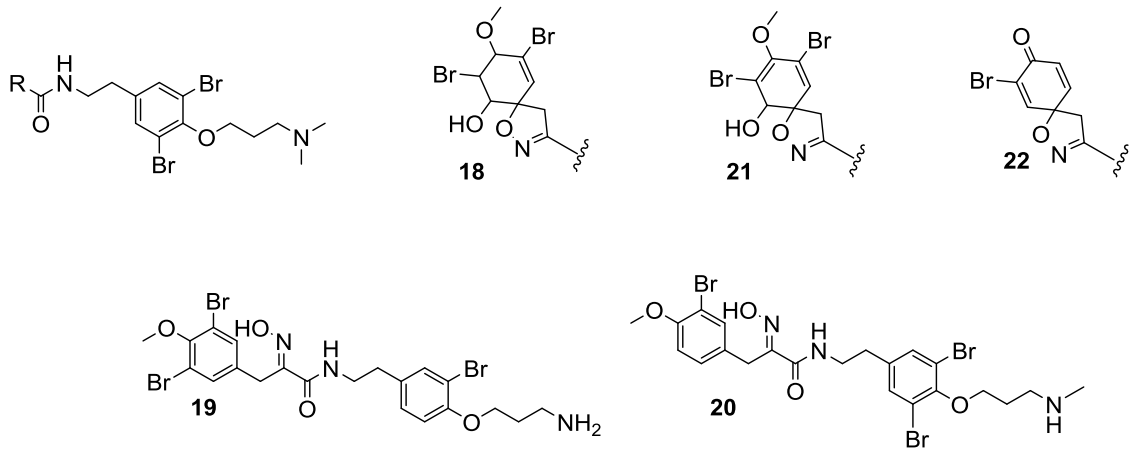



Figure 9. Purealidin Q **18**, purealidin B **21**, purealidin P **22,** 16-debromoaplysamine-4 **19** and purpuramine I **20**

Two bromotyramine family grew by two in 2012 when purpurealidin J **23** and purpurealidin I **24** were indentified from *Pseudoceratina purpurea* as a part of MAREX project (Figure 10) (Tilvi and D'Souza 2012). The biological activity of the crude extract was tested in University of Helsinki. The average inhibitions of HCV replicon with the used concentration 100µg/ml were 24% (methanol) and 47% (methanol/chloroform) (Lillsunde KE, unpublished observation 2014).

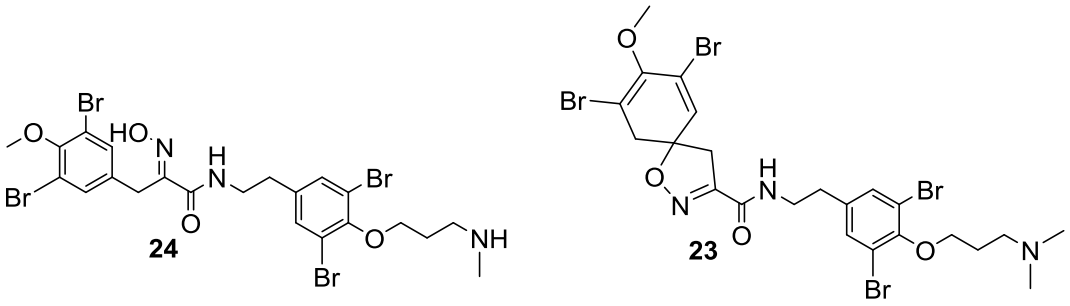



Figure 10. Purpurealidin I **24** and purpurealidin J **23**

### 4.1.2 Bromotyramines isolated from other sponges

Even though *Pseudoceratina purpurea* is one of the most important and studied sponges as a source of bromotyramines, there are other marine species where bromotyramines can be identified as well. Three halogenated tyramine derivatives **25**, **26**, and **27** were identified from non-Verondiga sponge *Iotrochorcl birotulata* (Figure 11) (Costantino *et al.* 1994). Identification of these novel compounds was interesting since bromotyramines or bromotyrosines were thought to be characteristic only for Verondiga family. Identification of these halogenated tyramines proved for the first time that bromotyramines can also be identified from other sponges.

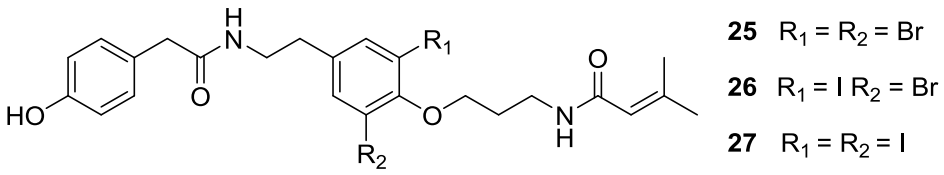



Figure 111. Bromotyramines **25**, **26** and iodotyramine **27** isolated from *Iotrochorcl birotulata*

Aplyzanzine A **28** was originally found from sponge *Aplysina* sp (Figure 12) (Evan *et al.* 2001). It was collected from Indo-pacific Ocean close to the Zanzibar coast. At the same year two very similar compounds, suberedamine A **29** and suberedamine B **30**, were identified from Okinawan marine sponge *Suberea* sp (Tsuda *et al.* 2001). Both of the sponges belong to Verondiga order. Tsuda's team (2001) made a narrow biological activity evaluation for **29** and **30.** They were found to be cytotoxic against murine leukemia L1210 cells and epidermoid carcinoma cells. Both of the compounds showed antibacterial activity against Gram-positive bacteria *Micrococcus luteus*.

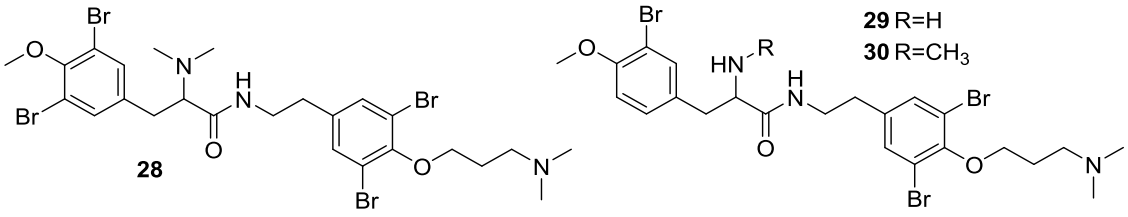



Figure 12. Aplyzanzine A **28** and suberedamines A **29** and B **30**

These compounds were synthesized later, and new biological activity tests were made (Kottakota *et al.* 2012). The synthesis of **30** is discussed in chapter "4.3 Synthesis of bromotyramines" (p. 23). All three compounds **28**, **29** and **30** were tested against Gram-negative bacteria showing only mild activity. Against Gram-positive bacteria compounds **28**, **29** and **30** had moderate activity. Aplyzanzine A **28** showed the most potent activity against Gram-positive bacteria and it also displayed moderate antifungal activity. Suberedamine B **30** showed cytotoxic activity against the NCI 60 cells and all the compounds had activity against *Mycobacterium bovis* and *Mycobacterium tuberculosis*.

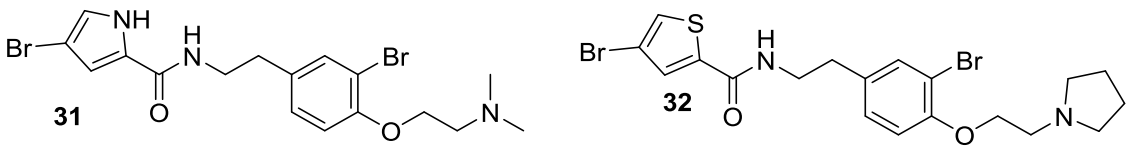
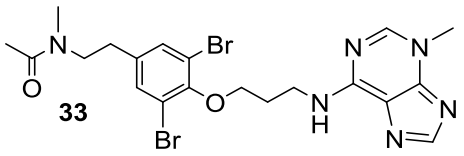



Figure 13. Dispyrin **31** and 4-bromo-*N*-[3-bromo-4-[2-(pyrrolidin-1-yl)ethoxy]phenethyl]thiophene-2-carboxamide **32**

Dispyrin **31** is a bromopyrrole tyramine that was identified in 2007 from a Caribbean sponge *Agelas dispar* (Figure 13) (Piña *et al.* 2007). Dispyrin **31** haven't shown any antibacterial or cytotoxic activity (Kennedy *et al.* 2008). However, it was found to be a potent ligand and antagonist of several therapeutically relevant GPCR including $\alpha_{1D}$ and $\alpha_{2A}$ adrenergic receptors and the $H_2$ and $H_3$ histamine receptors. Due to the potential nanomolar affinity for α-adrenergic receptors and low micromolar affinity for the H1 and H3 histamine receptors, a number of analogues from the bromotyramine unit were synthesized (Kennedy *et al.* 2009). Functional groups into the pyrrolidine ring lowered the affinity for H3 receptors. Especially electron withdrawing fluorine had a large effect. A shortened alkyl chain also lowered the affinity, whereas altering the halogen atom from Br to Cl on the benzene ring had little effect. Also the heavy bromine halogen was noticed to be unnecessary for H3 inhibition. Alteration of heterocyclic pyrrole ring to tiophene lead to synthesis of 4-bromo-*N*-[3-bromo-4-[2-(pyrrolidin-1-yl)ethoxy]phenethyl]thiophene-2-carboxamide **32** that increased the affinity to H3 receptors and it produced analogues up to 33-fold higher affinity for inhibition of H3 receptors compared to the natural product dispyrin **1**. Many of the compounds with bromotyramine core noticed to be uniformly active in H3 inhibition.




**33**

Figure 14. Aphrocallistin **33**

Aphrocallistin **33** was found from a sponge *Aphrocallistes beatrix* (Figure 14) (Wright *et al.* 2009). It was collected from Fort Pierce, Florida. Unlike most of the bromotyramines concentrated on this thesis, aphrocallistin has only a small acetyl substituent in the tyramine amine and a large purine in the phenol side of tyramine. The molecule was tested against *Candida albicans, Pseudomonas aeruginosa*, and methicillin-resistant *Staphylococcous aureus* and did not show significant inhibition. However, aphrocallistin showed cytotoxity against human tumor cells and it has been shown to induce cell cycle arrest in pancreatic carcinoma cells.

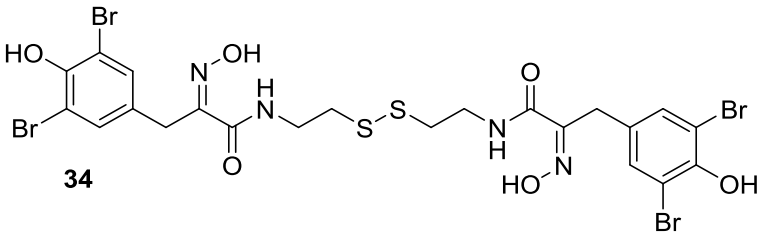



Figure 15. Bromopsammaplin A **34**

The focus in this thesis has been on bromotyramines without substituents between the tyramine aromatic ring and primary amine. Bromopsammaplin A **34** is an exception for this (Figure 15). It is a bromotyrosine that can be categorized to oxime-disulfides (Peng *et al.* 2005). It was originally isolated from two non-Verondiga sponge *Pwrillartra* sp. and *Jaspis* sp. (Park *et al.* 2003). Few years earlier a monobrominated analog of bromopsammaplin A **34** that was originally identified in 1995, had already shown antibacterial activity against methicillin resistant *Staphylococcus aureus* (Jung *et al.* 1995; Kim *et al.* 1999). In the tests made by Park's team, **34** had even greater antimicrobial activity against some of the used strains of *Staphylococcus aureus* than the well-known antibiotic meropenem (Park *et al.* 2003). Additionally, it showed cytotoxity against the tested human tumor cell lines.

Identification of bromotyramines **25**, **26** and iodotyramine **27** was not only interesting due to the fact that they proved that bromotyramines can be identified from non-Verondiga sponges. The other interesting finding was the presence of iodide. Even though iodide is a common mineral in the seas, it is relatively rarely seen in compounds identified from sponges (Peng *et al.* 2005). The finding suggested that iodization may depend on the capability of organism to concentrate iodide from seawater. Indeed, there has been observations that high concentrations of halogens correlates with the halogens found from the compounds (Costantino *et al.* 1994). It is possbile that there are high concentration of bromine inside the sponges where bromotyramines are identified. The concentrated amount of bromine could depend on sponges morphology. Another explanation could be, that tyramine derivatives are halogenated inside a microorganism. As discussed in chapter "3.1 Sponge morphology" (p. 7), microbes living in symbiosis with the sponges can be due to the incapability of sponge to consume the specific microbe as nutrition. There could be microbes that concentrates bromine and express

tyramine. This hypothesis would explain the small variations between the bromotyramines, since the metabolism of microbes is usually fast.

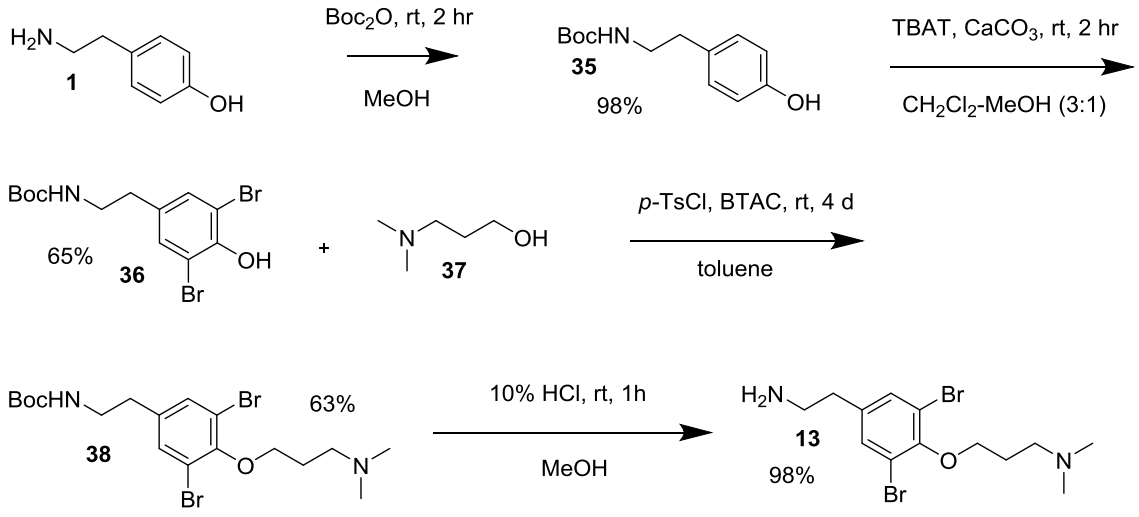
Table 1. Biological activity of bromotyramine derivates

| Number | Name of the compound | Antiviral activity | Antibacterial activity | Antifungal activity | Cytotoxity | Miscellaneous activity |
|---|---|---|---|---|---|---|
| **24** | Purpurealidin I (Crude extract of *pseudoceratina purpurea*) | X | | | | |
| **23** | Purpurealidin J (Crude extract of *pseudoceratina purpurea*) | X | | | | |
| **18** | Purealidin Q | | X | | X | EGF receptor kinase inhibition |
| **29** | Suberedamine A | | X | | X | |
| **30** | Suberedamine B | | X | | X | |
| **34** | Bromopsammaplin A | | X | | X | |
| **28** | Aplyzanzine A | | X | X | | |
| **21** | Purealidin B | | X | | | |
| **19** | 16-Debromoaplysamine-4 | | X | | | |
| **20** | Purpuramine I | | X | | | |
| **15** | Aplysamine 4 | | X | | X | |
| **16** | Aplysamine 3 | | X | | X | |
| **17** | Aplysamine 2 | | | | X | |
| **9** | Moloka'iamine | | | X | X | Parasympatholytic effects |
| **11** | Ceratinamine | | | X | X | |
| **14** | JBIR-44 | | | | X | |
| **22** | Purealidin P | | | | X | EGF receptor kinase inhibition |
| **33** | Aphrocallistin | | | | X | |
| **12** | Moloka'iakitamide | | | X | | Parasympatholytic effects |
| **31** | Dispyrin | | | | | GPCR antagonist |
| **32** | 4-bromo-*N*-[3-bromo-4-[2-(pyrrolidin-1-yl)ethoxy]-phenethyl]thiophene-2-carboxamide | | | | | H3 receptor inhibition |
| **13** | Purpurealidin E | | | | | |

## 4.2 Synthesis of purpurealidin E

In this master's thesis, purpurealidin E **13** was used to synthesize novel bromotyramine derivates. It was one of the most important compounds in order to synthesize new bromotyramines. Thus, the synthesize of **13** is discussed in this separate chapter.

Purpurealidin E **13** was used 2005 to study a possible synthesis route for bromotyramine metabolites by using coumarin as a reagent (Harburn *et al.* 2005). Hardburn's team (2005) used purpurealidin E **13** as a starting material but did not report how they synthesized it. For the first time **13** was synthesized from tyramine **1** was in 2008 (Scheme 2) (Yoshida and Yamaguchi 2008).

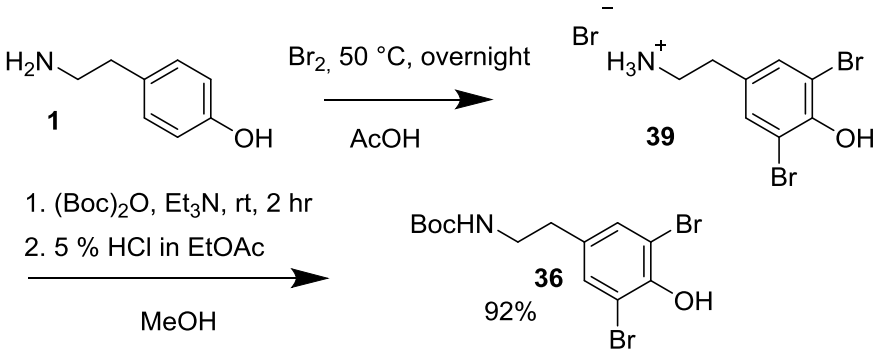



Scheme 2. Synthesis of purpurealidin E **13** (Yoshida and Yamaguchi 2008)

In the first step of synthesis a protective group (Boc) was attached to the primary amine of **1** giving the product **35** (Yoshida and Yamaguchi 2008). This is reasonable since the free electron pair of protected secondary amine is conjugated with the Boc-group. This prevents the formation of HBr-salt when is brominated. As source of bromine Yoshida and Yamaguchi (2009) used TBAT and the yield of bromination was relatively low (65%). In the third step *t*-butyl (3,5-dibromo-4-hydroxyphenethyl)carbamate **36** was treated with 3-dimethylamino-1-propanol **37** and the ether bond was formed. After the removal of protective group of **38**, purpurealidin E **13** was formed. The overall yield was 39%. Yoshida and Yamaguchi (2008) did not offer any analytical data from **13**, only from its acetylated derivative salt.

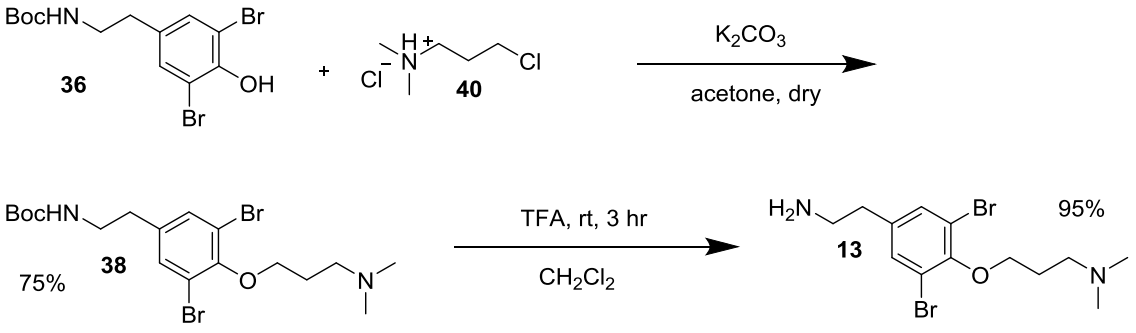
Four years later, a new synthesis route for **13** was reported (Scheme 4) (Kottakota *et al.* 2012). Kottakota's team (2012) used **1** as a starting material and the first two steps were made according to a recent publication (Scheme 3) (Kotoku *et al.* 2005). Kotoku's team

(2005) made the first two steps of the synthesis in different order than Yoshida and Yamaguchi (2008). First, **39** was synthesized by adding bromine and **1** in acidic conditions. In the second step a protective group (Boc) was added giving the product **36**. The overall yield of the first two steps was excellent (92%), especially if compared with the overall yield of the first two steps that Yoshida and Yamaguchi (2008) received (64%).




