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Stereoselectivity profiling of phase I mephedrone Metabolites
at the human serotonin reuptake transporter

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1.Introduction

1.1 Serotonin

Serotonin, also known as 5-Hydroxytryptamine, is a biogenic amine well-known for its role as neurotransmitter in most vertebrates. In humans, it modulates bodily functions such as gastrointestinal motility, emesis and vascular tones in the cerebrum and periphery, but also plays a major role in psychiatric and neurological disorders, mood and sleep.^{1,5}

The history of serotonin began in 1937, as the Italian scientist Vittorio Erspamer extracted a substance which was responsible for contracting smooth muscle tissue from the gastrointestinal tracts of animals. Said substance, subsequently named “enteramine” was later isolated and identified as 5-Hydroxytryptamine by scientists Maurice Rapport and Irvine Page in 1948.³ The ability of 5-Hydroxytryptamine to constrict blood vessels gave it the name “serotonin”, comprised of the Latin word “serum” and the Greek “tonic”.

Serotonin is mostly synthesized within the raphe nuclei in the brain, where the essential amino acid tryptophan is altered by two enzymes, tryptophan hydroxylase and amino acid decarboxylase.⁵ After synthesis, serotonin molecules are gathered in vesicles where they are stored until a presynaptic signal induces its release into the synaptic cleft between neurons via exocytosis.⁶ Following their release, serotonin molecules have three ways to proceed: They can either bind to auto-receptors on the presynaptic cell (inhibiting their own release), be removed via reuptake performed by the serotonin reuptake transporter (SERT) or they can bind to a group of serotonin-receptors. Said receptors are all G-protein coupled receptors with the ligand-gated ion channel 5-HT₃ being the exception. Any serotonin production outside of the central nervous system is limited to enterochromaffin cells and, to a lesser extent, platelets.¹⁰ After release from the enterochromaffin cells into the portal system, serotonin is quickly taken up by platelets and metabolized by the liver.

As mentioned beforehand, serotonin functions as one of the main neurotransmitters in the human central nervous system as it mediates behavioural processes such as mood, perception, anger, memory, appetite and sexuality.¹ Dysregulation of the serotonergic system is therefore associated to diseases like anxiety, schizophrenia, addiction or depression.¹⁰ Due to the prevalence of these diseases, drugs targeting the serotonin system are of high scientific interest.

1.2 Serotonin transporter

The first notion of the existence of the serotonin transporter (SERT) appeared in 1964 after experiments with the antidepressant drug imipramine demonstrated an ability to inhibit serotonin reuptake into the presynaptic terminal.⁷ Shortly after, the gene for the serotonin reuptake transporter (SLC6A4) was discovered and cloned.⁸ This so called SLC family constitutes to one of the

biggest gene families within the human genome. Over 65 subfamilies based on sequence similarity make up over 400 proteins with differing substrate specificity.¹²

SERT is a member of the SLC6-family (SLC6 being an abbreviation for “solute carrier 6”). This transport family comprises secondary active co-transporters which use an osmotic sodium (Na^+) gradient to combine transport of said ion with transport of their respective substrates. Some transport chloride (Cl^-) as well and are therefore labelled “ Na/Cl^- dependent transporters”.¹¹

SLC6 transporters can be spilt up in four different branches based on their substrate preferences: First, there is the family of monoamine neurotransmitter transporters that is populated by SERT, the dopamine transporter DAT and the norepinephrine transporter NET. Next to them is a family of neurotransmitter amino acid transporters, a family of nutrient amino acid transporters and a group consisting of transporters that translocate GABA, osmolytes, creatine and taurine.