Scheme 3. Synthesis of **36** (Kotoku *et al.* 2005)

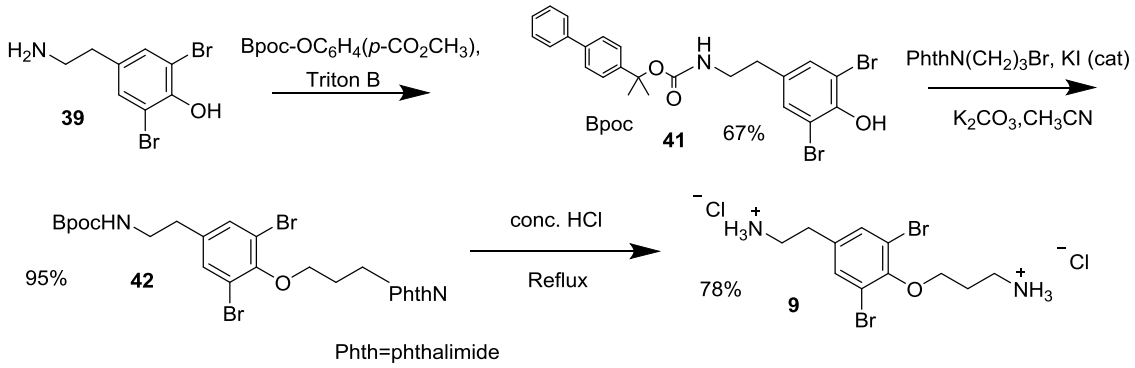
In the third step, 3-chloro-*N*,*N*-dimethylpropan-1-aminium chloride **40** was reacted with **36** (Scheme 4). The reaction conditions and use of **40** by Kottakota's team (2012) offered little better yield (75%) compared to the yield of third step that Yoshida and Yamaguchi (2008) received (63%). In the last step the protective group of **38** was cleaved in acidic conditions giving the product **13** excellent yield (95%).




Scheme 4. Synthesis of purpurealidin E **13** by Kottakota's team (2012)

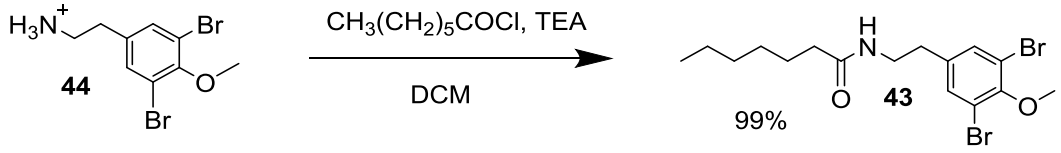
## 4.3 Synthesis of bromotyramines

Moloka'iamine **9** was synthesized in three steps as a reply to the growing interest against antifouling activity that bromotyramines had shown (Scheme 5) (Schoenfeld and Ganem 1998).




Scheme 5. Synthesis of moloka'iamine **9** (Schoenfeld and Ganem 1998)

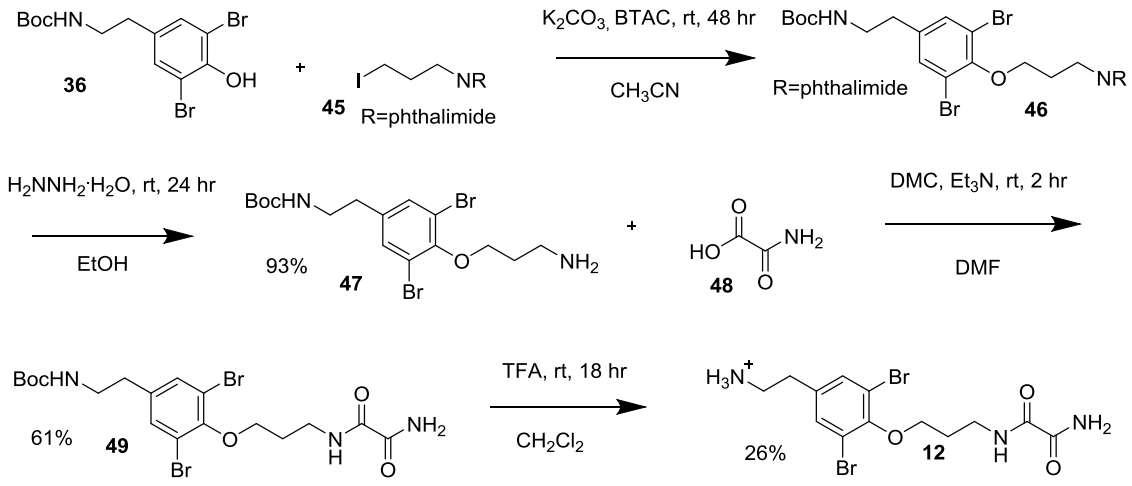
The tin-containing antifouling paints used to prevent the growth of marine species on ship hulls were banned in 1987 in many western countries due to their toxicity to marine mammals. In the first step the protective group 2-(biphenyl-4-yl)propan-2-yloxycarbonyl (Bpoc) was attached to the primary amine of **39** giving the product **41** (Scheme 5). In the next step, **42** was synthesized when the phthalimide protected propyl chain was attached to the phenol. Finally the protective groups were cleaved in acidic conditions. The total yield for the final product was 50%.




Scheme 6. Synthesis of bromotyramine derivate **43** with acyl chloride (Schoenfeld *et al.* 2002)

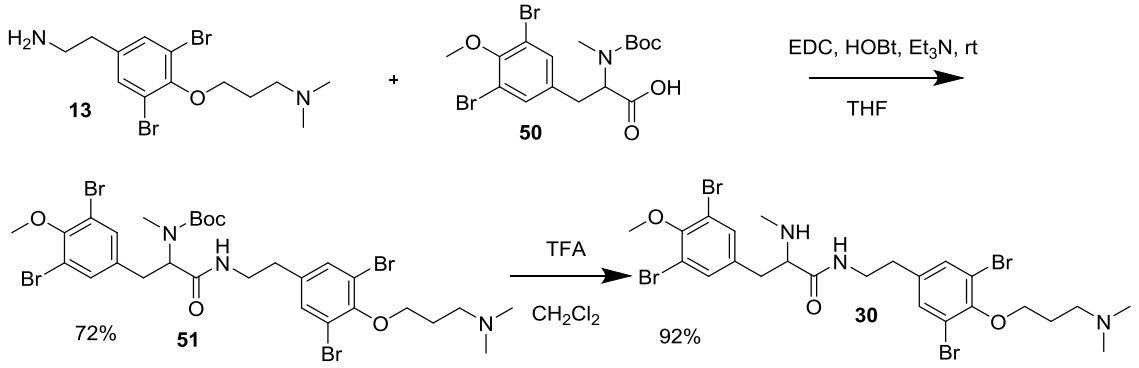
Acyl chlorides are well known to react with amines to form amide bonds. An example from this is a recent publication where a bromotyramine derivative with long alkyl

chain **43** was synthesized (Scheme 6) (Schoenfeld *et al.* 2002). A brominated tyramine with methylated hydroxyl group **44** was handeled with acyl chloride. The synthesis gave excellent yields (99%).



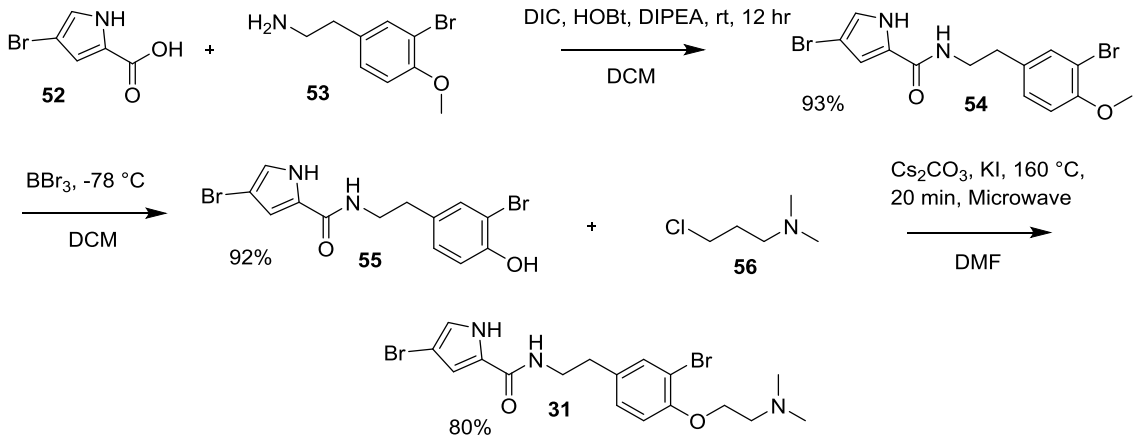

Scheme 7. Total synthesis of moloka'iakitamide **12** (Yoshida and Yamaguchi 2009)

Moloka'iakitamide **12** showed antifungal and parasymphatolytic activity reported by Badr's team (2008) and one year later the total synthesis was reported (Scheme 7) (Yoshida and Yamaguchi 2009). The used starting material was tyramine **1**. The first two steps were made as shown in Scheme 2 (p. 21). In the third step a phthalimide protected 3-iodopropan-1-amine **45** was attached to phenolic oxygen of **36** to give the product **46**. The protection of **45** amine was not necessary and there are examples in the literature where the reaction has been done without protective group (Scheme 2 and 4) (Yoshida and Yamaguchi 2008; Kottakota *et al.* 2012). In the next step phthalamite group was cleaved to give the product **47**. In order to make a amide bond, **47** was handeled with oxamic acid **48** in the precense of 2-chloro-1,3-dimethylimidazolinium chloride (DMC). DMC is a strong dehydrating agent that can be used in amidation reactions instead of dicyclohexylcarbodiimide (DCC) and *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC) (Isobe and Ishikawa 1999). Ultimately, TFA was used to cleave the Boc-group of **49** to give the product **12**. The overall yield was 26%.




Scheme 8. Synthesis of suberedamine B **30** by using purpurealidin E as starting material (Kottakota *et al.* 2012).

Aplyzanzine A **28** and suberedamines A **29** and B **30** were synthesized with a reasonable amidation reaction (Scheme 8) (Kottakota *et al.* 2012). For instance, **30** was synthesized by using **13** and carboxylic acid **50** as starting material. The used crosslinking agent for amidation reaction was EDC and hydroxybenzotriazole (HOBt) was used to activate the intermediate ester formed by the carboxylic acid. Since there was a protective group in **51,** the final step of the reaction was to cleave it with TFA and the product **30** was formed. The solvent used in this specific reaction was THF, but the yields were similar with the amidation reactions done in $CH_2Cl_2$. The synthesis of **30** was done with overall 72% yield.




Scheme 9. Synthesis of dispyrin **31** (Kennedy *et al.* 2008).

The synthesis of **31** offers another interesting route for synthesis of bromotyramine derivatives (Scheme 9) (Kennedy *et al.* 2008). Unlike Kottakota's team (2012), Kennedy's group (2008) first step of synthesizing **31** was the amidation reaction between carboxylic acid **52** and methylated bromotyramine derivative **53**. The used diisopropylcarbodiimide (DIC) is a crosslinking agent and HOBt was used to activate the intermediate ester. The amidation gave succesfully the product **54** with excellent yield (93%). In the second step the methoxy group was demethylated to hydroxyl with Lewis acid boron tribromide ($BBr_3$) to form **55**. The alkylation reaction was reported to be challenging from a reason that was not mentioned, but the use of *N*,*N*-dimethyl-3-chloropropylamine **56** finally gave the desired product **31**. The overall yield was good (68%).

# 5 THE AIM OF THE THESIS

The aim of this thesis was to synthesize new compounds by making amide bonds with the primary amine of purpurealidin E **13** and carboxylic acids. Carbodiimides, such as EDC, provide a versatile and well-known method to crosslink carboxylic acids and amines (Crosslinking Reagents Technical Handbook 2012). Amide bonds can also be synthesized with acyl chlorides. This master's thesis was continuum for the work done by other master's thesis student Paul Flemmich (Flemmich 2015). Flemmich synthesized successfully purpurealidin E **13**.

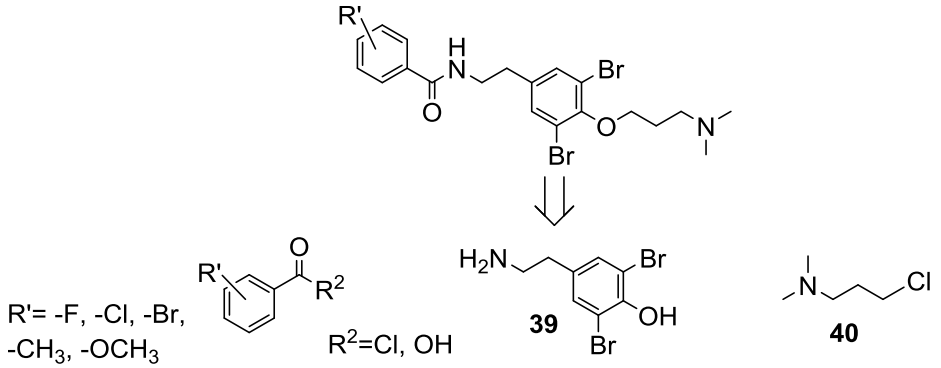
The crude extract of *Pseudoceratina purpurea* have shown potential activity against HCV (Lillsunde KE, unpublished observation 2014). HCV is a chronic virus disease that can ultimately cause cirrhosis and liver failure (Duodecim 2015). The treatment results of HCV have improved during the last years. Depending on the genotype of patient, HCV can be cured completely in some cases. However, the novel treatments do not accomplish to cure all the HCV infections. As a part of MAREX project, purpurealidin I **24** and J **23** were identified from the same crude extract that showed activity against HCV (Tilvi and D'Souza 2012). Thus, the aim was to design and synthesize novel compounds that were simplified analogs of **24** and **23** and test their

biological activity against HCV. Additionally, one goal of the thesis was to synthesize disulfide bromotyramine **57** that would mimic bromopsammaplin A **34**.

## 6 SYNTHESIS DESIGN

The synthesis design of new bromotyramine derivatives was divided into two sections. First, purpurealidin E **13** needed to be synthesized. After the synthesis of **13**, new bromotyramine derivatives were able to be synthesized through amidation reaction. In this chapter we will discuss the design of these routes.

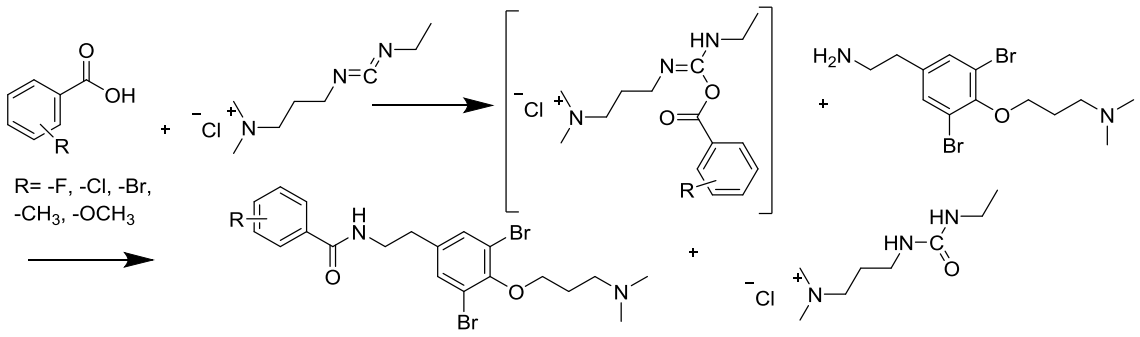
The synthesis of **13** was published recently (Scheme 2,3 and 4, p. 21-22) (Kottakota *et al.* 2012). The synthesis route published by Kottakota's team (2012) was repeated successfully in the University of Helsinki by master's thesis student Paul Flemmich (Flemmich 2015). This reaction route includes four simple steps and the yields of the reaction route have been moderate (Scheme 3 and 4) (Kottakota *et al.* 2012; Flemmich 2015). In the first step, bromine will undergo electrophilic aromatic substitution reaction replacing two hydrogens from the tyramine aromatic ring. The addition of protective group (Boc) to the primary amine is necessary in order to prevent substitution to the amine in the third step. After the alkylation, the protective group can be cleaved.




Scheme 10. Retrosynthetic route for bromotyramine derivatives

After purpurealidin E **13**, the amidation reactions to synthesize new bromotyramine derivatives were considered (Scheme 10). Amide bonds can be synthesized with multiple different reactions routes. For instance, Schotten–Baumann reaction is a well

known method to synthesize amides from acyl chlorides and primary amines (Scheme 6, p. 23). The advantage of using acyl chlorides to form amide bonds is that the reaction is volatile and it usually gives excellent yields (Schoenfelds *et al.* 2002). The other advantage is that acyl chlorides can be formed from carboxylic acids. For instance, carboxylic acids will undergo a nucleophilic acyl substitution reaction with oxalyl and thionyl chloride. After the nucleophilic acyl substitution reaction the acyl can be reacted with primary amine. The disadvantage of this reaction is that oxalyl and thionyl chlorides are vulnerable and they react easily with the moisture of air which leads to hydroxylation of the carbonyl carbon. Thus, often they need to be distilled before using.




Scheme 11. Reaction mechanism for bromotyramine derivatives using carbodiimide EDC to synthesize amide bonds

Amides can also be formed with carbodiimide crosslinking agents (Scheme 11). EDC is a carbodiimide that is widely used reagent that forms an O-acylisourea intermediate. It reacts with the amine giving the desired amine product and urea. Bromotyramine derivatives have been successfully synthesized with carboimiides (Scheme 8 and 9, p. 25) (Kennedy *et al.* 2008; Kottakota *et al.* 2012). Carbodiimides are more pleasant to handle than oxalyl and thionyl chloride. They are hygroscopic and vulnerable to moisture but less than oxalyl and thionyl chloride. The disadvantage is generally lower yields. The formed urea can be problematic to extract from the reaction mixture since purpurealidin E **13** has similar functionality (tertiary amine). The amidation reactions were decided to be made by using acyl chlorides or the by use of carboiimides as crosslinking agents.

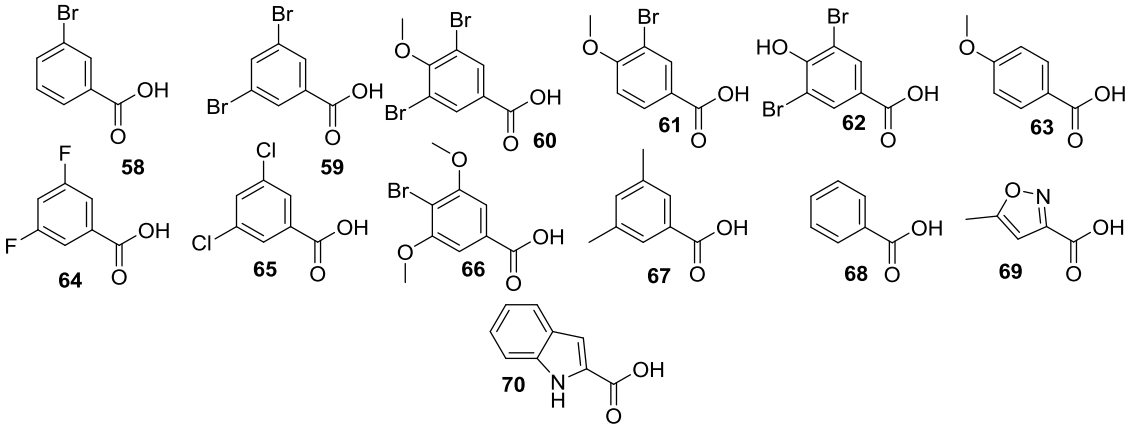
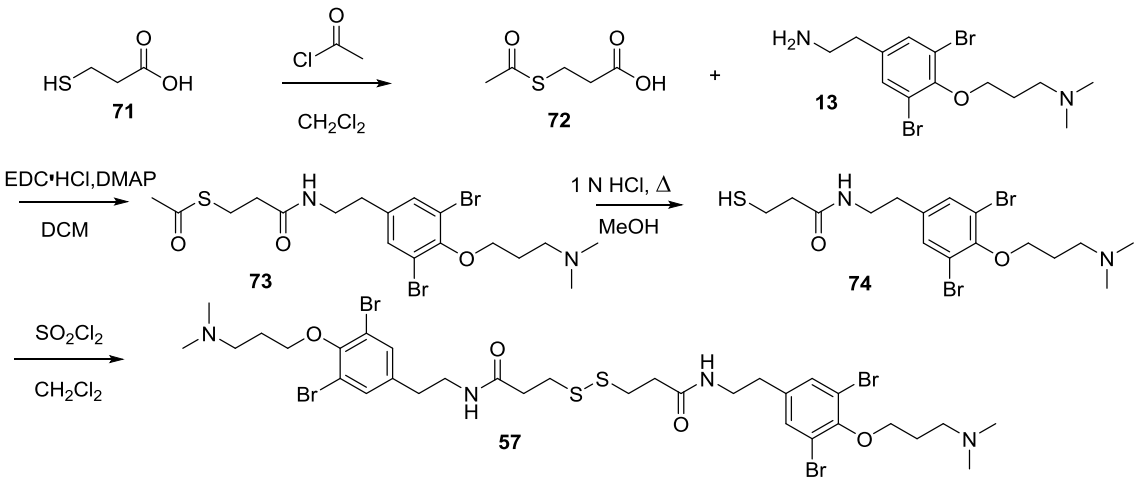



Figure 16. Carboxylic acids **58-70** that were chosen for the synthesis of bromotyramine derivatives

Both amide synthesis methods can be done with commercially available carboxylic acids. Since the aim of the thesis was to synthesize new bromotyramine derivates that were meant to mimic purpurealidin J **23**, it was reasonable to choose aromatic carboxylic acids. However, **23** is a relatively big and its isoxazole ring is attached to a non-aromatic cyclohexene with two bromine substituents and a methoxy group (Figure 10, p. 16). The molecular weight is over 700 g/mol which is not favorable in medicinal chemistry when considered the Lipinski's rule of 5. Additionally, the molecule is complex to synthesize since the heterocyclic isoxazole is attached direct to the cyclohexene. In the joint of two cyclic groups there is a chiralic carbon and thus **23** has an enantiomer. If the isoxazole ring is dropped out from the structure, the cyclic hexene is attached straight to the carbonyl carbon and the molecule is simpler. Thus, the chosen carboxylic acids were simple aromatic carboxylic acids **58-70** (Figure 16). These carboxylic acids are structurally close to each others, since the possible differences in biological activity (BA) would offer important information from the possible binding site and structure-activity relationship (SAR). The aromatic rings have different halogens (F, Cl, Br) since they have a large impact to the molecule weight. Halogens have also different steric properties due to the differences in the size of electron clouds. These properties may show alternate behavior in the BA evaluation.



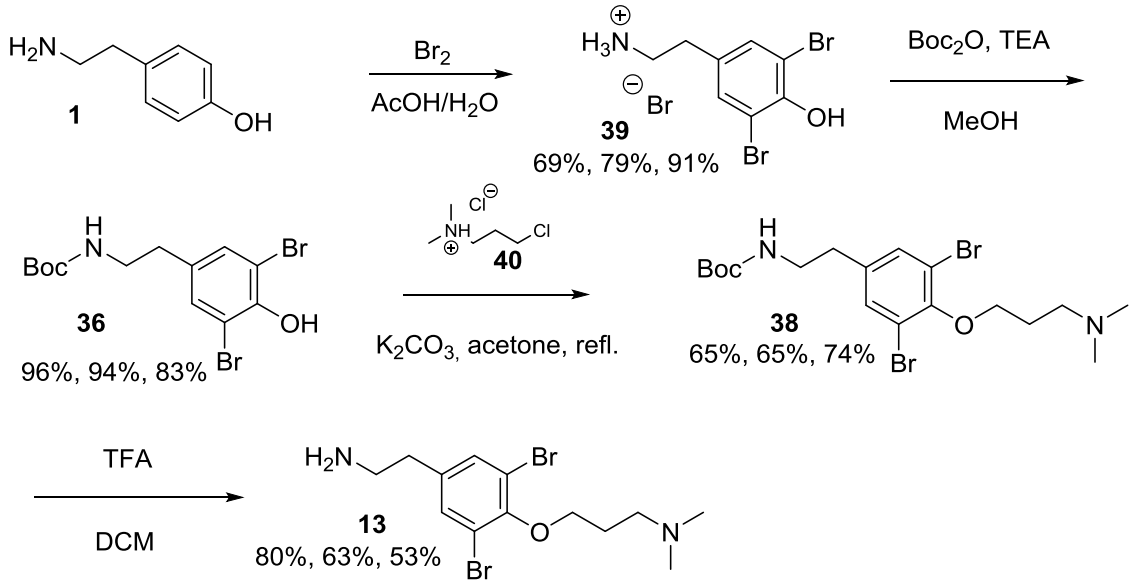

Scheme 12. Planned route for disulfide bromotyramine **57** derivative

Bromopsammaplin A **34** has shown interesting antimicrobial activity with lower MIC values than meropenem against some *Staphylococcus aureus* strains (Figure 15, p. 18) (Park *et al.* 2003). Thus, a synthetic route was designed for disulfide bromotyramine derivative 3,3'-disulfanediylbis[*N*-[3,5-dibromo-4-[3-dimethylamino)propoxy]-phenethyl]propanamide] **57** (Scheme 12). The synthesis was planned to start from the protection of 3-mercaptopropionic **71** thiol group. The thiol group of unprotected **71** could react with primary amine of **13** forming an unwanted thioether side product. Easy method to protect the thiol group is to let it react with acyl chloride giving 3-(acetylthio)propanoic acid **72** (Larsson and Ramström 2006). After protecting the thiol group, the amide bond can be formed between **13** and **72** by using carbodiimide giving the product *S*-[3-[[3,5-dibromo-4-[3-(dimethylamino)propoxy]phenethyl]amino]-3-oxopropyl] ethanethioate **73** (Kang *et al.* 2014). After synthesizing **73** the acetyl group could be removed with acid catalyzed reaction giving a bromotyramine with free thiol group *N*-[3,5-dibromo-4-[3-(dimethylamino)propoxy]phenethyl]-3-mercaptopropanamide **74** (Kang *et al.* 2014). Finally, the sulfur bridge can be synthesized with a method published recently giving the product **57** (Leino and Lönnqvist 2004)

# 7 RESULTS AND DISCUSSION

## 7.1 Purpurealidin E

### 7.1.1 Synthesis of purpurealidin E




Scheme 13. Synthesis of purpurealidin E **13**

Purpurealidin E **13** was successfully synthesized three times with the overall yields 35%, 30% and 29% (Scheme 13). The three separate times are indicated with alphabets **a**, **b** and **c**. The synthesis of **13** started from the bromination of tyramine. In the first two times the starting material was a HCl-salt of **1.** When the first step was made for the first time **39a** did not show satisfying bromination with the used ratio of bromine in the beginning. Even though the reaction was left overnight, TLC did not indicate dibromination. Thus, more bromine was added and the total amount of bromine in the end of reaction was 3.5 eq. The yield was still relatively low, only 69%. The second time this step was proceeded extra bromine was added again and the total amount of used bromine the reaction was 5.5 eq. The product **39b** was filtrated and the yield was poor (24%). The poor yield was mainly due to insufficient filtration, since there was a lot of product in the filtrate solvent that crystallized after couple days. The crystals were filtrated again giving a total yield 79% for **39b**. The third time **1** was brominated, the

used solvent was only AcOH. In the first two times there was water as co-solvent, but according to the literature this was not necessary (Kotoku *et al*. 2005; Wada *et al.* 2011). Also, the used starting material was a molecule form of tyramine **1**, not an HCl-salt. The reaction was left overnight. The next day TLC indicated that the reaction was completed even though the used amount of bromine was lower than in the second time (3.5 eq). The brominated tyramine **39c** was filtered with a sinter giving now a good yield (91%).

In the next step a protective Boc-group was added. In all the three times the reaction was left overnight and the TLC indicated full completion of reaction. The yields were excellent. However, there was color variations between the reactions done and products received. In the first time, the product **36a** was off-white solid (96%). In the second time, after TEA was added, the color of the reaction solvent turned black. After the extraction the received product **36b** was dark green (94%). In the third time, the received solid **36c** was mainly white but there were light brown areas amongst the white solid. The product **36c** was purified with flash chromatography. There was light brown polar impurity that stayed in the top of silica column. The product **36c** was now white homogenous solid and the yield stayed relatively good (83%). Thus, the purification with flash chromatography after the second step was a good method to remove the gummy impurity.

The third step was the addition of 3-chloro-*N*,*N*-dimethylpropan-1-aminium chloride **40**. The first time the reaction was made, after the work-up the received product **38a** was an oil. The $^{1}$H-NMR-spectrum showed impurities. Due to this, the product was recrystallized. During the reflux dark solid/gum appeared to the bottom of the flask. It was assumed to be impurity and the flask was changed leaving the impurity to the old flask. The product **38a** was white solid. The second time this step was performed, after adding all the reagents to the flask to color of the solvent was dark brown. In the extraction and washing procedure there was no separation between the layers. The product **38b** was first brown oil, but after keeping the product in oil pump for 11 hr the product was hard dark brown solid. Again, the product was recrystallized and the received product **38b** was light yellow. It seemed that there was impurity that was able

to be removed by recrystallization. The third time this step was made, the starting material **36c** had already been purified with flash chromatography. The reaction solvent was light yellow. The work-up including the washing procedure was performed easily and no difficulties in phase separation appeared. The product **38c** was off-white solid and the yield was 74%. The yields were moderate but they correlate, especially the third time, with the literature (Kottakota *et al.* 2012).

The removal of protective Boc-group was the final step of the synthesis. The first time this final step was performed a miscalculation was made and the amount of added TFA was 70 eq when the right amount of TFA would have been 6 eq. However, yellow oily product **13a** was received with yield 80%. The second time, this step was performed the correct amount of TFA (6 eq) was added. However, $^{1}$H-NMR-spectrum and TLC from the product indicated that the reaction wasn't completed and there was still starting material left. The amount of used TFA was same and the reaction time was longer compared to literature but from some reason the reaction was not finished (Kottakota *et al.* 2012). Due to this the reaction was made again. TFA was added in two batches and the total amount was 40 eq. Now the yellow oily product **13b** was received with yield 63%. The third time this final step was performed the added amount of TFA was straight 40 eq. The reaction was left overnight and washed with high amount of NaOH. The product **13c** was again yellow oil and the yield was 53%. The yield was low for **13c** probably due to insufficient work-up.

In the first time purpurealidin E was synthesized, the total amount of used TFA was highly excessive (70 eq). Fortunately, it did not damage the final product. Theoretically, there was a possibility for ether cleavage through $S_N2$ and nucleophilic substitution of $H_2O$ but since the reaction was made with anhydrous solvent under argon this did not occur. However, it is unclear why wasn't the amount of TFA (6 eq) used in the second time enough to cleave the Boc-group since in the literature, 1.2 eq was enough to detach the protective group with excellent yield (Kottakota *et al*. 2012). One possibility could be that the used TFA was not currant. The big amounts of used TFA caused problems in the extraction of the product. Even though careful washing procedure a small amount of TFA was probably left amongst the product in **13c** since there was an extra peak seen

on $^{13}$C-NMR-spectrum. The final step and the removal of protective group should be repeated in heated conditions and the used amount of TFA should be lower. This would reduce the problems in extraction caused by to the acidity.

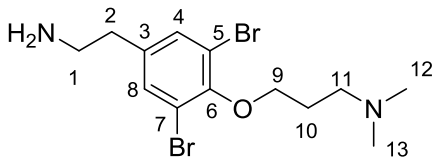
### 7.1.2 Characterisation of purpurealidin E

The $^{1}$H-NMR-spectra of synthesized **13a-c** were all similar. However, the FTIR-spectrum showed difference in the values 3200-3500. This area is characteristic for amines. A free amine should show broad peak with value 3500 when the protonated amine shows broad peak in the value 3030. The free amine peak of **13a** seen in FTIR was obvious. However, there was no proper free amine peak seen for **13b** in the FTIR-spctrum and there was a reason to doubt that the amine of **13b** was protonated.

Thus, a cation exchange was made with ISOLUTE SCX-2-ion exchange column. $^{1}$H-NMR or the FTIR did not show any changes for **13b** after the cation exchange suggesting that the product **13b** was not a salt. To confirm this information, the "cation exchanged" purpurealidin E was dissolved in 4 M HCl dioxane and the **13b HCl-salt** was filtered. The result is compared in the table (Table 2). Kottakota's team (2012) had noticed before that the NMR-data of Tilvi's team (2004) published earlier was most likely from salt form of purpurealidin E **13**.

There is no clear correlation with either of the articles published (Table 2). One possibility that could explain the different values is that **13b HCl-salt** was protonated from both primary and tertiary amine. The fact that hydrogens attached to the carbon 1 and 11 have both high ppm values supports this idea. Also, the ppm values of -N($CH_3$)$_2$ are relatively similar with the results reported by Tilvi's team (2004). Surprisingly, the FTIR-spectrum for **13b HCl-salt** shows a wide peak with value 3370. This argues with the data known that the protonated salt should be seen with value 3000. Also, since the peak of the **13b HCl-salt** was more similar with **13a** than with the molecule form **13b**, it can be thought that it was more likely that **13a** was a salt. However, since the NMR-data was so similar in all the three purpurealidin E **13a-c** synthesized, this is not likely.

Table 2. NMR-data of **13b HCl-salt** compared with the literature



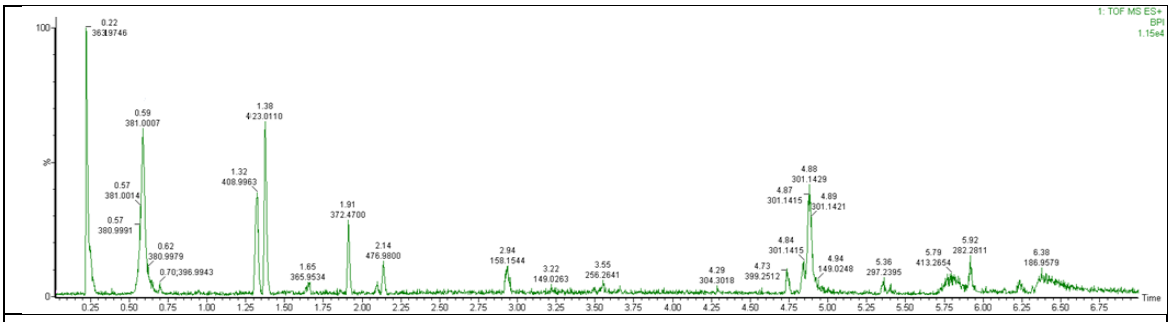

| | H-NMR δ ($CD_3OD$) | | | C-NMR δ ($CD_3OD$) | | |
|---|---|---|---|---|---|---|
| Position | **13b HCl-salt** | Tilvi *et al.* 2004 | Kottakota *et al.* 2012 | **13b HCl-salt** | Tilvi *et al.* 2004 | Kottakota *et al.* 2012 |
| 1 | 3.52 (m) | 2.73 (t, 13.2 Hz, 6.6 Hz) | 2.86 (t, 7.3 Hz) | 41.40 | 40.0 | 44.0 |
| 2 | 2.93 (m) | 3.24 (t) | 2.70 (t, 7.3 Hz) | 33.11 | 33.6 | 38.8 |
| 3 | | | | 137.73 | 130.3 | 140.5 |
| 4 | 7.58 (s) | 7.43 (s) | 7.43 (s) | 134.46 | 133.0 | 134.3 |
| 5 | | | | 119.31 | 117.3 | 119.2 |
| 6 | | | | 153.02 | 150.7 | 152.9 |
| 7 | | | | 119.31 | 117.3 | 119.2 |
| 8 | 7.58 (s) | 7.43 (s) | 7.43 (s) | 134.46 | 133.0 | 134.3 |
| 9 | 4.14 (t, 5.6 Hz) | 4.05 (t, 5.6 Hz) | 4.03 (t, 6.2 Hz) | 71.14 | 69.8 | 72.8 |
| 10 | 2.31 (m) | 2.23 (m) | 2.07 (m) | 26.41 | 25.0 | 29.1 |
| 11 | 3.18 (m) | 2.44 (t, 5.6 Hz) | 2.64 (t, 5.7 Hz) | 57.00 | 55.8 | 57.6 |
| 12 | 2.97 (s) | 2.90 (s) | 2.28 (s) | 43.70 | 42.7 | 45.9 |
| 13 | 2.97 (s) | 2.90 (s) | 2.28 (s) | 43.70 | 42.7 | 45.9 |

After the first synthesis of **13a** a small amount was purified with flash chromatography to receive a MS-spectrum. Purpurealidin E **13** has earlier shown interesting pseudo molecular ion peaks and this case was no different (Table 3) (Tilvi *et al.* 2004). The MS-spectrum (SIM/ESI) showed two peaks with different retention times, 0.25 and 0.50 for **13a**. The ratio of the peaks area is almost 1:1. Calculated mass value of **13** is 379.0021 and the two peaks had values 379.0024 and 379.0003. The peaks have almost identical MS-spectra with similar isotope pattern. Theoretically, it is not possible to have 0.0021 difference in the mass. However, there was a clear separation in the HPLC-retention times suggesting that there might two different compounds. One explanation for the different retention times could be that a part of the product **13a** was still a salt. However, this is idea is not supported by the FTIR-spectrum where there was a wide peak with value 3393 nor by the $^1$H-NMR-spectrum, since they both suggest that **13a** wasn't a salt. Other possibility is that the formic acid used as co-solvent in the HPLC

formed a salt with **13a**. The counter ion could have been detached and the measured mass ended up being almost identical with two different retention times.

To study the pseudo molecular peaks more, MS-spectra were taken from **13b**, "cation exchanged" **13b** and **13b HCl-salt** (Table 3). However, the spectra were now even more complicated. In all three spectra there were now even more pseudo molecular peaks. The difference 42 in mass value might be due to possible acetonitrile adduct since it was used as eluent.