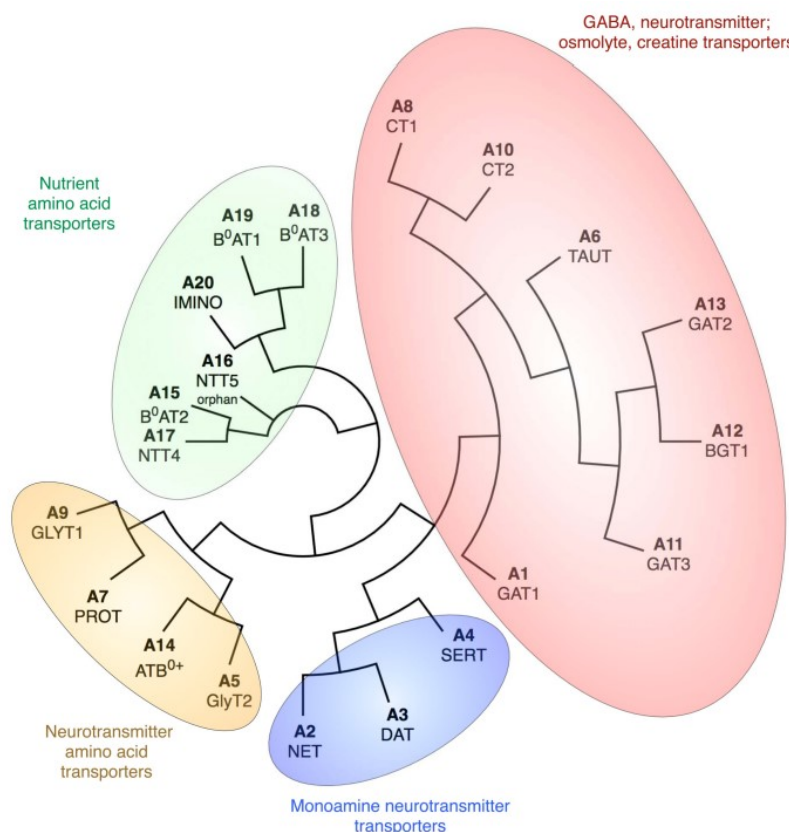


Figure 1: Phylogenetic tree of human SLC6-transporters. Image adapted from Bala et al., 2013

The discovery that reuptake-inhibitors are able to alter synaptic activity caused monoamine reuptake transporters to become an important research topic. Different members of SLC6 transporters have thus been used in cloning and animal studies to determine their substrate specificity.¹³ As research had advanced, the physiological implication of NSS-modulation and their role as target for antidepressant medication has been widely understood. However, the three-dimensional structure of monoamine transporters had only been rudimentary explored. Studies to understand NSS topology have focused on methods like partial proteolysis, mutation studies, cysteine accessibility

studies and hydrophobicity analyses. First results concluded that NSS transporters consist of 11-13 transmembrane helices and have a high level of sequence similarity, thus allowing information acquired about one transporter to be extrapolated to the others. This insight was of great importance as the bacterial homologue LeuT, a leucine transporter found in *Aquifex aeolicus*, was found to form complexes with antidepressant drugs.¹⁴ After crystallized structures of LeuT bound to different substrates were obtained, a first glimpse of the binding mechanism and three-dimensional structure of their mammalian counterparts were given.

1.3 Structure of SERT

The human serotonin transporter is composed of 632 amino acids, forming a total of 12 transmembrane helices. Both N- and C-termini are located intercellularly and a large loop between helices III and IV is glycosylated.¹⁶ 10 of the 12 helices constitute the core of the transporter, arranged in a so-called “5+5 inverted repeat”. Two bundles of five helices (TM1-6 and TM6-10) are aligned antiparallel to each other. This yields a pseudo two-fold axis of symmetry. The remaining two helices (TM-11 &-12) reside next to the bundles and play a less critical role in substrate transport.

SERT can also form oligomeric structures.⁶¹ It is reported that SERT can build oligomers of up to 5 and more individual transporters. Oligomerization shows different functions in SERT and other monoamine transporters, ranging from cellular trafficking to and from the endoplasmatic reticulum, functional cooperativity and suggested roles in reverse transport.⁶²