Table 3. The TOF MS/ESI pattern that represents the result from **13b**, "cation exchanged" **13b** and **13b HCl-salt**. The difference between calculated value and received value with the possible peak explanation

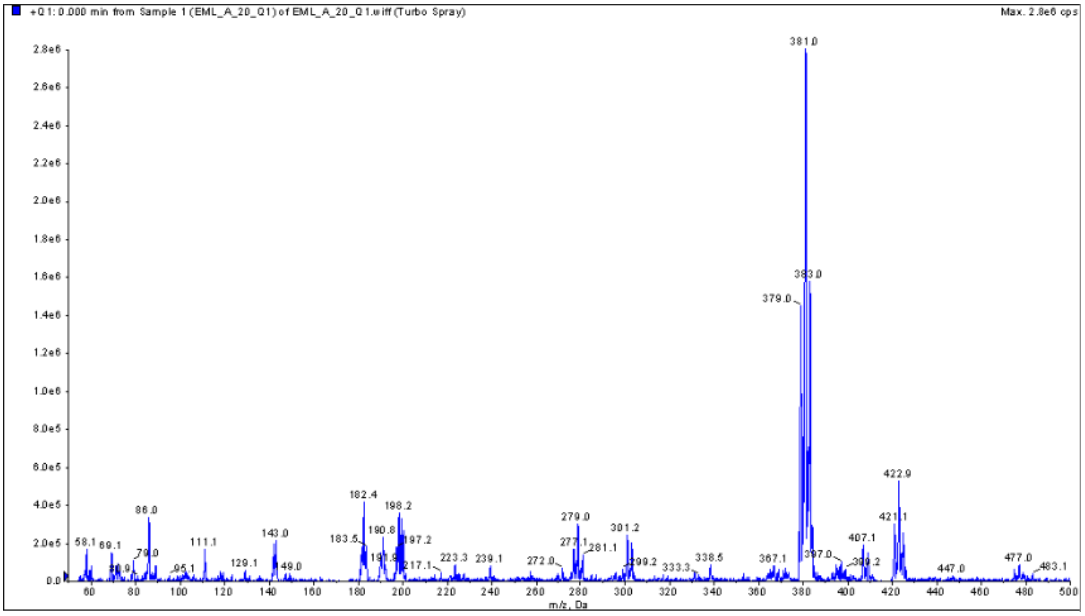



Calculated value for **13** is 379.0021 (Due to the isotope pattern for bromine the values in the picture are +2). The peaks on the right side do not origin from the compound.

| Pseudo molecular peak | purpurealidin E peak | Pseudo molecular peak - purpurelidin E | Possible peak |
|---|---|---|---|
| 476 | 381 | 95 | Unknown |
| 372 | 381 | -9 | Unknown |
| 423 | 381 | 42 | Acetonitrile adduct? |
| 408 | 381 | 27 | Unknown |

Due to the possibility that **13** makes adducts with the used HPLC co-solvents, a direct mass without HPLC was taken (Picture 17). The major mass peak was now 379 but the peak 421 with difference 42 could still be seen. This argues with the claim that 421 is an acetonitrile adduct. However, MS-pattern was now clearer and most of the pseudo molecular peaks were not seen. This suggests that the "impurity" peaks are not actually impurities. More likely, the peaks are due to breakdown of **13** and reformation of the breakdown pieces. This can be also explained by two observations. First of all, there

would have been more impurities seen in the NMR-results if all the peaks seen in MS-spectra are due to impurities. Secondly, since the yields of crude products after the crosslinking reactions were up to 85%, the starting materials **13a** and **13b** had to be quite pure.

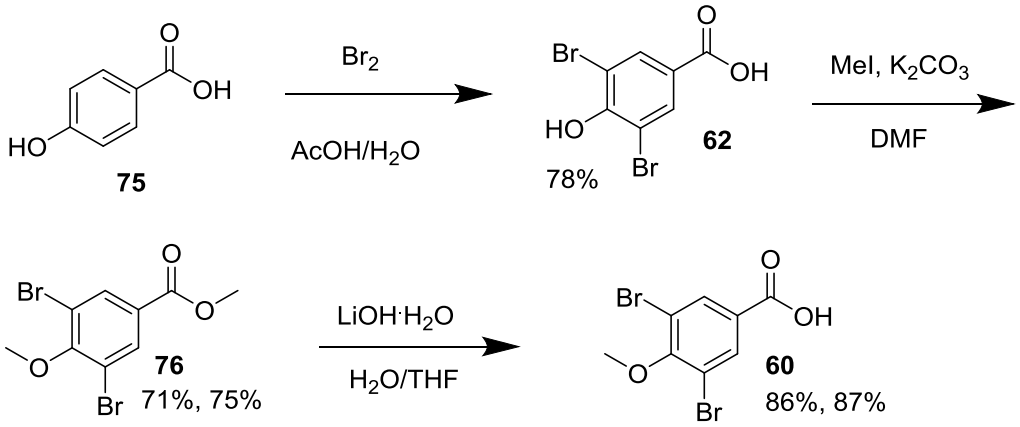



Picture 1. The MS-spectrum of **13 HCl-salt** without chromatography.

Tilvi's team (2004) reported that the isolated purpurealidin E **13** from *Psammaplysilla purpurea* was colorless oil. This oil was noticed to be most likely a salt later on (Kottakota *et al.* 2012). The synthesized purpurealidin E **13** was an orange gum reported by Kottakota's team (2012) similar than in this work. Similar compounds moloka'iamine **9** and moloka'iakitamide **12** reported by Hamann and Scheuer (1993) and Badr's team (2008) were white solids. Thus, the color and composition of pure purpurealidin E **13** is still unclear. Purpurealidin E **13** does not have any extended conjugations that could explain the orange color. Since the isolated salt of **13** by Tilvi's team (2004) was colorless oil, it is possible that the orange color is due to the synthesis procedure. It would be useful to purify **13** more or try alternate reaction routes and reagents in order to find out the composition of pure **13**.

## 7.2 Carboxylic acids

To synthesize bromotyramine derivatives it was necessary to synthesize some of the carboxylic acids that were not commercially available. The total synthesis of 3,5-dibromo-4-methoxybenzoic acid **60** using 4-hydroxybenzoic acid **75** as starting material was succeeded in total three steps (Scheme 14).

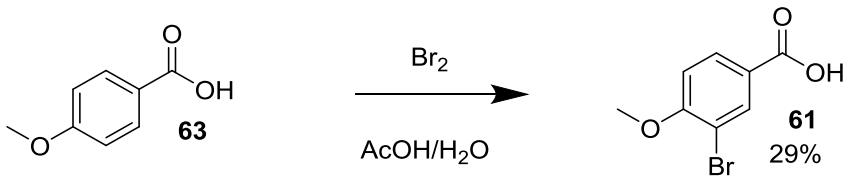



Scheme 14. Synthesis of 3,5-dibromo-4-Methoxybenzoic acid **60**

In the first step **75** was brominated. The mechanism and the reaction conditions were same than when **39a** was synthesized (Scheme 13, p. 31). The product **62** was white solid and the yield was 78%. The last two steps were made twice. In the second step of the reaction methyliodide was added and the used solvent was DMF. Methyliodide is an easy target for nucleophilic attacks and it is used in substitution reactions since iodide is cleaved easily from the methyl group. Thus, both of the hydroxyl groups were methylated giving the products **76a** and **76b**. The yields were 71% and 75% and the product was white powder in both times. In the last step of the reaction lithium hydroxide was used to cleave the methoxy group and white solid carboxylic acids **60a** and **60b** were formed. The overall yields of the reaction were 47% and 51%.

Since 4-methoxybenzoic acid **63** is commercially available, it was tried to be brominated and the synthesis of **60** would have been done in one step (Scheme 15). This reaction was not found from the literature. The reaction conditions were similar than in the synthesis step of **62** (Scheme 14). Excessive amount of bromine was added to the

reaction solvent. The product **61** was surprisingly a monobromine with low yield 29% (Scheme 15).




Scheme 15. The monobromation of **63** giving the product **61**

The synthesis of monobromine **61** was surprising when the chemistry of aromatic hydroxyl, carboxyl acid and methoxy is considered. Aromatic carboxyl acid is moderately deactivating substituent since it withdraws the electrons from the aromatic ring and it directs substituents to *meta* position. Vice versa, hydroxyl and methoxy are strongly activating substituents that donate electrons to the aromatic ring and thus they are *ortho-para* directing. Halogens are weakly deactivating substituents that direct electrophiles to *ortho-para* positions. When the first bromine is attached to aromatic ring, it prevents the substitution of second bromine to the aromatic ring. The directive effect of aromatic hydroxyl and carboxylic acid overrules the monobromines preventive effect and the ring is dibrominated. From some reason this was not the case with **63** and the attached bromine overrules the ring activating effect of methoxy and the ring deactivating effect of carboxylic acid.

## 7.3 Bromotyramine derivates

The amidation reactions between purpurealidin E **13** and carboxylic acids **58**-**70** were started by the synthesis of bromotyramine derivatives **77** and **78** (Figure 17). In **77** the used carboxylic acid was **62** and to synthesize **78** the used acid was **64**. The used reagents were EDC as cross-linking agent, DIPEA and the solvent was DMF.

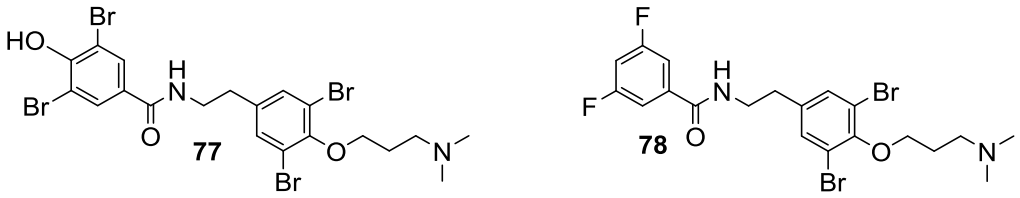
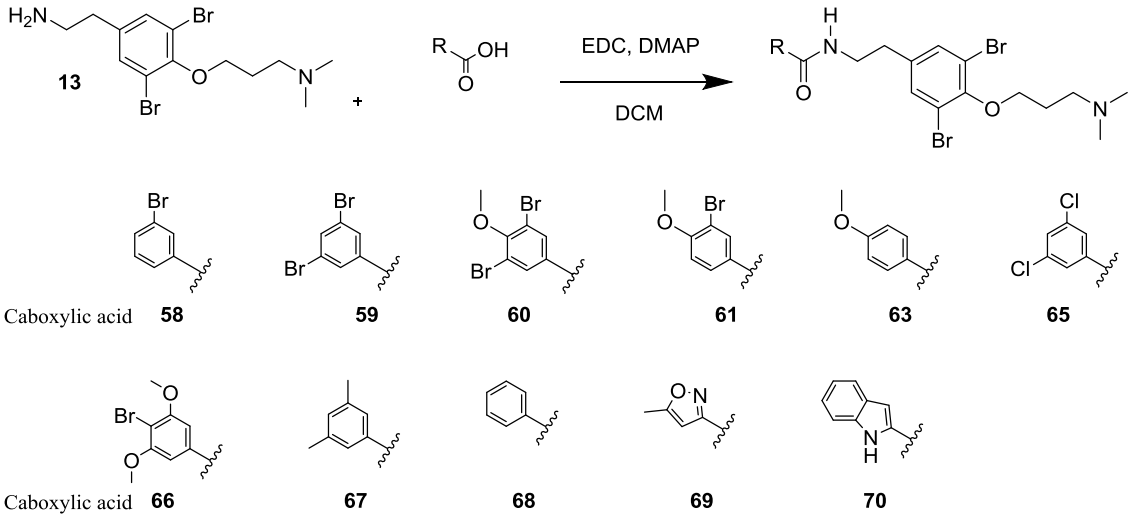



Figure 17. Bromotyramine derivatives **77** and **78**

Even though both of the reactions were completed according to TLC, there were no products received. In the case of **77** the product couldn't be extracted. The problems to extract **77** with organic and water phase was due to the zwitterion functionality seen for tertiary amine and phenolic hydroxyl group. The pKA values of protonated tertiary amine and phenolic hydroxyl groups are approximately 10. This means, that it is not possible to have a pH where majority of molecule is free of charge. Thus, **77** was most likely trapped to the water phase when the product was extracted. In the case of **78** the product was lost when it was tried to be purified with flash chromatography.




Scheme 16. Synthesis of bromotyramine derivatives (general procedure 1) and the used carboxylic acids

After the failed synthesis of **77** and **78** the crosslinking amidation reaction was optimized and general procedure 1 was made (Scheme 16). The details of the general procedure are told in the experimental chapter (p. 61). Kottakota's team (2012) published recently a reasonable amidation procedure with crosslinking agent EDC

(Scheme 8, p. 25). Even though DMF was most probably a suitable solvent for amidation reaction of **77** and **78**, DCM was used as the reaction solvent due to the lower boiling point and easier evaporation. The weak nucleophilic base DIPEA was changed to similar base DMAP in the general procedure 1 due to a recently published article that showed reasonable yields (Morkunas *et al.* 2012).

The synthesis of bromotyramine derivatives using the chosen carboxylic acids **58-70** and purpurealidin E **13** as starting material was succeeded with the general procedure 1 (Scheme 8). Total amount of 11 bromotyramine derivatives were synthesized and their compositions were identified by $^{1}$H-NMR (Figure 18).

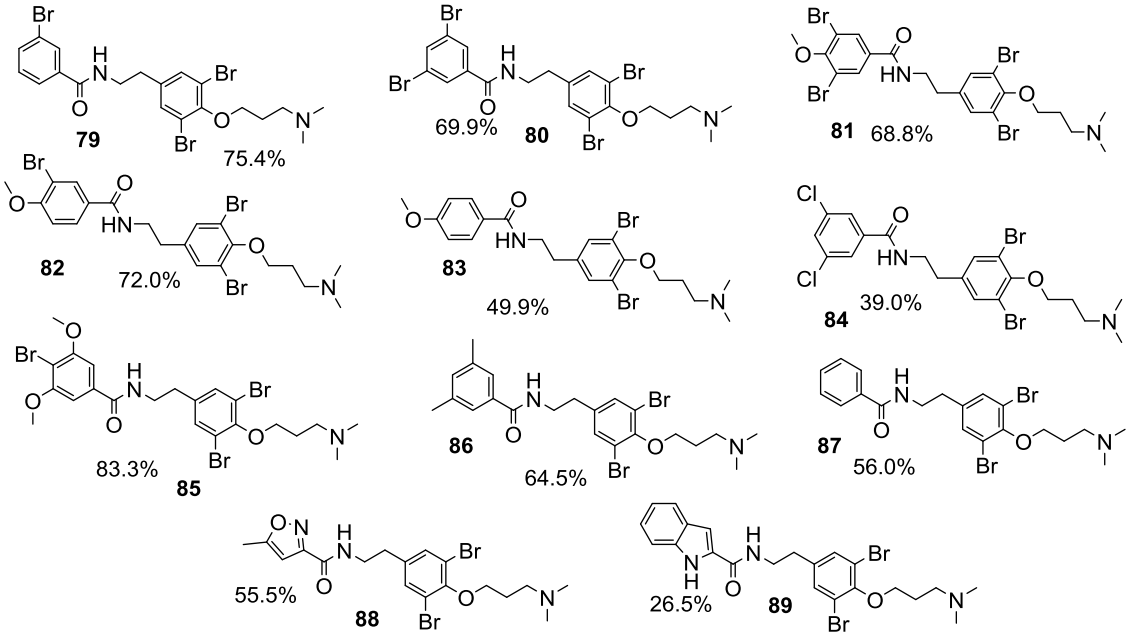
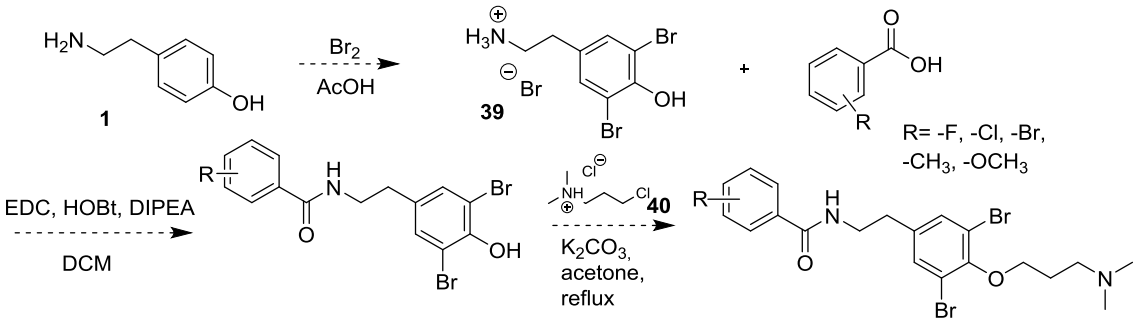



Figure 18. 11 new bromotyramine derivatives **79-89**. The yields are from the crude products

Based on the yields, reaction optimization and different ratios of reagents were tried during the work. However, there were quite large variations between the yields of crude products (Figure 18). The yields remained also relatively low compared to the literature (Kennedy *et al.* 2008; Kottakota *et al.*2012). However, the yields are only estimations since the amount of impurity may vary and it is not known. It would be useful to continue the optimization of general procedure 1. For instance, in this work DMAP was

used as poor nucleophilic base even though the role of DMAP is often to catalyze the amidation reaction with small equivalence (Neises and Steglich 1978). The use of base is essential since only deprotonated acids can attack as a nucleophile to the carbodiimide carbon. However, the reaction conditions should not be too basic, since EDC is noticed to work best in mildly acidic conditions (Crosslinking Reagents Technical Handbook 2012). The amount of used DMAP varied from 1.3 eq to 1.5 eq. Instead of using DMAP as the "main base", it would be useful to try to use DIPEA as poor nucleophilic base and add only small amount of DMAP to catalyze the reaction.

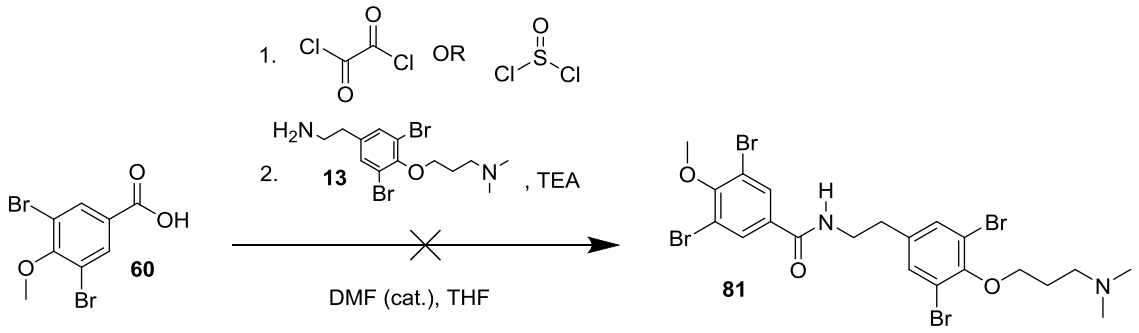
In the synthesis of **79**, the amidation reaction between **58** and **13** was done by using different crosslinking agent HBTU. Otherwise the reaction conditions were similar than in general procedure 1 (Scheme 16). The yield of the crude product was similar than with the reactions done with EDC, 75%. There were no advantages noticed in the use of HBTU instead of EDC. Thus, the use of EDC as crosslinking agent was continued in latter amidation reactions. However, in the literature there are other crosslinking agents than EDC and HBTU that haven given excellent yields of amidation reactions (Kennedy *et al.* 2008). Thus, it would be useful to test alternate crosslinking agents. Additionally, small amounts of HOBt could be added to the reaction to improve the yields.




Scheme 17. Possible synthesis route for bromotyramine derivatives

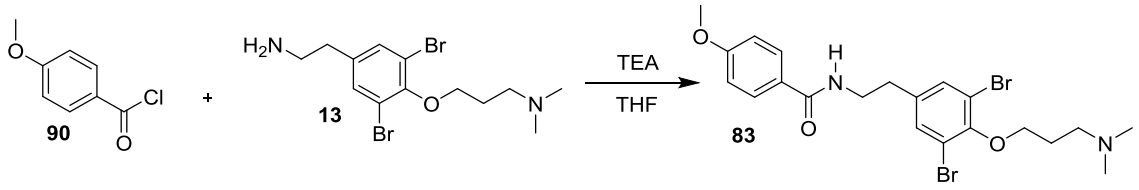
The reaction route and order could be changed (Scheme 17). The first step of synthesis of **31** done by Kennedy's team (2008) was the amide formation (Scheme 9, p. 25). The reaction gave excellent yields considering that it was a crosslinking reaction. There are many advantages in this reaction route. For instance, as a first step a large amount of **1**

could be brominated (Sheme 17). In the second step brominated tyramine **39** could be reacted with the desired carboxylic acid and the amide bond could be formed with large scale (Mahindroo *et al*. 2009). Thus, a bromotyramine derivative could be formed only in two steps. After this, in the third step the aminopropyl chain could be attached by using **40** and the desired bromotyramine derivative could be formed. The amount of the reaction steps could be reduced in this way. Additionally, the amidation reaction could be done with larger scale and it could be optimized since the starting materials would be faster to make.




Scheme 18. The designed synthesis route for acyl chlorides did not work

The other designed route to synthesize amides was to use acyl chlorides (Scheme 18). Thus, bromotyramine derivate **81** was tried to be synthesized by using **60** and **13** as starting materials. The carboxylic acid **60** was first acetylated with oxalyl or thionyl chloride. A small amount of DMF was added to catalyze the reaction. After a while, **60** was assumed to be converted to acyl chloride. Purpurealidin E **13** was added with TEA and the reaction was left over the weekend. The synthesis of **81** by using oxalyl or thionyl chloride surprisingly failed. It is possible, that oxalyl and thionyl chloride had been in touch with moisture. The synthesis of **83** was done successfully by using **13** and commercially available 4-methoxybenzoyl chloride **90** as starting material (Scheme 19). The reaction conditions were similar. THF was used as solvent and TEA was added as a reagent. The product was able to be synthesized and the yield of the crude product was 50%. Unfortunately, the yield after purification of **83** was only 6%. However, successfull reaction of **83** suggests that the reason for failed reactions with oxalyl and thionyl chloride were mostly due to bad reagents.




Scheme 19. Synthesis of **83** by using **90** and **13** as starting material

Three of the 11 novel bromotyramines were able to be purified (Figure 19). Two of the molecules, **84** and **81** were successfully recrystallized. One of the compounds, **82** was successfully crystallized as HCl-salt, **82 HCl-salt**. Considering that the recrystallizations were done with small amount of product, less than 100 mg, the yields were satisfying. The biological activity evaluation was made for all the three compounds with a **13b HCl-salt**. The results are discussed in chapter 7.6 (p. 48).

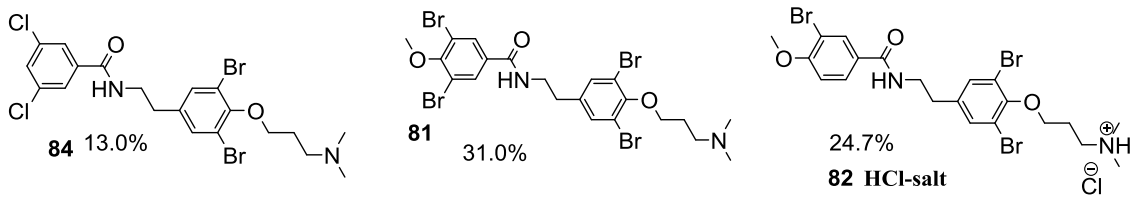



Figure 19. Novel bromotyramines **84**, **81** and **82 HCl-salt** with overall yields

## 7.4 Purification of bromotyramines

Purification of the novel bromotyramines was one of the challenges faced in the work. When **78** was tried to be purified with automated flash chromatography, the product was lost in the column. Thus, the flash chromatography was considered to unsuitable purification method and an effort was made to find and optimize a suitable recrystallization solvent and procedure. One of the first recrystallizations that were made was successful and the product **80** was filtered as white solid. Even though the MS-spectrum of **80** did not suggest that the compound was pure, recrystallization was considered to be a suitable purification method.

All the crude products were orange gummy oils, which made the recrystallization challenging. The hypothesis was that the orange gum was polar impurity that was

implemented to **13** maybe as early as in the second or first step of its synthesis (Scheme 13, p. 31). This impurity seemed to bind efficiently the product. This was problematic, since the recrystallization solvent needed to be enough polar to dissolve the orange gum. If the gum did not dissolve, the amount of the product was too small in the solvent to be crystallized. On the other hand, if the used solvent was too polar and all the orange gum dissolved, the product never crystallized. A large number of solvents and solvent mixtures were tested including: hexane, 2-propanol, methanol, acetone, hexane/methanol, hexane/DCM, hexane/toluene and hexane/2-propanol. In the mixtures a small amount of hexane was added to the crude product and the heated to reflux. Hexane and drops of more polar co-solvent were added till the most of the gum was dissolved. This method led to a successful recrystallization two times giving the pure products **84** and **81**. The used solvents were hexane with drops of methanol or DCM. These used solvents failed in many other recrystallizations that was tried to be made. This provokes an interesting question: Why did the recrystallization happen for **84** and **81** but not for the other pure compounds? One explanation could be the molecule weight. **84** and **81** are both dihalogenated from the "non-tyramine" aromatic ring. High molecule weights tend to increase the melting point and thus the solid could be more easily achieved.

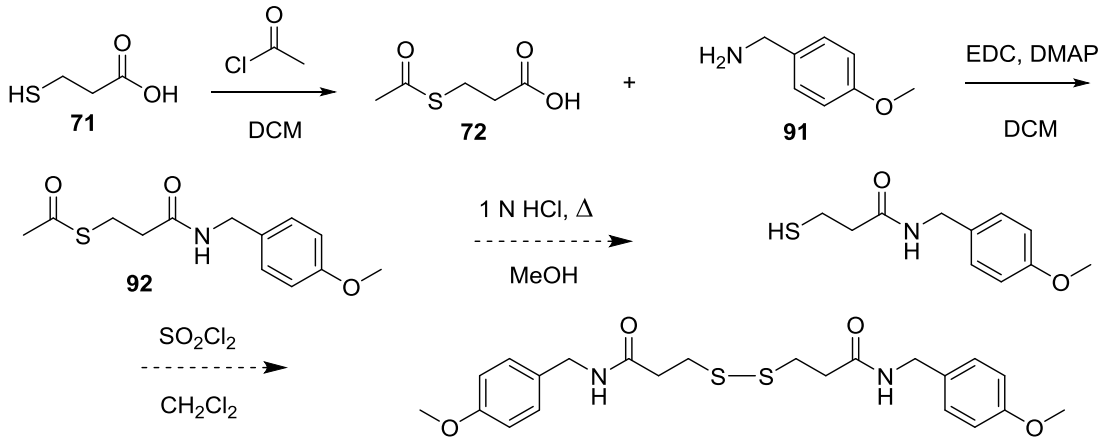
Due to the complicate recrystallization, **87** and **86** were tried to be purified with manual flash chromatography regardless of the challenges faced when **78** was tried to be purified. If the hypothesis from the impurity was right, the polar orange gum should have stayed in the stationary phase. Indeed, when **87** and **86** were implement to the silica column an orange layer was able to be seen to stay in the top part of silica. However, there were still some challenges faced by use of flash chromatography. The problem was that bromotyramine derivates tended to move slowly in the silica column. Stronger eluents were used to receive the product out from the stationary phase but this probably made the impurity to stick with the product. The use of TEA in the eluents reduced the tailing of the products but still no pure compound was received from the flash chromatography. Thus, more optimization should be done in order to find the proper flash chromatography method.

The salt formation as purification method was tried after recrystallization and flash chromatography. It is well known that salts have higher melting point and worse solubility to organic solvents. Thus, it was hoped that the bromotyramine derivative salt form would have been solid and able to be filtered. The compound **82** was purified by salt formation and the product **82 HCl-salt** was a white solid. After the successfull salt formation of **82 HCl-salt**, same procedure was tried for bromotyramine derivatives **80**, **87** and **85**. The compounds **80, 85** and **87** were handeled with 4 *M* HCl in 1,4-dioxane. Since the **85 HCl-salt** was not able to be filtered it was handeled with oxalic acid in order to make oxalate salt. The **85 oxalate-salt** was not soluble for the used NMR-solvents and thus the structure or the purity were not confirmed. The filration of **87 HCl-salt** failed and the filtration of **80 HCl-salt** wasn't done due to lack of time. However, salt formation might be a suitable method for the purification of novel bromotyramine derivatives if a suitable procedure is found.

The composition of the novel bromotyramine derivatives at ambient room temperature needs to be discussed in order to understand and overcome the challenges in purification of these compounds. It is possible that the synthesized bromotyramines can't be crystallized in room temperature since their composition might be oily instead of being a crystal. This could be due to the freely spinning alkyls and thus insufficient intermolecular forces to form a solid. This is supported by the observations made couple times that there were a solid in the flask after recrystallization when the product was in a freezer. When the product was tried to be filtered in room temperature it melted and went through the sinter. It is possible that in lower temperatures the intermolecular forces of bromotyramine derivates are strong enough to from a solid.

## 7.5 Disulfide bromotyramine

The synthesis of disulfide bromotyramine **57** started from the protection of **71** (Scheme 20). The reaction was done in large scale to have enough protected thiol **72** for the formation of disulfide. The mechanism of the reaction was same than seen above with 4-methoxybenzylchloride **90**, $S_N2$ reaction where the nucleophilic free electron pair of sulfur attacks to the acetyl chloride (Scheme 19, p. 44). The used solvent was DCM.




Scheme 20. The first two successful steps of synthesis of disulfide. The reaction route was tested using **91** as a model compound.

The reaction was succeeded and the yield of purified product **72** was 63%. Even though the reaction succeeded and the compound **72** was able to be purified with flash chromatography, the choice of protective group was not optimal. This was due to the similar boiling points for 3-mercaptopropionic acid **71** (111 °C), AcOH (118 °C) and 3-(acetylthio)propanoic acid **72** (127 °C). The AcOH was not able to be evaporated in rotavapor since the boiling point with the product **72** was so similar. Due to similar properties there wasn't a great difference seen in the TLC $R_f$ values either. However, the product was able to be purified with large volume manual flash chromatography. Hindsight, it would have been more convenient to protect the thiol with larger substituent that would have increased the boiling point. The next step was decided to be tested with 4-methoxybenzylamine **91** (Scheme 20). This would show if the designed route worked without using purpurealidin E **13**. A different crosslinking agent CDI was used with DIPEA as a base and the solvent was DCM. The idea was to test different reagents and their function in the amidation reaction. Since the NMR-data did not show assumed product **92a** the reaction was repeated with different solvent, THF. Again, the NMR-spectrum did not show assumed product **92b**. Thus, it was assumed that CDI and DIPEA were not suitable reagents for the reaction. The reaction was made third time with EDC and DMAP. Again, the NMR-spectrum of **92c** did not suggest that the reaction was completed, since the integrals of the spectrum was not logic. Now, the possibility of mixture of **91** starting material and the product **92** was considered. The compound was purified with automated flash chromatography with yield 28% and

NMR-spectrum showed the assumed product **92c**. Since the amidation was managed to be done with **91**, the amidation between **13** and **72** was done in similar reaction conditions than **92c** (general procedure 1). The $^{1}$H-NMR identified crude product of **73** was orange oil and the yield of the reaction was 29%. Unfortunately, there was no time to finish the designed reaction route of **92c** or **73**.

## 7.6 Biological activity

The compounds **84** and **81** activity against potassium channel (EAG) were tested as part of MAREX project. The activity test was made due to the request of Leuven University. Potassium EAG channels play important role in actio potential and neuronal excitability. The activity of clathrodin analogs against sodium channels was recently studied in master's thesis work done in University of Helsinki (Leino 2013). The compounds did not show any activity with the tested concentrations up 5 µM.

The activity test made against HCV replicons with compounds **84**, **81** and **82 HCl-salt** showed potential antiviral activity (Figure 20) (Table 4). The salt from of purpurealidin E **13b HCl-salt** did not show significant activity against HCV. The test was made with two different concentrations 25µM and 50 µM. Both of the used concentrations of bromotyramine derivatives **84**, **81** and **82 HCl-salt** showed potential activity against HCV replicon. Additionally, neither of the used concentrations showed HCV activity for **13b HCl-salt**. The standard deviations were small. The cytotoxity of the compounds will be screened in the near future in University of Helsinki.

Table 4. HCV activity with average inhibition (AVG), standard deviation (SD), relatively standard deviation (RSD) and standard error of the mean (SEM)

| | 6-azau. 60 µM | **13b HCl-salt** 50 µM | **13b HCl-salt** 25 µM | **84** 50 µM | **84** 25 µM | **82 HCl-salt** 50 µM | **82 HCl-salt** 25 µM | **81** 50 µM | **81** 25 µM |
|---|---|---|---|---|---|---|---|---|---|
| AVG | 49.03 | 36.52 | 15.47 | **94.04** | **72.87** | **96.16** | **63.51** | **92.64** | **82.37** |
| SD | 0.15 | 2.81 | 6.59 | 0.97 | 0.33 | 1.56 | 0.93 | 1.67 | 2.49 |
| RSD | 0.3 | 7.7 | 42.61 | 1.03 | 0.46 | 1.62 | 1.47 | 1.8 | 3.02 |
| SEM | 0.09 | 1.62 | 3.81 | 0.56 | 0.19 | 0.9 | 0.54 | 0.96 | 1.44 |

The results are linear with biological activity tests found from literature. Purpurealidin E **13** and similar compounds such us moloka'iamine **9** haven't showed as much biological activity than larger bromotyramines derivatives. The activities of the novel compounds **84**, **81** and **82 HCl-salt** are almost three fold higher compared to **13b HCl-salt**. This suggests that the carboxylic acid part is critical for the biological activity. The SAR could be studied more by using aromatic carboxylic acids such as **60** as positive control. The carboxylic acid could be methylated to ester in order to prevent the effects of free acid functionality. If the methylated carboxylic acid hasn't any activity against HCV by itself, it would provide information that purpurealidin E region is needed for HCV inhibition. This again would provide information from the possible SAR.

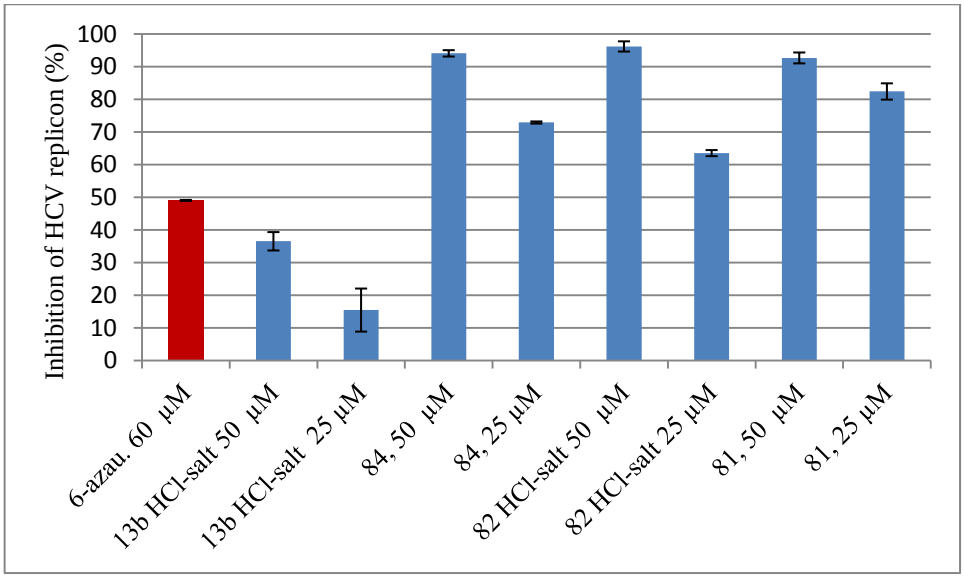



Figure 20. Activity of the tested compounds against HCV replicons

All of the three synthesized novel compounds have almost equally strong activity against HCV replicons at sample concentration 50 µM (Figure 20). At the used concentration 25µM, **81** has slightly stronger activity compared to **84** and **82 HCl-salt**. The gap of inhibition percent between two different concentrations is smallest for **81**. Even though the differences in inhibitions are small, it might be that large substituents in carboxylic acid part are favorable for BA against HCV. Synthesis of bromotyramine derivatives with large substituents in the carboxylic acid part could provide more information about this.

The tested compounds against HCV replicon showed potential antiviral activity. However, the cytotoxity needs to tested as well. For instance, Hamann and Scheuer (1993) noticed antiviral activity for the small aromatic aldehyde **10**, but the compound was found out to be cytotoxic as well. The possibility that the activity against HCV is due to cytotoxity needs to be excluded. If the compounds aren't cytotoxic, the activity against HCV might be due to an unknown mechanism. This would make bromotyramine derivatives highly interesting compounds that would require more research.

# 8 CONCLUSION

The research of marine species as novel lead compounds have developed greatly in the last decades and the work has lead to first-in-class medicines approved by FDA or EMEA. However, due to the limitations and challenges in the marine exploring there are still great potential for new discoveries. Sponges and the microbes living in symbiosis with them have been one of the most interesting marine specie for many researchers.

The aim of this thesis was to use purpurealidin E **13** as starting material for bromotyramine derivatives that would mimic **23**. Purpurealidin E **13** and total amount of 11 bromotyramine derivatives **79-89**, were successfully synthesized. Three of the synthesized bromotyramine derivatives, **84**, **81** and **82 HCl-salt**, were able to purified and their biological activity were evaluated with **13b HCl-salt**. Purpurealidin E **13b HCl-salt** did not have any biological activity but all the three purified compounds **84**, **81** and **82 HCl-salt** showed potential activity against HCV. The cytotoxity of the compounds needs to be screened. If the compounds are not cytotoxic, the activity against HCV might be due to an unknown mechanism.

In order to study the activity more, a proper purification method for novel bromotyramine derivatives needs to be developed. Additionally, the reaction route could be changed and the "three step" synthesis of bromotyramine derivatives should be

tested (Scheme 17, p. 42). The synthesis of disulfide bromotyramine derivate should be carried to the end since the **57** might have interesting biological activity.

# 9 EXPERIMENTAL

## 9.1 Reagents and devices used in thesis

The reagents and solvents used in the experiments are listed below (Table 5). All the reagents listed were commercially available. The purity and the manufacturer are listed if the data was available.

The NMR-spectrums were measured with Varian Mercury 300 MHz -spectrometer (CA, USA). The used NMR-solvents $d_1$-chloroform ($CDCl_3$), $d_4$-methanol ($CD_3OD$), $d_6$-dimethyl sulfoxide ($d_6$-DMSO) and heavy water ($D_2O$) were all purchased from Cambridge Isotope Laboratories, Inc. (MA, USA). Chemical shifts ($\delta$) are given in parts per million (ppm) relative to the NMR reference solvent signals ($CDCl_3$: 7.26 ppm; $CD_3OD$: 3.31 ppm; $d_6$-DMSO: 2.50 ppm). Multiplicities are indicated by s (singlet), br s (broad singlet), d (doublet), dd (doublet of doublet), t (triplet), dt (doublet of triplets), q (quartet) and m (multiplet). The NMR-spectra from the crude products included impurity peaks for example from solvents that are not reported.