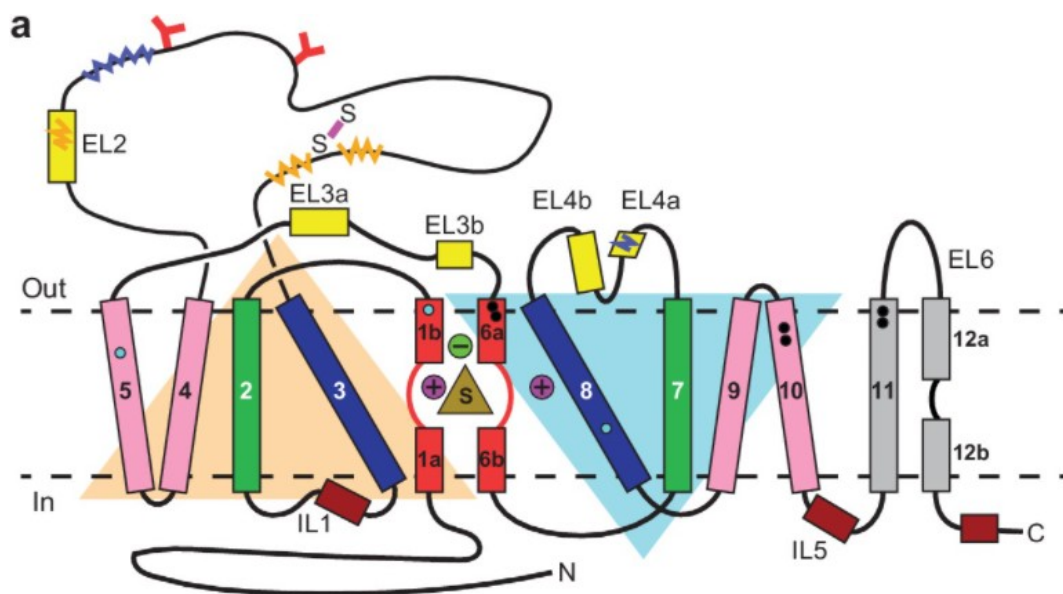


Figure 2: Topology of the human Serotonin Reuptake Transporter. The substrate (triangle, sand coloured) bound in the central binding site, flanked by two sodium ions (purple spheres) and one chloride ion (green sphere) are depicted. The light orange and blue triangles display the pseudo two-fold helical repeats, which are aligned antiparallel to each other. Image adapted from Coleman et al., 2020

SERT contains three distinct ion and two substrate binding sites – one central and one allosteric. The following section will describe these sites in detail.

The central binding site (referred to as “s1”) is made up by amino acid residues from TMs 1, 3, 6 and 8. Two binding sites for sodium atoms (referred to as Na1 and Na2) can be found in proximity to s1, also inside the central cavity of SERT.

The Chloride binding site consists of residues of TM7, TM6 and TM2. As it is close to Na1, it is assumed that the positive charge of one sodium atom needs to be compensated by one negative charge. Research suggests that an abundance of chloride is decreasing serotonin transport.¹⁹

Interestingly, chloride-independent transporter LeuT is shown to have one negatively charged glutamic acid residue (E290) in the same region as the Cl⁻ site in chloride dependent NSS-transporters.¹⁹ This reinforces the assumption that one negative charge in proximity to Na1 is necessary for substrate transport. Site s1 is also separated from the intracellular and extracellular medium by two gating regions, which are highly conserved amongst all SLC6 members.

Sodium sites Na1 and Na2 have each essential roles in either transporter stabilisation or substrate transport:

Sodium bound at Na1 was found to be responsible for stabilizing unwound segments in TM1 and TM6. In LeuT, it is also in contact with the substrates' carboxyl-group. As monoamines lack such a functional group, an aspartates acidic side chain is believed to interact with it instead. As this aspartate is highly conserved among monoamine transporters, it is believed to function as a replacement for the carboxy-group of leucine in LeuT-mediated transport.²¹ Initial binding of sodium stabilizes the transporters outward-open conformation as well, offering easy access to the s1 site.²⁵ A second sodium bound at Na2 is assumed to be the one that is co-transported with every transport cycle. Amino acid Serine-438 (Ser-438) coordinates sodium in Na2 when substrate is not bound. However, when substrate is bound to the s1-site, the side chain of Ser-438 rearranges and no longer coordinates any sodium bound at Na2, therefore allowing it to be released.²¹

The s2 site is located in the so called extracellular vestibule¹⁶, a pathway that connects extracellular solvent to the s1 site.²³ It is above the central binding site and embedded by TMs 1, 6a, 10, 11 and extracellular loops EL2, 4 and 6. That second binding site is suggested to trigger conformational changes, allowing substrates in the s1 site to be released into the cell. As s2 and s1 are separated by an extracellular gate, allosteric mechanisms are suggested for this site. Experiments conducted with citalopram, an antidepressant drug known to inhibit SERT-mediated serotonin transport, shows reduced dissociation rates at the s1 site when s2 is occupied.²²

As binding mechanisms of the s1 site are discussed more thoroughly in this thesis, a closer and more detailed look into its topography is necessary:

The S1 binding site can be divided into three different subsites named A, B and C, based on their properties and interaction with ligands.^{6,24,25} Important residues in subsite A are Tyr-95 and Asp-98, the latter being absolutely conserved among monoamine transporters. Both TM1 and TM6 form this subsite. Asp-98 is forming a salt-bond with either the substrates or inhibitors protonated amine group, an essential interaction for monoamine transport but also for inhibitor binding. Subsite B is described as a mostly hydrophobic pocket, lined by Ala-173, Tyr-176, Asn-177 and Thr-439 whereas Subsite C is also lined by hydrophobic side chains of Ile-172, Phe-334 and -341.²⁶

The S1 binding site with its gating regions is very conserved across all SLC6 members. A 55-67% sequence similarity underlines the importance of these structural surroundings and their therefore implied role for substrate transport.

1.4 Transport Mechanism

Apart from passive diffusion, there are three distinct ways for molecules to be moved across cellular membranes:

First, there are ATP-powered pumps that actively use energy in form of ATP to move molecules or ions against a chemical gradient.²⁷ Second, there are channel proteins which form a protein lined passage allowing water and/or ions to move across the membrane with the chemical gradient. And third, there are transporters. Contrary to channel proteins, transporters bind only one or few substrates at a time and undergo conformational changes in order to move exclusively these molecules or ions against their concentration gradient across the membrane.²⁷ The essential mechanism for these conformational changes is called “alternating access”: Here, the substrate binding site is made accessible from only one side of the membrane. After substrate has bound, the transporter undergoes conformational changes. As a result, the binding site reopens to the other side of the membrane and allows for substrate and any co-substrate to be released. This model suggests that the binding site can be accessed from either side of the membrane via two distinct pathways, of which only one of them is open at a time.³¹

Transporters can further be classified by the direction they facilitate transport of small molecules or ions. “Uniporters” transport just one molecule or ion per transportation cycle. This happens passively as only the electrochemical potential is directing substrate flow.³⁰ Symporters couple transport of one molecule against its concentration gradient with one or more ions down their respective electrochemical gradient. They utilize the concentration gradient of specific ions to move molecules across membranes.²⁷ Therefore symporters can be called “secondary active transporters”.

“Antiporters” function in a similar manner to symporters, only the relative direction of substrate transport is opposite to those of symporters. They exchange substrate for ion or vice versa rather than transporting both in the same direction.³⁰

The key aspect of symport is that neither substrate nor driving ions can be transported separately. Only together can they be moved across the membrane. As mentioned before, SERT couples substrate transport to the sodium gradient and can therefore be described as a secondary active transporter – hence it's affiliation with the “Neurotransmitter Sodium Symporter Group” (NSS).

SERT belongs to a group of transporters sharing the LeuT fold.²⁸ Their alternating access-mechanism is called the “rocking-bundle model” or also “gated-pore mechanism”.³⁰ Regardless of their naming schemes, both models suggest that the central binding site is enclosed by two gates allowing alternating access to the binding site from either side of the membrane. In addition, the transporter is split up into two distinct domains: scaffold and bundle.