Table 5. Reagents and solvents, their purity and supplier used in the experimental.

| **Reagent or solvent** | **Purity** | **Supplier** |
|---|---|---|
| 1,1'-Carbonyldiimidazole (CDI) | ≥97% | Aldrich |
| 1,4-Dioxane, extra dry | 99.8% | Acros |
| 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide•HCl (EDC• HCl) | ≥98.0% | Fluka or ABCR |
| 2-Propanol | ≥99.9% | Sigma-Aldrich |
| 7 *N* $NH_3$/MeOH | | Sigma-Aldrich |
| 3,5-Dibromobenzoic acid | 98.0% | Alfa Aesar |

| | | |
|---|---|---|
| 3,5-Dichlorobenzoic acid | 97.0% | Aldrich |
| 3,5-Difluorobenzoic acid | 97.0% | Sigma-Aldrich |
| 3,5-Dimethylbenzoic acid | 99.0% | Aldrich |
| 3-Bromobenzoic acid | 98.0% | Aldrich |
| 3-Dimethylamino-1-propyl chloride hydrochloride | 96.0% | Sigma-Aldrich |
| 3-Mercaptopropionic acid | ≥99.0% | Fluka |
| 4 *M* HCl in 1,4-dioxane | | Sigma-Aldrich |
| 4-Bromo-3,5-dimethoxybenzoic acid | 97.0% | Aldrich |
| 4-Bromobenzoic acid | ≥98.0% | Aldrich |
| 4-Dimethylaminopyridin (DMAP) | 99.0% | Aldrich |
| 4-Hydroxybenzoic acid | ≥98.0% | Merck/ Fluka/ Sigma-Aldrich |
| 4-Methoxybenzoyl chloride | ≥98.0% | TCI America |
| 4-Methoxybenzylamine | 98.0% | Aldrich |
| 5-Methylisoxazole-3-carboxylic acid | ≥98.0% | Alfa Aesar |
| Acetic acid | 100.0% | Merck |
| Acetic acid | ≥98.0% | Sigma-Aldrich |
| Acetone | 99.8% | Sigma-Aldrich |
| Acetyl chloride | 98.0% | Aldrich |
| Ammonia ($NH_3$) min. 25% | | VWR Chemicals |
| Benzoic acid | | University pharmacy |
| Bromine | ≥99.5% | Fluka |
| Dichloromethane | 99.8% | Sigma-Aldrich |
| Dichloromethane (anhydrous) | ≥99.8% | Sigma-Aldrich |
| Diethyl ether | 99.8% | Sigma-Aldrich |
| Di-*t*-butyl dicarbonate | 98.0% | Fluka |
| Ethyl acetate | ≥99.5% | Sigma-Aldrich |
| Hexane | ≥97.0% | Sigma-Aldrich |
| Hydrochloric acid | ≥37% | Sigma-Aldrich |
| Indole-2-carboxylic acid | 98% | Sigma-Aldrich |
| Iodomethane | ≥99.0% | Sigma-Aldrich |
| Lithium hydroxide monohydrate | ≥99.0% | Sigma-Aldrich |
| Methanol | ≥99.9% | Sigma-Aldrich |

| | | |
|---|---|---|
| Methanol anhydrous | ≥99.8% | Sigma-Aldrich |
| *N*,*N*-Diisopropylethylamine (DIPEA) | ≥98.0% or ≥99.5% | Fluka/ Sigma-Aldrich |
| *N*,*N*-dimethylformamide | 99.8% | Acros |
| O-(Benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU) | ≥98.0% | Aldrich |
| Oxalic acid | 98% | Aldrich |
| Oxalyl chloride (in a two-neck flask, supplier and purity unknown) | | |
| *p*-anisoyl chloride | ≥97% | Fluka |
| Potassium carbonate | ≥99.5% | Merck |
| Sodium hydrogen carbonate | 100.0% | VWR Chemicals |
| Sodium hydroxide | ≥99.0% | Merck |
| Sodium sulfate | ≥99.0% | Merck |
| Tetrahydrofuran | ≥99.9% | Sigma-Aldrich |
| Tetrahydrofuran (anhydrous) | ≥99.9% | Sigma-Aldrich |
| Thionyl chloride (in a two-neck flask, supplier and purity unknown) | | |
| Triethylamine | ≥99.5% | Sigma-Aldrich |
| Triethylamine (anhydrous) | ≥99.5% | Sigma-Aldrich |
| Trifluoroacetic acid | ≥99.0% | TCI |
| Tyramine | ≥98% | Alfa Aesar |
| Tyramine•HCl | 99.0% | Merck |

FTIR (ATR) measurements were made with Bruker Vertex 70 FT-IR (Ettlingen, Germany). All the spectrums were taken from the used NMR-solvents and the solvents were evaporated before making the measurement. Used resolution was 4, number of scans 64 and the measurement area was 4000-650. Wide or broad peaks are indicated with "(w)". The melting points were measured with MP Electrothermal Engineering IA9100 (Essex, Great Britain) and are uncorrected. The used rotavapor was Heidolph Laborota 4003 control (Schwabach, Germany). pH-paper used was made by Macherey-nagel (Duren, Germany).

For the HRMS-spectrums the compounds were ran through Waters Acquity UPLC-system (MA, USA). The mobile phase consisted of $H_2O$ (A) and acetonitrile (B) (Chromasolv grade, Steinheim, Germany) both containing 0.1% HCOOH (Steinheim, Germany). The composition of the mobile phase varied (gradient elution) in the following way: 0-6 min A 95% -> 5%, 1 min equibl. = total 7 min. The compounds were separated on a Acquity UPLC BEH C18 column (1.7 µm, 50 x 2.1 mm, Waters, Ireland) in 40°C. The injection volume was 2 µl and the flow-rate of the mobile phase was 0.6 ml/min. Tray temperature was 10 °C. The used MS-detector was Waters Synapt G2 HDMS (MA, USA) with the ESI (+), high resolution mode. Samples were analyzed in positive ion mode, with capillary voltage at 3.0 kV. The source temperature was 120 °C desolvation temperature was set to 350 °C. Gas flow rate was from 20 L/h to 800 L/h. The direct MS (without LC) of **13b HCl-salt** was taken with PE SCIEX API 300 mass spectrometer (Concord, Canada).

Manual flash chromatography was made with silica ordered from Merck (Silica gel 60, 0.040-0.063mm). The automated flash chromatography was made with Biotage SP1 flash chromatography purification system with 254 nm UV-detector (Uppsala, Sweden). The used TLC-plates were provided by Merck (Silica gel 60-$F_{254}$). CAMAG dual wavelenght (254/366) was used for TLC detection (Muttenz, Switzerland). Some of the TLC-plates were colored with ninhydrin (1.5g ninhydrin in 100 ml of ethanol), dinitrophenylhydrazine (12.0 g of 2,4-dinitrophenylhydrazine, 60.0 ml of conc. sulfuric acid and 80.0 ml of water in 200.0 ml of 95.0% ethanol) or bromocresol green (0.04 g of bromocresol green in 100.0 ml of absolute ethanol. 0.1 *M* NaOH till blue color). The cation exchange was made with Biotage - ISOLUTE SCX-2 (Uppsala, Sweden).

## 9.2 Synthesis

**2-(3,5-Dibromo-4-hydroxyphenyl)ethan-1-aminium bromide (39a) (39b) (39c)** This reaction was performed three times:

**39a**: Hydrochloride salt of **1** (2.00 g, 11.5 mmol) was dissolved in 50 ml of AcOH and 10 ml $H_2O$. After 10 minutes, bromine (1.49 ml, 29.0 mmol, 2.5 eq) was added

dropwise. The solution was stirred at ambient room temperature overnight (24 hr). TLC did not indicate that the reaction was completed. More bromine (0.60 ml, 11.5 mmol) was added to the reaction till the solution was dark red. After a while, 60 ml of $EtO_2$ was added to the system. The product was filtered and washed with $EtO_2$. The filtration provided white powder, 3.01 g (69.2% yield).

**39b**: Hydrochloride salt of **1** (4.00 g, 23.0 mmol) was dissolved in 100 ml of AcOH and 20 ml $H_2O$. After 10 minutes, bromine (4.15 ml, 81.0 mmol, 3.5 eq) was added dropwise. The solution was stirred at ambient room temperature overnight (24 hr). TLC did not indicate that the reaction was completed. After adding more bromine (2.37 ml, 46.0 mmol, 2 eq) product was filtrated with similar way than earlier. Reaction time was 19 hr. White powder 6.02 g. (79.0% yield).

**39c**: Tyramine **1** (2.00 g, 15.0 mmol) was dissolved to 30 ml AcOH (Wada *et al.* 2011). Bromine (2.63 ml, 51.1 mmol, 3.5 eq) was added dropwise. TLC (9:1 DCM:MeOH/ninhydrin colored) suggested reaction completion after 24 hr. The product filtrated with sinter and was washed with $EtO_2$. The filtration provided white powder 5.04 g (91.8% yield)

$^1$H-NMR (300 MHz, $d_6$-DMSO) $\delta$ 7.83 (s, 1H), 7.46 (s, 2H), 3.04 (t, $J$ = 7.3 Hz, 2H), 2.79 (t, $J$ = 7.4, 2H). $^{13}$C-NMR (75 MHz, $d_6$-DMSO) $\delta$ 149.43, 132.47, 131.62, 111.94, 39.58, 39.52, 31.14.

$^1$H-NMR (300 MHz, $CD_3OD$) $\delta$ 7.54 (s, 2H), 3.25 (t, $J$ = 7.5 Hz, 1H), 2.97 (t, $J$ = 7.6 Hz, 2H). $^{13}$C-NMR (75 MHz, $CD_3OD$) $\delta$ 150.18, 132.28, 130.54, 111.13, 40.29, 31.47.

***t*-Butyl (3,5-Dibromo-4-hydroxyphenethyl)carbamate (36a) (36b) (36c).** This reaction was performed three times:

**36a**: Compound **39a** (2.00 g, 5.32 mmol) and $Boc_2O$ (1.39 g, 6.38 mmol, 1.2 eq) were dissolved to anhydrous MeOH (50 ml) under argon atmosphere. TEA (2.23 ml, 16.0 mmol, 3 eq) was added slowly after a while. TLC (3:1Hex:EtOAc) indicated near

completion of reaction. Reaction time was 19.5 hr at ambient room temperature. MeOH was concentrated *in vacuo* with rotavapor. The product was extracted with EtOAc (40 ml) and washed with 40 ml of 1 *M* HCl. The 1 *M* HCl phase was re-extracted with 20 ml of EtOAc. Combined EtOAc (60 ml) phase was washed with water (~ 50 ml) till the pH was neutral. Organic phase was dried over anhydrous $Na_2SO_4$, filtrated with cotton wool and evaporated *in vacuo* with rotavapor. For more sufficient evaporation the product was dissolved in DCM. When DCM was evaporated *in vacuo* with rotavapor white solid appeared 2.02 g (96.3% yield).

**36b**: Compound **39b** (5.90 g, 15.7 mmol) and $Boc_2O$ (4.10 g, 18.8 mmol, 1.2 eq) were dissolved to anhydrous MeOH (110 ml) under argon atmosphere. TEA (6.58 ml, 47.2 mmol, 3 eq) was added similarly. TLC (2:1 Hex:Acetone + 2% TEA) suggested completion of reaction after 21 hr at ambient room temperature. Reaction work-up was done similarly than in the previous time. Dark green solid powder appeared after evaporation of organic phase, 5.80 g (93.5% yield).

**36c**: Compound **39c** (5.04 g, 13.4 mmol) and $Boc_2O$ (3.51 g, 16.1 mmol, 1.2 eq) were dissolved to anhydrous MeOH (75 ml) under argon atmosphere. TEA (5.61 ml, 40.2 mmol, 3 eq) was added similarly. TLC (3:1 Hex:EtOAc/ninhydrin colored) suggested completion of reaction after being overnight at ambient room temperature. Work-up similarly than earlier. The rest of the organic solvents was evaporated in oil-pump. White powder with orange spots. The product was purified by flash chromatography (8:2 Hex:EtOAc). The used amount of silica was 100 ml. Tubes containing the wanted product were combined and evaporated *in vacuo* with rotavapor. White powder, 4.36 g (82.5% yield).

$^1$H-NMR (300 MHz, $CDCl_3$) $\delta$ 7.28 (s, 2H), 5.79 (s, 1H), 4.52 (s, 1H), 3.31 (q, $J$ = 6.8 Hz, 2H), 2.70 (t, $J$ = 7.0 Hz, 2H), 1.44 (s, 9H). $^{13}$C-NMR (75 MHz, $CDCl_3$) $\delta$ 155.92, 148.16, 133.86, 132.39, 110.19, 109.97, 41.81, 34.95, 28.55. $^1$H-NMR is in accordance with literature (Kotoku *et al.* 2005).

***t-B*utyl [3,5-dibromo-4-[3-(dimethylamino)propoxy]phenethyl]carbamate, (38a) (38b) (38c)** This reaction was performed three times:

**38a:** Compound **36a** (2.00 g, 5.06 mmol), **40** (0.96 g, 6.07 mmol, 1.2 eq) and $K_2CO_3$ (1.75 g, 12.3 mmol, 2.5 eq) were dissolved in anhydrous acetone (50 ml) under argon atmosphere and heated to reflux (77 °C) overnight (19 hr). TLC (3:1 Hex:EtoAc) indicated near completion of reaction. The solution was filtered with *Büchner funnel in vacuo*. The filtered organic phase was concentrated *in vacuo* with rotavapor. The remained acetone was extracted with 45 ml DCM and washed with 30 ml 0.1 *M* NaOH and with water (3x30 ml). Organic phase was dried over anhydrous $Na_2SO_4$, filtrated with cotton wool and evaporated *in vacuo* with rotavapor. Due to the impurities seen in TLC (9:1 DCM:MeOH) the product was recrystallized. It was dissolved in 15 ml hexane and heated till the boiling point to reflux. During the recrystallization brown solid was formed. The solution was transferred to new flask without the brown solid. The solution was cooled down slowly first at room temperature then in ice-bath. The appeared white solid was filtered with *in vacuo*, 1.59 g (65.3% yield).

**38b:** Compound **36b** (5.66 g, 14.3 mmol), **40** (2.72 g, 17.2 mmol, 1.2 eq) and $K_2CO_3$ (4.95 g, 35.8 mmol, 2.5 eq) was dissolved in anhydrous acetone (80 ml) under argon atmosphere and heated to reflux (77 °C). TLC (2:1 Hex:Acetone + 2% TEA) 5 hr later did not suggest completion of reaction so it was leaved overnight. After 23 hr reaction work-up similarly than earlier. The rest of the solvent was dissolved with oil-pump, dark brown powder appeared. Recrystallization similarly than earlier. White powder 4.49 g (65.3% yield).

**38c:** Compound **36c** (4.36 g, 11.0 mmol), **40** (2.10 g, 13.3 mmol, 1.2 eq) and $K_2CO_3$ (3.81 g, 27.6 mmol, 2.5 eq) were dissolved in anhydrous acetone (95 ml) under argon atmosphere and heated (77 °C) to reflux. TLC (3:1 Hex:EtOAc/ninhydrin colored) suggested near completion of reaction after 19 hr. Work-up similarly than earlier. White powder, 3.91 g (74.0% yield).

$^1$H-NMR (300 MHz, $CDCl_3$) $\delta$ 7.32 (s, 2H), 4.04 (t, $J$ = 6.5 Hz, 2H), 3.32 (q, $J$ = 6.8 Hz, 2H), 2.71 (t, $J$ = 7.0 Hz, 2H), 2.58 – 2.49 (m, 2H), 2.27 (s, 6H), 2.09 – 1.98 (m, 2H), 1.44 (s, 9H). $^1$H-NMR is in accordance with literature (Kottakota *et al.* 2012).

**3-[4-(2-Aminoethyl)-2,6-dibromophenoxy]-*N*,*N*-dimethylpropan-1-amine, purpurealidin E (13a) (13b) (13c)** This reaction was performed three times:

**13a**: Compound **38a** (1.59 g, 3.30 mmol) was dissolved in 35 ml anhydrous DCM under argon atmosphere. By miscalculation, too much TFA (17.8 ml, 232.5 mmol, 70.4 eq) was added slowly, when the amount should have been (1.50 ml, 19.5 mmol, 6.0 eq). TLC (9:1 DCM:MeOH) indicated the completion of reaction after 3 hr. The solution was concentrated *in vacuo* with rotavapor. The product was extracted with 15 ml EtOAc and washed with 15 ml 2 *M* NaOH. To the used NaOH was added more NaOH till the pH~7 and it was re-extracted with 15 ml EtOAc. Finally, combined EtOAc phase was washed with water. Organic phase was dried over anhydrous $Na_2SO_4$, filtrated with cotton wool and evaporated *in vacuo* with rotavapor. The water phase was re-extracted again with total amount 75 ml of EtOAc and 15 ml of 5 *M* NaOH in three steps. TLC (9:1 DCM:MeOH) indicated that at this time the extraction was fine. Organic phase was dried over anhydrous $Na_2SO_4$, filtrated with cotton wool and evaporated *in vacuo* with rotavapor. The product was kept in oil-pump till there was no EtOAc left. Orange, oily gum 1.01 g (80.4% yield). Small amount of the product (50 mg) was purified by using flash chromatography (9:1 DCM:MeOH) for the HRMS.

FT-IR: 3393 (w), 2956, 1691, 1460, 1265, 1051, 739 HRMS (ESI+): Calculated value 379.0021 ($C_{13}H_{21}N_2OBr_2$), found value 379.0024 and pseudomolecular ion peak 379.0003.

**13b:** Compound **38b** (4.49 g, 9.35 mmol) was dissolved in 40 ml anhydrous DCM under argon atmosphere. TFA (4.30 ml, 56.0 mmol, 6 eq) was added slowly. TLC (2:1 Hex:Acetone + 2% TEA) 2 hr later did not suggest completion of reaction so it was leaved overnight. After 18 hr DCM was concentrated in rotavapor. The product was extracted with 20 ml EtOAc and washed with 20 ml 2M NaOH. NaOH was re-extracted

with total 40 ml EtOAc and washed with 2 *M* NaOH till the pH≥10. Organic phase was dried over anhydrous $Na_2SO_4$, filtrated with cotton wool and evaporated *in vacuo* with rotavapor. TLC (2:1 Hex:Acetone + 2% TEA) suggested that the reaction didn't work as wanted. The product was dissolved again in 25 ml anhydrous DCM under argon atmosphere. TFA (7.2 ml, 96.8 mmol, 40 eq) was added slowly. TLC (2:1 Hex:Acetone + 2% TEA) suggested now that the reaction was completed after 19.5 hr at ambient room temperature. Work-up similarly than earlier. The product was kept in oil-pump till there was no solvent left. Orange, oily gum 2.22 g (62.5% yield).

$^1$H-NMR (300 MHz, $CDCl_3$) $\delta$ 7.34 (s, 2H), 4.04 (t, $J$ = 6.4 Hz, 2H), 2.94 (t, $J$ = 6.8 Hz, 2H), 2.65 (t, $J$ = 7.0 Hz, 2H), 2.59 (t, $J$ = 7.5 Hz, 2H), 2.31 (s, 6H), 2.06 (m, 2H), 1.55 (br s, 2H). FT-IR: 2955, 2820, 2772, 1547, 1460 , 1265, 1051, 741. HRMS (ESI+): Calculated value 379.0021 ($C_{13}H_{21}N_2O\ Br_2$), found value 379.0022. There was other peaks with different mass value found as well.

Small amount of **13b** (50 mg) was dissolved to MeOH and it was inserted to ISOLUTE SCX-2 cation exchange column. The sorbent was first washed with MeOH and then with 3.5 *N* $NH_3$/MeOH solution. The combined solution from tubes was evaporated with rotavapor. Orange, oily gum 50 mg (100% yield). No significant changes in the NMR nor in the FTIR was noticed.

**13c:** Compound **38c** (3.00 g, 6.25 mmol) was dissolved in 30 ml anhydrous DCM under argon atmosphere. TFA (19.1 ml, 250.0 mmol, 40 eq) was added slowly. TLC (9:1 DCM:MeOH/ninhydrin colored) suggested that the reaction was completed after 19 hr at ambient room temperature. DCM was washed multiple times with 2 *M* NaOH till pH >7. Re-extraction from water phase was done with DCM. Organic phase was dried over anhydrous $Na_2SO_4$, filtrated with cotton wool and evaporated *in vacuo* with rotavapor. The remained solvent was evaporated with oil-pump. Orange, oily gum 1.25 g (52.5% yield).

$^1$H-NMR (300 MHz, $CDCl_3$) $\delta$ 7.32 (s, 2H), 4.03 (t, $J$ = 6.4 Hz, 2H), 2.93 (t, $J$ = 6.9 Hz, 2H), 2.64 (t, $J$ = 6.8 Hz, 2H), 2.61 – 2.54 (m, 2H), 2.29 (s, 6H), 2.10 – 1.97 (m, 2H).

$^{13}$C-NMR (75 MHz, $CDCl_3$) $\delta$ 151.75, 138.68, 133.00, 118.28, 110.15 (Peak from TFA), 71.98, 56.49, 45.52, 43.27, 38.89, 28.30.

**2-[3,5-Dibromo-4-[3-(dimethylamino)propoxy]phenyl]ethan-1-aminium chloride (13b HCl-salt):**

The product treated with cation exchange sorbent **13b** (50 mg) was dissolved in 2 ml of 1,4-dioxane. Excessive amount of 4 *M* HCl in 1,4-dioxane was added (0.80 ml). The appeared orange solid was filtered and washed with $EtO_2$, 29.3 mg (53.5% yield).