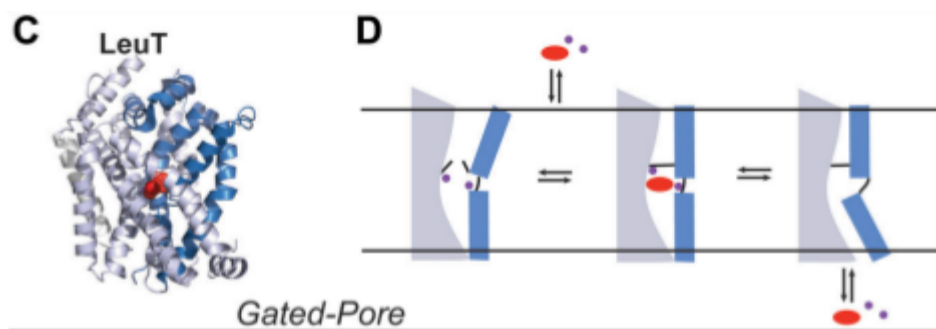


Figure 3: The gated pore mechanism of transporters utilizing the LeuT fold. Scaffold domain (light blue), bundle domain (blue), substrate (red) and ions (purple spheres). Image adapted from Colas et al., 2016

During a transport cycle, the scaffold domain remains static. The bundle domain undergoes conformational changes to bind and release the substrate.³⁰ In SERT, the helices TM1, TM2, TM6 and TM7 are believed to comprise the bundle domain undergoing conformational changes.²⁸ TM3-5 and TM8-10 comprise the scaffold region. The gating regions are located within the extra- and intracellular pathways. Two gates can be found in the extracellular vestibule: Glu-493 (TM10) and Arg-104 (TM1b) can form a salt-bridge and Tyr-176 (TM3) and Phe-334 (TM6a) form a hydrophobic lid, shielding the bound substrate from the extracellular side. For the intracellular pathway, a group of residues from TM5, TM6, TM8, TM9 and the N-terminus are responsible for regulating the opening and closing of said path.²⁸

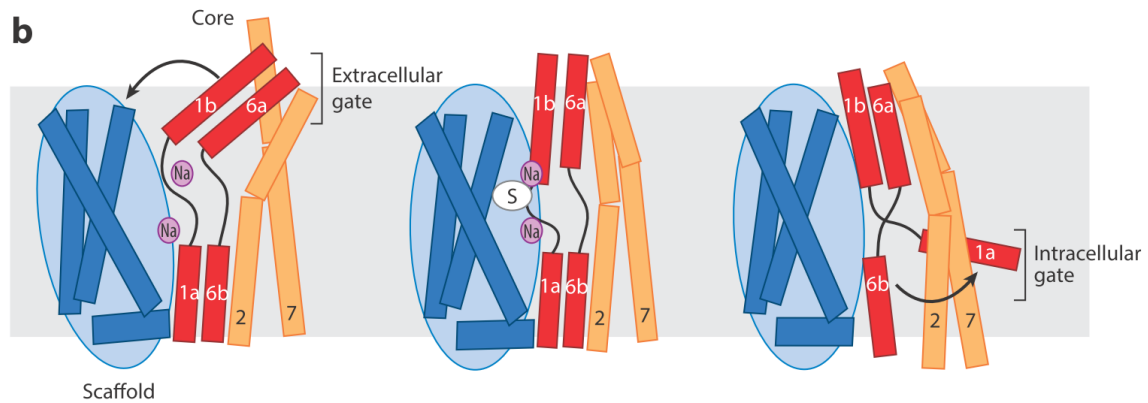


Figure 4: Example of the rocking bundle alternating access-mechanism. The core domain is synonymous with the bundle domain explained in Figure 3. TM1 and TM6 are displayed in red, the substrate is shown as the white sphere. During one transport cycle, the transporter adopts different conformations. The outward open (left) Intermediate occluded (centre) and inward-open (right) conformations show the distinctive states of the rocking bundle mechanism. Image adapted from Drew et al. (2016)

SERT and other transporters utilizing the LeuT fold undergo a succession of different states when transporting their respective substrates. A variety of procaryotic structures with the same 5+5 inverted repeat fold became the baseline for research on this field as they provided snapshots of different transporter conformations.¹⁶

One transport cycle of SERT begins with the transporter in the outward open state. Two sodium ions bind to their respective Na1 and Na2 sites. Then, serotonin binds to the central s1 site and causes conformational changes.¹⁶ The extracellular parts of TM1 and TM6 move towards the scaffold domain, closing the extracellular gates. This so called outward-occluded state is the first intermediate state after substrate binding. It secondly moves on to the fully occluded, and subsequently to the inward occluded state. The last state is the inward-open state. After this has been reached, 5-HT and one sodium ion are released into the cytosol.²⁸

One potassium ion then binds to the transporter, causing it to reset its conformational stages and gets “antiported” out of the cell into the synaptic cleft. Where and how potassium binds within the transporter is still unknown.²⁸

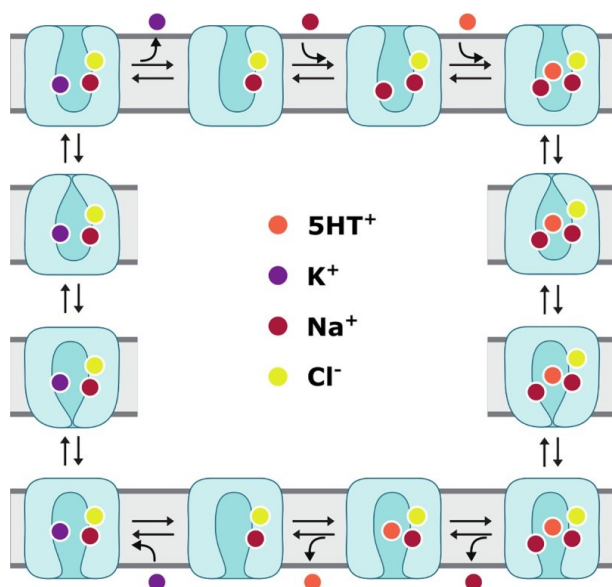


Figure 5: One transport cycle of hSERT displayed schematically. The respective ions and substrates are displayed in the middle using different coloured spheres. (Image adapted from Hellsberg et. al, 2019)

1.5 Cathinone derivatives

1.5.1 Introduction on Amphetamine and Cathinone

Cathinone derivatives are phenylethylamines, synthetically produced analogues of cathinone, the natural psychoactive compound found in the *Catha edulis* plant.³² In turn, cathinone is the β -keto variant of amphetamine, a drug discovered in 1910 and first synthesized in 1927.³³ As derivatives of these two substances are studied in this thesis, this section, offering an overview of said substances, is included.

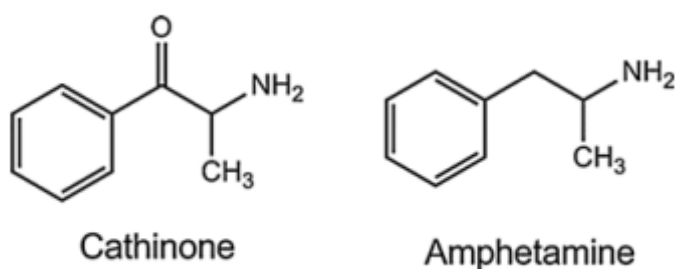


Figure 6: Chemical structures of Cathinone and Amphetamine. Image adapted from Valente et al., 2013

Amphetamine quickly became a substance of interest due to its stimulant properties and its ability to counter drug-induced anaesthesia and to treat narcolepsy. The company *Smith, Kline and French* soon marketed it under the name “Benzedrine” as a treatment for a plethora of diseases, including epilepsy, narcolepsy, fatigue and depression.³⁴ Due to its stimulant properties, amphetamine was also included as effective ingredient of so called “Energy Tablets” and distributed among British Forces in World War 2. The US forces distributed Benzedrine inhalers, whereas Japanese and German forces supplied their troops with the derivative metamphetamine.⁴² Amphetamine was also used to treat children suffering from attention deficit/hyperactivity disorder (ADHD) and it was commonly