$^1$H-NMR (300 MHz, $CD_3OD$) $\delta$ 7.58 (s, 2H), 4.14 (t, $J$ = 5.6 Hz, 2H), 3.56 – 3.49 (m, 2H), 3.22 – 3.13 (m, 2H), 2.97 (s, 6H), 2.96 – 2.89 (m, 2H), 2.36 – 2.25 (m, 2H). $^{13}$C-NMR (75 MHz, cd3od) $\delta$ 153.02, 137.73, 134.46, 119.31, 71.14, 57.00, 43.70, 41.40, 33.11, 26.41. FT-IR: 3370 (w), 2940 (w), 2480 (w), 1464, 1267, 1051, 736. $R_f$ = 0,44 (1:1 DCM:MeOH + TEA 1%). HRMS (ESI+): Calculated value 379.0021 ($C_{13}H_{21}N_2O Br_2$), found value 379.0025. There were other peaks with different mass value found as well e.g. m/z: 379.0

**3,5-Dibromo-*N*-[3,5-dibromo-4-[3-(dimethylamino)propoxy]phenethyl]-4-hydroxybenzamide (77)**

Carboxylic acid **62** (93.0 mg, 0.316 mmol, 1.2 eq), EDC·HCl (60.5 mg, 0.316 mmol, 1.2 eq), DIPEA (0.055 ml, 0.316 mmol, 1.2 eq) and **13a** (100 mg, 0.263 mmol, 1.0 eq) were dissolved in DMF under argon atmosphere. After 25 hr at ambient room temperature TLC (4:1 DCM:MeOH) indicated completion of reaction. DMF was concentrated in rotavapor. The product was extracted with EtOAc and washed with water and sat. $NaHCO_3$. While washing the organic phase, pH was adjusted with 2 *M* $H_2SO_4$. No product was managed to get out from the extraction.

***N*-[3,5-Dibromo-4-[3-(dimethylamino)propoxy]phenethyl]-3,5-difluorobenzamide (78)**

Carboxylic acid **64** (50.0 mg, 0.316 mmol, 1.2 eq), EDC·HCl (60.5 mg, 0.316 mmol, 1.2 eq), DIPEA (0.055 ml, 0.316 mmol, 1.2 eq) and **13a** (100 mg, 0.263 mmol, 1.0 eq) were dissolved in DMF under argon atmosphere. After 24 hr TLC (9:1 DCM:MeOH/ninhydrin colored) indicated completion of reaction. DMF was concentrated in rotavapor. The product was dissolved in EtOAc and washed with $NH_3$ and water. Organic phase was dried over anhydrous $Na_2SO_4$, filtrated with cotton wool and evaporated *in vacuo* with rotavapor. The product was yellow gum and yield of the crude product was 100 mg (73.0%). $^1$H-NMR-spectrum indicated multiple impurities and the peaks of the possible product were not recognized. The product was purified with Biotage flash chromatography (weak solvent 9:1 DCM:MeOH, strong solvent: MeOH). The eluents collected to the tubes were combined to two sections and the eluents were evaporated with rotavapor. The yield of first section was 37.3 mg (27.2%) and the yield of the second section was 28.3 mg (20.7%). $^1$H-NMR did not show any expected product in either of the sections.

**General procedure 1:** Synthesis of amides from **13** and carboxylic acids

Carboxylic acid (1.1 or 1.2 eq), EDC·HCl (60.5 mg, 0.316 mmol, 1.2 eq) and DMAP (38.6 mg, 0.342 mmol, 1.3 eq) were added in anhydrous DCM (5-10 ml) under argon atmosphere. While the solids were dissolving, **13a-c** (100 mg, 0.263 mmol, 1.0 eq) was dissolved in 1-2 ml anhydrous DCM in separate flask under argon atmosphere. After 15 minutes, dissolved **13** was added to the reaction. The reaction was kept at ambient room temperature and it was followed by TLC. The reaction times varied. After the completion of reaction, 15 ml of DCM was added to dilute the concentration. Organic phase was washed with the following method: 1. 20 ml Brine/$H_2O$ 1:1, 2. 20 ml brine/$NH_3$ 25% 1:1, 3. 20 ml brine/HCl 1 *M* 1:1, 4. 20 ml brine/$NH_3$ 25%, 5. 2x20 ml brine. Organic phase was dried over anhydrous $Na_2SO_4$, filtrated with cotton wool and evaporated *in vacuo* with rotavapor. The remained products were dried with in an oil-pump.

**3,5-Dibromo-*N*-[3,5-dibromo-4-[3-(dimethylamino)propoxy]phenethyl]benzamide (80)**

Carboxylic acid **59** (81.0 mg, 0.289 mmol, 1.1 eq) and other reagents were added as in procedure 1. Reaction was left over the weekend since the TLC (2:1 Hex:Acetone + 2% TEA) indicated incomplete reaction. Extra 0,3 eq of DMAP was added since the TLC did not indicate completed reaction. Reaction time was 95 hr. Unlike in procedure 1, the product was washed with EtOAc. The product was purified by recrystallization by using hexane and drops of methanol. White powder, 38.2 mg (22.6% yield).

$^{1}$H-NMR (300 MHz, $CDCl_3$) $\delta$ 7.79 (t, $J$ = 1.7 Hz, 1H), 7.76 (d, $J$ = 1.7 Hz, 2H), 7.37 (s, 2H), 4.07 (t, $J$ = 6.4 Hz, 2H), 3.64 (q, $J$ = 6.8 Hz, 2H), 2.85 (t, $J$ = 7.0 Hz, 2H), 2.63 (t, $J$ = 6.6 Hz, 2H), 2.33 (s, 6H), 2.09 (m, $J$ = 6.7 Hz, 2H). $^{13}$C-NMR (75 MHz, $CDCl_3$) $\delta$ 137.91, 137.32, 137.13, 133.07, 129.03, 123.55, 118.66, 110.19, 72.01, 56.48, 45.45, 41.31, 34.52, 28.19, 26.41. Mp. 100-102 °C. $R_f$ = 0.44 (1:1 DCM:MeOH + TEA 1%). HRMS (ESI+): Calculated value 638.8493 ($C_{20}H_{23}N_2O_2Br_4$), found value 638.8487.

Due to impurity peak seen in MS-spectrum and H-NMR, flash chromatography was made for a small amount (6.3 mg) of the product. Used eluent was 3:1 DCM:MeOH. Combined solvents from tubes were evaporated in rotavapor. The yield was 5.2 mg of product **80** (82.5%). No improvements in purity were seen in $^{1}$H-NMR.

***N*-[3-[2,6-Dibromo-4-[2-(3,5-dibromobenzamido)ethyl]phenoxy]propyl]-*N*-methylmethyliumaminium chloride (80 HCl-salt)**

The whole amount of the **80** was dissolved in 1,4-dioxane. Excessive amount of 4 *M* HCl in 1,4-dioxane was added (0.50 ml). Extra dioxane was evaporated with running airflow. White powder appeared to dioxane but there was not enough time to try filter it.

**3,5-Dichloro-*N*-[3,5-dibromo-4-[3-(dimethylamino)propoxy]phenethyl]benzamide (84)**

Carboxylic acid **65** (55.0 mg, 0.289 mmol, 1.1 eq) and other reagents were added as in procedure 1 with the exception that the used amount of DMAP was 1,5 eq. Reaction was completed according to TLC (2:1 Hex:Acetone + 2% TEA) after 20 hr. The product was purified by recrystallization by using hexane and DCM as solvents. Filtration gave white solid 19.0 mg (13.0%),

$^{1}$H-NMR (300 MHz, $CDCl_3$) $\delta$ 7.57 (d, $J$ = 1.9 Hz, 2H), 7.49 (t, $J$ = 1.9 Hz, 1H), 7.37 (s, 2H), 6.09 (s, 1H), 4.06 (t, $J$ = 6.4 Hz, 2H), 3.65 (q, $J$ = 6.7 Hz, 2H), 2.85 (t, $J$ = 7.0 Hz, 2H), 2.63 (t, $J$ = 7.7 Hz, 2H), 2.33 (s, 6H), 2.09 (m, 2H). $^{13}$C-NMR (75 MHz, $CDCl_3$) $\delta$ 165.22, 152.28, 137.48, 137.32, 135.77, 133.06, 131.64, 125.70, 118.65, 72.00, 56.48, 45.45, 41.30, 34.52, 28.19. FT-IR: 3305 (w), 2941, 2866, 2829, 2781, 1647, 1562, 1546, 1452, 1250, 1050, 808, 744. $R_f$ = 0.42 (1:1 DCM:MeOH + TEA 1%). HRMS (ESI+): Calculated value 550.9503 ($C_{20}H_{23}N_2O_2Cl_2Br_2$), found value 550.9503.

**3-[2,6-Dibromo-4-[2-(3-bromo-4-methoxybenzamido)ethyl]phenoxy]-*N*,*N*-dimethylpropan-1-aminium chloride (82)**

Carboxylic acid **61** (66.9 mg, 0.289 mmol, 1.1 eq) and other reagents were added as in procedure 1 with the exception that the used amount of DMAP was 1,5 eq. Reaction was not completed according to TLC (2:1 Hex:Acetone + 2% TEA) after 44 hr, extra **61** (0.2 eq) was added. After 2 hr TLC (2:1 Hex:Acetone + 2% TEA) suggested completion of reaction. Reaction time was 46 hr. $^{1}$H-NMR was taken from the crude product. The product was purified by recrystallization by using hexane and DCM. When the cold product was tried to be filtrated, it melted and went through the sinter. The solvent was evaporated in rotavapor in order to make salt from the product.

$^{1}$H-NMR (300 MHz, $CDCl_3$) $\delta$ 7.91 (d, $J$ = 2.2 Hz, 1H), 7.67 (dd, $J$ = 8.6, 2.2 Hz, 1H), 7.37 (s, 2H), 6.91 (d, $J$ = 8.6 Hz, 1H), 6.07 (s, 1H), 4.06 (t, $J$ = 6.5 Hz, 2H), 3.94 (s,

3H), 3.64 (q, *J* = 7.0 Hz, 2H), 2.84 (t, *J* = 7.0 Hz, 2H), 2.57 (t, *J* = 7.4 Hz, 2H), 2.29 (s, 6H), 2.14 – 1.98 (m, 2H).

**3-[2,6-Dibromo-4-[2-(3-bromo-4-methoxybenzamido)ethyl]phenoxy]-*N*,*N*-dimethylpropan-1-aminium chloride (82 HCl-salt)**

The product **82** was dissolved in 10 ml 1,4-dioxane. Excessive amount of 4 *M* HCl in 1,4-dioxane was added (0.50 ml). Extra dioxane was evaporated with running airflow. White solid appeared to dioxane. The salt was filtrated and washed with $Et_2O$. White powder 44.8 mg (24.7% yield).

$^1$H-NMR (300 MHz, $CD_3OD$) $\delta$ 7.97 (t, *J* = 2.2 Hz, 1H), 7.77 (dd, *J* = 2.2, 8.6 Hz, 1H), 7.51 (s, 2H), 7.09 (d, *J* = 8.7 Hz, 1H), 4.12 (t, *J* = 5.6 Hz, 2H), 3.94 (s, 3H), 3.58 – 3.47 (m, 4H, assumed to be two triplets with two -$CH_2$-), 2.97 (s, 6H), 2.86 (t, *J* = 7.1 Hz, 2H), 2.34 – 2.23 (m, 2H). $^{13}$C-NMR (75 MHz, $CD_3OD$) $\delta$ 168.47, 160.03, 152.16, 140.58, 134.51, 133.39, 129.28, 128.99, 118.74, 112.70, 112.40, 71.16, 57.17, 56.97, 43.73, 41.97, 35.22, 26.37. FT-IR: 3390 (w), 2940 (w), 2490 (w), 2370, 1640, 1535, 1460, 1271, 1058, 756. $R_f$ = 0.66 (1:1 DCM:MeOH + TEA 1%). HRMS (ESI+): Calculated value 590.9494 ($C_{21}H_{26}N_2O_3Br_3$), found value 590.9496.

**3,5-Dibromo-*N*-[3,5-dibromo-4-[3-(dimethylamino)propoxy]phenethyl]-4-methoxybenzamide (81)**

Carboxylic acid **60b** (97.9 mg, 0.316 mmol, 1.2 eq) ) and other reagents were added as in procedure 1. After 42 hr TLC (2:1 Hex:Acetone + 2% TEA) suggested completion of reaction. The product was recrystallized with hexane and few drops of methanol. White powder, 54 mg (31.0% yield).

$^1$H-NMR (300 MHz, $CDCl_3$) $\delta$ 7.86 (s, 2H), 7.36 (s, 2H), 6.11 (s, 1H), 4.06 (t, *J* = 6.4 Hz, 2H), 3.92 (s, 3H), 3.63 (q, *J* = 6.7 Hz, 2H), 2.84 (t, *J* = 7.0 Hz, 2H), 2.58 (t, *J* = 7.4 Hz, 2H), 2.30 (s, 6H), 2.06 (m, 2H). $^{13}$C-NMR (75 MHz, $CDCl_3$) $\delta$ 164.71, 157.07, 152.32, 137.35, 133.05, 132.79, 131.62, 118.71, 118.65, 72.13, 60.92, 56.50, 45.56,

41.30, 34.55, 28.33. FT-IR: 3288 (w), 3084, 2956, 2879, 2831, 2791, 1651, 1547, 1474, 1317, 1267, 742. Mp.: 83-85 °C. $R_f$ = 0.59 (1:1 DCM:MeOH + TEA 1%). HRMS (ESI+): Calculated value 668.8599 ($C_{21}H_{25}N_2O_3Br_4$), found value 668.8609.

**3-Bromo-*N*-[3,5-dibromo-4-[3-(dimethylamino)propoxy]phenethyl]benzamide (79)**

Carboxylic acid **58** (63.5 mg, 0.316 mmol, 1.2 eq) and other reagents were added as in procedure 1 with the exception that the used crosslinking agent was HBTU (41.9 mg, 0.342 mmol, 1.3 eq). After 42 hr TLC (2:1 Hex:Acetone + 2% TEA) suggested completion of reaction. Recrystallization was made bu using multiple different solvents (hexane, 2-propanol, MeOH, acetone) but no solid appeared that could have been filtered. $^1$H-NMR was taken before recrystallization from the crude product, orange gum 111.6 mg.

$^1$H-NMR (300 MHz, $CDCl_3$) $\delta$ 7.86 (t, $J$ = 1.8 Hz, 1H), 7.63 (t, $J$ = 1.9 Hz, 1H), 7.61 (t, $J$ = 1.9 Hz, 1H), 7.37 (s, 2H), 7.30 (t, $J$ = 7.8 Hz, 1H), 4.05 (t, $J$ = 6.4 Hz, 2H), 3.65 (q, $J$ = 6.8 Hz, 2H), 2.89 – 2.82 (m, 2H), 2.61 – 2.52 (m, 2H), 2.29 (s, 6H), 2.06 (dt, $J$ = 6.5, 13.5 Hz, 2H).

***N*-[3,5-Dibromo-4-[3-(dimethylamino)propoxy]phenethyl]-5-methylisoxazole-3-carboxamide (88)**

Carboxylic acid **69** (40.1 mg, 0.316 mmol, 1.2 eq) and other reagents were added as in procedure 1. After 42 hr TLC (2:1 Hex:Acetone + 2% TEA) suggested completion of reaction. Recrystallization was done with hexane and drops of MeOH. No solid appeared that could have been filtered when cooled down. $^1$H-NMR was taken before recrystallization from the crude product, orange gum with small white spots 71.6 mg.

$^1$H-NMR (300 MHz, $CDCl_3$) $\delta$ 7.36 (s, 2H), 6.43 (d, $J$ = 1.0 Hz, 1H), 4.05 (t, $J$ = 6.4 Hz, 2H), 3.69 – 3.58 (m, 2H), 2.83 (t, $J$ = 7.2 Hz, 2H), 2.58 (t, $J$ = 7.4 Hz, 2H), 2.47 (d, $J$ = 0.9 Hz, 3H), 2.30 (s, 6H), 2.06 (dt, $J$ = 6.5, 13.3 Hz, 2H).

***N*-[3,5-Dibromo-4-[3-(dimethylamino)propoxy]phenethyl]benzamide (87)**

Benzoic acid **68** (38.6 mg, 0.316 mmol, 1.2 eq) and other reagents were added as in procedure 1. After 68 hr TLC (3:1 DCM:MeOH) suggested completion of reaction. The crude product (71.1 mg) was purified with flash chromatography (3:1 DCM:MeOH). The evaporated solvent from the combined tubes provided orange gum. The yield from the flash chromatography was 63 mg (49.6%). Recrystallization with hexane and 2-propanol did not provide white crystals when cooled down. The $^1$H-NMR was not taken after the flash chromatography, since it was assumed that the pure product was solid. Thus, the $^1$H-NMR is from crude product before the flash chromatography.

$^1$H-NMR (300 MHz, $CDCl_3$) $\delta$ 7.74 – 7.69 (m, 2H), 7.46 – 7.42 (m, 2H), 7.40 (t, $J$ = 1.6 Hz, 1H), 7.38 (s, 2H), 4.06 (t, $J$ = 6.4 Hz, 2H), 3.73 – 3.62 (m, 2H), 2.86 (t, $J$ = 7.0 Hz, 2H), 2.59 (t, $J$ = 7.4 Hz, 2H), 2.31 (s, 6H), 2.13 – 2.00 (m, 2H).

***N*-[3-[4-(2-benzamidoethyl)-2,6-dibromophenoxy]propyl]-*N*-methylmethylium-aminium chloride (87 HCl-salt)**

The solvents were evaporated and the product **87** was dissolved to 4 ml 1,4-dioxane. Excessive amount of 4 *M* HCl in 1,4-dioxane was added and white solid appeared to solvent. The solid was tried to be filtered without success.

***N*-[3,5-Dibromo-4-[3-(dimethylamino)propoxy]phenethyl]-3,5-dimethylbenzamide (86)**

Carboxylic acid **67** (47.4 mg, 0.316 mmol, 1.2 eq) and other reagents were added as in procedure 1. After three days TLC (3:1 DCM:MeOH) suggested completion of reaction. The crude product **86** (87.1 mg) was purified with the same way and the same result than **87**. The yield from the flash chromatography was 44.9 mg and the product was clear gum. The $^1$H-NMR was taken from the crude product before the flash chromatography since it was assumed that the pure product needed to be solid.

$^1$H-NMR (300 MHz, $CDCl_3$) $\delta$ 7.38 (s, 2H), 7.30 (s, 2H), 7.14 – 7.10 (m, 1H), 4.05 (t, *J* = 6.5 Hz, 2H), 3.68 – 3.60 (m, 2H), 2.84 (t, *J* = 6.9 Hz, 2H), 2.64 – 2.48 (m, 2H), 2.35 (s, 6H), 2.29 (s, 6H), 2.06 (dt, *J* = 6.5, 13.2 Hz, 2H).

**4-Bromo-*N*-[3,5-dibromo-4-[3-(dimethylamino)propoxy]phenethyl]-3,5-dimethoxybenzamide (85)**

Carbocylix acid **66** (63.5 mg, 0.316 mmol, 1.2 eq) and other reagents were added as in procedure 1. After 47 hr TLC (3:1 DCM:MeOH/ninhydrin colored) suggested completion of reaction. Orange gum 136.7 mg, the $^1$H-NMR is from the crude product.

$^1$H-NMR (300 MHz, $CDCl_3$) $\delta$ 7.38 (s, 2H), 6.90 (s, 2H), 4.04 (t, *J* = 6.5 Hz, 2H), 3.93 (s, 6H), 3.65 (q, *J* = 6.7 Hz, 2H), 2.87 (t, *J* = 6.9 Hz, 2H), 2.59 – 2.51 (m, 2H), 2.28 (s, 6H), 2.05 (dt, *J* = 6.6, 13.9 Hz, 2H).

***N*-[3-[2,6-Dibromo-4-[2-(4-bromo-3,5-dimethoxybenzamido)ethyl]phenoxy]-propyl]-*N*-methylmethyliumaminium) chloride (85 HCl-salt)**

The product **85** was dissolved to small amount of 1,4-dioxane (1.5 ml) and 0.5 ml 4 *M* HCl in 1,4-dioxane was added. The solvent turned cloudy. No solid was appeared after cooled down. The 1,4-dioxane was evaporated.