taken to prevent weight gain.⁴² Today, amphetamine is FDA-approved only for the treatment of ADHD and narcolepsy. Due to its euphoric effects, recreational usage is still prevalent.³⁵ The β -keto variant of amphetamine, cathinone, stems from the plant *Catha edulis*.³⁶ The plant commonly known under the name “Khat” is cultivated in the Republic of Yemen and other countries of eastern Africa. Chewing its young buds and leaves causes the consumer to attain a state of euphoria and stimulation.³⁷ Khat chewing seems to be a cultural and social activity, supposed to enhance social interaction. Some students chew khat because of its stimulating properties, causing states of “increased alertness and ability to concentrate”.³⁷ These effects are caused by the major naturally occurring compound cathinone, which is also a member of the phenylethylamine type of substances. It was isolated from the plant in 1975 and became the basis for further synthesis of derivatives. In a similar manner to amphetamines, some synthetic derivatives of cathinone such as bupropion or diethylpropion were developed for therapeutic purposes.³⁹ They were intended to treat diseases like anorexia or depression.

1.5.2 Addictive Qualities and Substance Abuse

The addictive qualities of amphetamine, cathinone and their later synthesized derivatives raised interest for recreational usage. After World War 2, a surplus of amphetamines created for the war effort entered the black market. In the 1950s, the addictive and abuse potential was recognized³³ and the widespread negative health consequences became more evident in the 1960s.⁴²

Later research reports psychiatric adverse effects in some cases such as psychotic states, erratic or aggressive behaviour and paranoia.⁴¹ These side effects, together with the upcoming of tricyclic monoamine reuptake inhibitors like Imipramine⁴⁰ in the 1950s, questioned the safety of amphetamine prescription. These new tricyclic monoamine reuptake inhibitors (or tricyclic antidepressants, TCA) were meant for replacement of amphetamines. Still, it was well until the 1970s that amphetamine prescription rates started to drop significantly in the United States, as the new group of TCAs were not as popular with patients and showed lower compliance.

Cathinone on the other hand has also shown addictive qualities, albeit not as strong as in amphetamine.⁴³ In 1985, the WHO Committee on Drug Dependence issued a report stating that cathinone is about half as potent as amphetamine. Further research in that time period showed signs of dependence or tolerance not necessarily on a physical but rather on a psychic level. More recent research conducted in a city of Somalia suggests a correlation between khat usage and psychotic disorders but doesn't prove that the usage of khat itself is the only cause.⁴⁴ Nevertheless, there is a lack of data on khat usage outside of its social context, as it is a drug more commonly consumed in the previously mentioned eastern African states or within their respective immigrant groups elsewhere.⁴⁵

1.5.3 Emergence of Synthetic Derivatives

To stress the wide and common usage, the World Drug Report of 2021, issued by the United Nations' Office on Drugs and Crime, estimates 27 million people aged between 15-64 used amphetamine or amphetamine-like substances, including structural deviations and non-medically used pharmaceutical stimulants.⁴⁶ The trend report shows that amphetamine and amphetamine-like substances have become prevalent on the worldwide drug market on almost all continents. But as cathinone remained a drug not commonly used among the broader public in Europe and amphetamine almost disappeared as a prescription medication, how can the reappearance of this type of substances be explained?

To put it briefly, the trend of new "designer drugs" emerged after new legislation had passed in the 1970s.⁴⁷ In the United States, chemical substances were classified on a scale based on their risk and abuse liability. Following the passing of that legislation, a new set of substances were created to mimic and abuse the effects of popular illicit prescription drugs. The goal was to bypass legislation as these new compounds were not yet scheduled. They were given the name "designer drugs", often describing molecules synthesized in backdoor laboratories in smaller scales. Some of these designer drugs were also synthesized by pharmaceutical companies decades ago with the prospect of using them medicinally. MDMA for example was originally synthesized by Merck in 1912 as an appetite suppressor, then it was "rediscovered" in the 1970s for recreational usage.^{47, 48}

It is a similar story for mephedrone (4-methylmethcathinone) and methcathinone: mephedrone was first synthesized in 1929 and methcathinone in 1928.⁴⁹ Both are synthetic cathinones with stimulant properties. Methcathinone was intended as antidepressant at first but was then subsequently banned due to reports of widespread abuse.^{50,51} After that it was until the 2000s that this type of substances reappeared, albeit in a bigger way: they reappeared as ingredients of several "legal highs" in certain countries.⁵¹ Methylone emerged around 2004 in Japan and the Netherlands, derivatives with an alpha-pyrrolidinophenone structure appeared in Germany around the same time⁵² and Mephedrone itself entered the Israeli market around 2007. They were primarily marketed as party drugs and alternatives to MDMA and Cocaine.⁴⁷ Interest in these synthetic cathinones had returned in the 21st century, as mephedrone was someday prevalent in 20 European countries which forced legislators to act.⁵³