***N*-[3-[2,6-Dibromo-4-[2-(4-bromo-3,5-dimethoxybenzamido)ethyl]phenoxy]-propyl]-*N*-methylmethyliumaminium] carboxyformate (85 oxalate-salt)**

The product **85 HCl-salt** was dissolved in 2-propanol and assumed equivalent amount of oxalic acid (19.8 mg) was added. The solvent turned cloudy and it was kept in freezer overnight. The formed solid was filtered and washed with $Et_2O$. Brown solid 10.1 mg. The product did not dissolve in $CD_3OD$ nor in $D_2O$, so no NMR spectra were able to run.

***N*-[3,5-Dibromo-4-[3-(dimethylamino)propoxy]phenethyl]-1H-indole-2-carboxamide (89)**

Carboxylic acid **70** (38.6 mg, 0.316 mmol, 1.2 eq) and other reagents were added as in procedure 1. The reaction was left over weekend. TLC (9:1 DCM:MeOH) suggested completion of reaction after 91 hr. Yellow solid appeared 36.6 mg. The product should have been purified, but the time ran out. The $^1$H-NMR is from the crude product.

$^1$H-NMR (300 MHz, $CDCl_3$) $\delta$ 9.42 (s, 1H), 7.64 (d, $J$ = 8.0 Hz, 1H), 7.44 (dq, $J$ = 1.0, 8.4 Hz, 1H), 7.39 (s, 2H), 7.36 – 7.26 (m, 1H), 7.14 (ddd, $J$ = 1.0, 7.0, 8.0 Hz, 1H), 6.78 (dd, $J$ = 0.9, 2.0 Hz, 1H), 6.27 (t, $J$ = 6.1 Hz, 1H), 4.06 (t, $J$ = 6.5 Hz, 2H), 3.70 (q, $J$ = 6.8 Hz, 2H), 2.87 (t, $J$ = 7.1 Hz, 2H), 2.56 (dd, $J$ = 6.5, 8.3 Hz, 2H), 2.28 (s, 6H), 2.05 (dt, $J$ = 6.6, 13.5 Hz, 2H).

***S*-[3-[(4-methoxybenzyl)amino]-3-oxopropyl]ethanethioate (92a), (92b), (92c)**

This reaction was made three times:

**92a**: Thiol acid **72** (0.30 g, 2.02 mmol 1.0 eq) and CDI (0.43 g, 2.63 mmol, 1.3 eq) were dissolved in DCM under argon atmosphere and DIPEA (0.46 ml, 2.63 mmol, 1.3 eq) were added. After 25 min, **91** (0.34 ml, 2.64 mmol, 1.3 eq) was added. The reaction was left over weekend. After 72 hr, TLC (8:2 Hex:EtOAc) indicated completion of reaction. The reaction work-up as in procedure 1. The organic phase was filtered, since there was white crystals. The yield was 191 mg, but the $^1$H-NMR did not show assumed product.

**92b:** The reaction was repeated with the exception that the used solvents was THF. After 26 hr 25 ml of EtOAc was added. The organic phase was washed with 1 *M* $KHSO_4$, sat. $NaHCO_3$ and water. The solvent was evaporated with rotavapor. The product was dissolved in EtOAc rewashed with 1 *M* HCl and sat $NaHCO_3$. The solid in organic phase was filtered away, the organic phase was evaporated with rotavapor and dried in oil-pump. White solid 342.3 mg. $^1$H-NMR did not indicate the assumed product.

$^{1}$H-NMR (300 MHz, $d_6$-DMSO) $\delta$ 7.17 (d, $J$ = 8.6 Hz, 4H), 6.96 – 6.81 (m, 4H), 4.17 (dd, $J$ = 5.9, 12.8 Hz, 4H), 3.73 (s, 6H), 3.02 (t, $J$ = 7.0 Hz, 2H), 2.42 (t, $J$ = 7.0 Hz, 2H), 2.31 (s, 3H).

**92c:** Carboxylic acid **72** (0.30 g, 2.02 mmol 1.0 eq), EDC·HCl and DMAP were added as in procedure 1. The used solvent was anhydrous THF. After 15 min, **91** (0.34 ml, 2.64 mmol, 1.3 eq) was added. The reaction was left over weekend. After 92 hr TLC (9:1 DCM:MeOH) indicated completion of reaction. THF was concentrated in rotavapor. The product was extracted with EtOAc and washed with 1 *M* HCl, sat. $NaHCO_3$ and brine. The product was purified with Biotage chromatography (1:1 Hex:EtOAc). The solvent from the combined tubes was evaporated in rotavapor and oil-pump. White solid 153.60 mg (28.4% yield).

$^{1}$H-NMR (300 MHz, $d_6$-DMSO) $\delta$ 7.23 – 7.10 (m, 2H), 6.97 – 6.79 (m, 2H), 4.19 (d, $J$ = 6.0 Hz, 2H), 3.73 (s, 3H), 3.02 (t, $J$ = 7.0 Hz, 2H), 2.42 (t, $J$ = 7.0 Hz, 2H), 2.31 (s, 3H). $^{13}$C-NMR (75 MHz, $d_6$-DMSO) $\delta$ 195.23, 169.76, 158.16, 131.26, 128.49, 113.62, 55.01, 41.51, 34.84, 30.44, 24.49.

**_S_-[3-[[3,5-Dibromo-4-[3-(dimethylamino)propoxy]phenethyl]amino]-3-oxopropyl] ethanethioate (73)**

Carboxylic acid **72** (234.0 mg, 1.58 mmol, 1.2 eq) and other reagents were added like in procedure 1. The reaction was left over weekend. TLC (9:1 DCM:MeOH) suggested near completion of reaction after 92 hr. Orange oil, 194.7 mg (28.9% yield).

$^{1}$H-NMR (300 MHz, $CDCl_3$) $\delta$ 7.33 (s, 2H), 4.04 (t, $J$ = 6.4 Hz, 2H), 3.46 (q, $J$ = 6.9 Hz, 2H), 3.12 (t, $J$ = 6.9 Hz, 2H), 2.85 (t, $J$ = 6.9 Hz, 2H), 2.74 (t, $J$ = 7.0 Hz, 2H), 2.63 – 2.53 (m, 2H), 2.45 (t, $J$ = 6.9 Hz, 2H), 2.33 (s, 3H), 2.30 (s, 6H), 2.07 (dd, $J$ = 6.5, 14.3 Hz, 2H).

**_N_-[3,5-Dibromo-4-[3-(dimethylamino)propoxy]phenethyl]-4-methoxybenzamide (83)**

Acyl chloride **90** (50.0 mg, 0.289 mmol, 1.1 eq) and **13** (100.0 mg, 0.263 mmol, 1.0 eq) were dissolved in anhydrous THF in argon atmosphere. TEA (0.055 ml, 0.395 mmol, 1.5 eq) was added. The reaction was left over weekend. The reaction time was 66 hr. The solvent was concentrated in rotavapor, extracted with EtOAc and washed with 25% $NH_3$ and water till the pH was ~7. The water phase was re-extracted with EtOAc. Organic phase was dried over anhydrous $Na_2SO_4$, filtrated with cotton wool and evaporated *in vacuo* with rotavapor. The product was purified with Biotage chromatography (Weak solvent: 2:1 Hex:Acetone + 2% TEA, Strong solvent: DCM + 2% TEA). The solvent of combined tubes was evaporated in rotavapor and finally in oil-pump, orange gum 10.4 mg (5.9% yield).

$^1$H-NMR (300 MHz, $CDCl_3$) $\delta$ 7.67 (dd, $J$ = 4.7, 8.9 Hz, 2H), 7.37 (s, 2H), 6.91 (dd, $J$ = 4.7, 8.9 Hz, 2H), 6.08 (s, 1H), 4.05 (t, $J$ = 6.5 Hz, 2H), 3.84 (s, 3H), 3.64 (q, $J$ = 6.8 Hz, 2H), 2.84 (t, $J$ = 7.0 Hz, 2H), 2.55 (t, $J$ = 7.2 Hz, 2H), 2.28 (s, 6H), 2.04 (m, $J$ = 7.4, 13.9 Hz, 2H). $^{13}$C-NMR (75 MHz, $CDCl_3$) $\delta$ 167.24, 162.42, 152.16, 137.79, 133.07, 128.77, 126.85, 118.53, 113.99, 72.15, 56.54, 55.56, 45.62, 41.03, 34.78, 28.42. HRMS (ESI+): Calculated value 513.0388 ($C_{21}H_{27}N_2O_3Br_2$), found value 513.0390.

**3,5-Dibromo-_N_-[3,5-dibromo-4-[3-(dimethylamino)propoxy]phenethyl]-4-methoxybenzamide (81)**

Carboxylic acid **60a** (98.0 mg, 0.316 mmol, 1.2 eq) was dissolved in anhydrous THF in argon atmosphere. Oxalyl chloride (0.034 ml, 0.395 mmol, 1.5 eq) and five drops of DMF were added. After 50 min **13a** (100 mg, 0.261 mmol, 1.0 eq) was dissolved in anhydrous THF in argon atmosphere and TEA (0.058 ml, 0.421 mmol, 1.6 eq) was added. The reaction was left over weekend. After 66 hr the solvents were evaporated with rotavapor, extracted with EtOAc and washed with 25% $NH_3$ and water/brine. Organic phase was dried over anhydrous $Na_2SO_4$, filtrated with cotton wool and

evaporated *in vacuo* with rotavapor and oil-pump. According to $^{1}$H-NMR there was no desired product.

The reaction was repeated with the difference that the used acid was thionyl chloride (0.029ml, 0.395 mmol, 1.5 eq) instead of oxalyl chloride and the solvent was anhydrous DCM. Before adding the amine, the formation of acyl chloride was followed with TLC (9:1 DCM:MeOH + 1% TEA). After 11 hr it suggested near completion of the acyl chloride formation and **13a** (100 mg, 0.261 mmol, 1.0 eq) was dissolved in anhydrous DCM and TEA (0.058 ml, 0.421 mmol, 1.6 eq) was added. TLC suggested near completion of the reaction after 24 hr. The solvents was evaporated with rotavapor, extracted with EtOAc, washed with 25% $NH_3$ and water/brine and the water phase was re-extracted with EtOAc. Organic phase was dried over anhydrous $Na_2SO_4$, filtrated with cotton wool and evaporated *in vacuo* with rotavapor and oil-pump. The product was purified with Biotage chromatography (Weak solvent: DCM + 2% TEA, Strong solvent: 9:1 DCM:MeOH). The solvent of combined tubes was evaporated in rotavapor and finally in oil-pump, but the $^{1}$H-NMR did not show any product assumed.

**3,5-Dibromo-4-hydroxybenzoic acid (62)**

Carboxylic acid **75** (2.00 g, 14.5 mmol, 1.0 eq) was dissolved in 50 ml of AcOH and 10 ml $H_2O$. After 10 minutes, bromine (2.60 ml, 50.8 mmol, 3.5 eq) was added dropwise. TLC (1:1 Hex:EtOAc) suggested completion of the reaction after 2 hr stirred at ambient room temperature. The product was filtered and the filtrate was washed water. The solvent residues were evaporated with oil-pump. White solid 3.36 g (78.3% yield).

$^{1}$H-NMR (300 MHz, $d_6$-DMSO) $\delta$ 8.01 (s, 2H). $^{13}$C-NMR (75 MHz, $d_6$-DMSO) $\delta$ 164.82, 154.72, 133.31, 124.58, 111.28. NMR-data is in accordance with literature (Spectral Database System of Organic Compounds 2015).

**Methyl-3,5-dibromo-4-methoxybenzoate (76a), (76b)** The reaction was done twice:

**76a:** Carboxylic acid **62** (1.00 g, 3.40 mmol, 1.0 eq) and $K_2CO_3$ (0.93 g, 6.80 mmol, 2.0 eq) were dissolved in DMF under argon atmosphere and MeI (2.11 ml, 33.8 mmol, 10.0 eq) was added. TLC (9:1 DCM:MeOH) suggested completion of reaction after 3.5 hr. The product was extracted with EtOAc and washed with water. Organic phase was dried over anhydrous $Na_2SO_4$, filtrated with cotton wool and evaporated *in vacuo* with rotavapor and oil-pump, white powder 0.78 g (70.9% yield).

**76b:** Carboxylic acid **62** (0.70 g, 2.37 mmol, 1.0 eq), $K_2CO_3$ (0.65 g, 4.73 mmol, 2.0 eq), MeI (1.47 ml, 23.7 mmol, 10.0 eq) and DMF were added similarly. TLC (2:1 Hex:Acetone + 2% TEA) suggested near completion of reaction after 2 hr. $K_2CO_3$ was filtered away with cotton wool. DMF was concentrated with rotavapor. Otherwise similar work-up gave white solid 0.57 g (74.8% yield).

$^1$H-NMR (300 MHz, $CDCl_3$) $\delta$ 8.18 (s, 2H), 3.94 (s, 3H), 3.91 (s, 3H). $^{13}$C-NMR (75 MHz, $CDCl_3$) $\delta$ 164.49, 158.17, 134.24, 128.47, 118.36, 60.90, 52.75. NMR-data is in accordance with literature (Grimster *et al.* 2013).

**3,5-Dibromo-4-methoxybenzoic acid (60a), (60b)** The reaction was made in two separated times:

**60a:** Methylated carboxylic acid **76a** (0.78 g, 2.41 mmol, 1.0 eq) and lithium hydroxide monohydrate (0.25 g, 6.01 mmol, 2.5 eq) were dissolved in 1:1 $H_2O$/THF solution. The reaction was first kept at ambient room temperature and eventually heated to 60 °C. After 24 hr TLC (1:1 Hex:EtOAc) indicated completion of reaction. 1 *M* HCl (10 ml) was added and the water phase was extracted with EtOAc. Organic phase was dried over anhydrous $Na_2SO_4$, filtrated with cotton wool and evaporated *in vacuo* with rotavapor. The rest of the solvents was evaporated with oil-pump. White solid 0.64 g (86.2% yield).

**60b:** Methylated carboxylic acid **76b** (0.57 g, 1.77 mmol, 1.0 eq) and Lithium hydroxide monohydrate (0.19 g, 4.43 g, 2.5 eq) were dissolved as earlier. The reaction

was left over weekend, TLC (8:2 Hex:EtOAc) indicated completion of reaction after 66 hr. After similar work-up, white solid 0.48 g (87.2% yield).

$^{1}$H-NMR (300 MHz, $d_6$-DMSO) $\delta$ 13.45 (s, 1H), 8.10 (s, 2H), 3.87 (s, 3H). $^{13}$C-NMR (75 MHz, $d_6$-DMSO) $\delta$ 164.44, 157.00, 133.54, 129.53, 117.65, 60.58, 39.52.

**3-Bromo-4-methoxybenzoic acid (61)**

Carboxylic acid **63** (0.50 g, 3.29 mmol, 1.0 eq) was dissolved in 10 ml AcOH and 2 ml water. Bromine (0.60 ml, 11.5 mmol, 3.5 eq) was added dropwise. The reaction was left over weekend. After 71 hr TLC (9:1 DCM:MeOH) indicated only one spot. The product was filtered while washing with $Et_2O$, white solid 0.30 g (29.6% yield).

$^{1}$H-NMR (300 MHz, $d_6$-DMSO) $\delta$ 12.90 (s, 1H), 8.06 (d, $J$ = 2.1 Hz, 1H), 7.94 (dd, $J$ = 2.1, 8.6 Hz, 1H), 7.21 (d, $J$ = 8.7 Hz, 1H), 3.93 (s, 3H), 3.29 (s, 1H) HRMS (ESI+): Calculated value 230.9657 ($C_8H_7BrO_3$), found value 230.9651.

**3-(Acetylthio)propanoic acid (72)**

Thiol acid **71** (10.0 ml, 115.0 mmol, 1.0 eq), 20 ml DCM and 30 ml AcOH were added into cooled argon atmosphere. Acetyl chloride (24.5 ml, 344.0 mmol, 3 eq) was added dropwise. The reaction was heated up to ambient room temperature. After 23 hr TLC indicated completion of reaction. The solvents were concentrated carefully in rotavapor. The product was purified with flash chromatography, used amount of silica was 300 ml (8:2 Hex:EtOAc). For better detection, the used TLC-plates from the tubes were colored with dinitrophenylhydrazine or bromocresol green. The combined solvents from tubes were evaporated carefully in rotavapor, clear solid 10.8 g (63.3% yield).

$^{1}$H-NMR (300 MHz, $CDCl_3$) $\delta$ 3.10 (t, $J$ = 6.9 Hz, 2H), 2.68 (t, $J$ = 6.9 Hz, 2H), 2.33 (s, 3H). $^{13}$C-NMR (75 MHz, $CDCl_3$) $\delta$ 195.60, 177.72, 34.29, 30.63, 23.96. NMR-data is in accordance with literature (Ekeroth *et al.* 2002).

## 9.3 Biological activity tests

The compounds activity to EAG channels were tested by Prof Jan Tytgat, Toxicology and Pharmacology, Catholic University of Leuven, Belgium. *Xenopus laevis* frogs oocytes were used to study the expression of the voltage gated potassium channels (hKv1.3, hKv10.1). The linearized plasmids were transcribed using the T7 or SP6 mMESSAGE-mMACHINE transcription kit (Ambion, USA). The stage V-VI oocytes were harvested from anasthetized female *Xenopus laevis* frog. The oocytes were Oocytes were injected with 50 nl of cRNA at a concentration of 1 ng/nl using a micro-injector (Drummond Scientific, USA). The oocytes were incubated in a solution containing (in mM): NaCl, 96; KCl, 2; $CaCl_2$, 1.8; $MgCl_2$, 2 and HEPES, 5 (pH 7.4), supplemented with 50 mg/l gentamycin sulfate. Two-electrode voltage-clamp recordings were performed at room temperature (18-22°C) using a Geneclamp 500 amplifier (Molecular Devices, USA) controlled by a pClamp data acquisition system (Axon Instruments, USA). Whole cell currents from oocytes were recorded 1-4 days after injection. Bath solution composition was ND96 (in mM): NaCl, 96; KCl, 2; $CaCl_2$, 1.8; $MgCl_2$, 2 and HEPES, 5 (pH 7.4) or HK (in mM): NaCl, 2; KCl, 96; $CaCl_2$, 1.8; $MgCl_2$, 2 and HEPES, 5 (pH 7.4). Voltage and current electrodes were filled with 3 *M* KCl. Resistances of both electrodes were kept between 0.7-1.5 MΩ. The elicited currents were filtered at 2 kHz and sampled at 500 Hz using a four-pole low-pass Bessel filter. Leak subtraction was performed using a -P/4 protocol. Kv1.3 currents were evoked by 500 ms depolarizations to 0 mV followed by a 500 ms pulse to -50 mV, from a holding potential of -90 mV. Current traces of Kv10.1 channels were elicited by applying a 0 mV pulse for 2 s from a holding potential of -90 mV. All data represent at least 3 independent experiments ($n \geq 3$) and are presented as mean ± standard error.

Compounds activity against hepatitis C virus (HCV) was screened in Päivi Tammela's group by Katja-Emilia Lillsunde, University of Helsinki. The antiviral screening was done with CV-Huh7 cell line that expresses HCV replicon and the Firefly luciferase as a reporter. Firefly luciferase produces light by catalysing luciferin oxidation using ATP and Mg2+ as a cosubstrate. The seeded 96-plate cells and samples were incubated overnight. The expression of the HCV replicon was measured after 24 hr incubation.

This was done by using Firefly luciferase (FFluc) assay kit from Promega (Luciferase Assay System) (WI, USA). The inhibition of the HCV replicon and the activity of the samples were calculated by comparing the luminescense with the DMSO vehicle control. Additionally, the used positive control was 60 µM 6-azauridine, that should show approx. 50% inhibition. Each sample was tested in triplicate, and the average inhibition and standard deviations were calculated.

## 10 LITERATURE

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