1.5.4 Chemical Structure of Cathinone Derivatives

Synthetic cathinones are phenylalkylamine derivatives. The structural difference between them and amphetamine lies in a keto-group located at the β -position of the amino-alkyl chain bound to the phenyl ring.³⁸ Using the structure of cathinone as a template for further synthesis, different substituents can be added at different positions of either the phenyl ring, α -position of the alkyl-side chain, the methyl-group adjacent to said α -carbon or the nitrogen-atom. This has led to a diversity of different structures with over 30 identified.

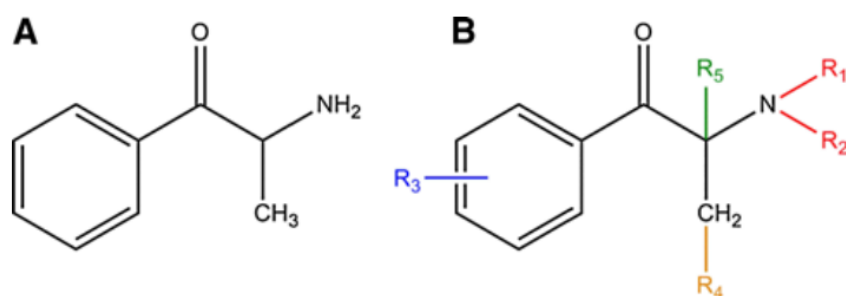


Figure 7: Chemical structure of cathinone (A) and general structure of synthetic cathinones (B) with options for further substitution (R1-5) highlighted in different colours. Image adapted from Valente et al., 2013

Synthetic derivatives can be divided in 4 groups, based on their chemical similarities:

The group investigated in this thesis are N-alkylated cathinones, some of which with substituents at the ring. Members include mephedrone, diethylpropion, and ephedrone. This group includes derivatives originally designed as either anorectics (diethylpropion and dimethylpropion), as antidepressants (bupropione, which is still prescribed today⁵⁴) or recreational drugs (like mephedrone, 4-MEC, MEPH).

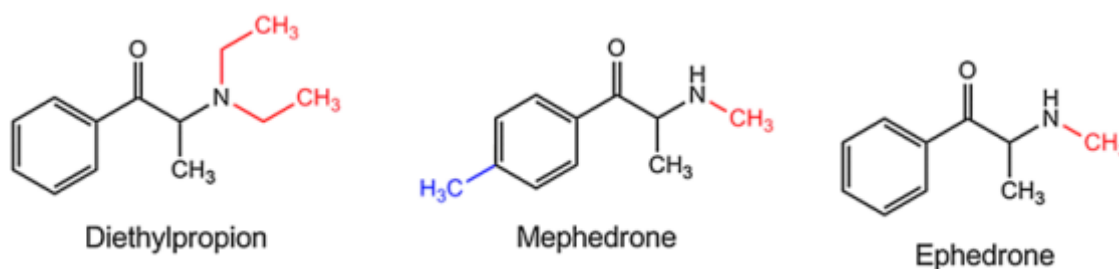


Figure 8: Example chemical structures of N-alkylated cathinone derivatives. Chemical modifications to cathinone structure highlighted in red and blue. N-alkylation highlighted in red, substitution at the ring highlighted in blue. Image adapted from Valente et al., 2013

The other groups are 3,4-methylenedioxy-N-alkylated derivatives (like methyldone, butylone or MDMA), a pyrrolidinophenone-like group (characterized by pyrrolidinyl substitution at the N-atom with members like MPPP, α -PVP or MPBP) and a group of 3,4-methylenedioxy-N-pyrrolidine cathinones, essentially combining properties of the two aforementioned groups. Members include

compounds like MDPPP and MDPBP. These groups were not of interest during the completion of this thesis and are just mentioned for the sake of completion.

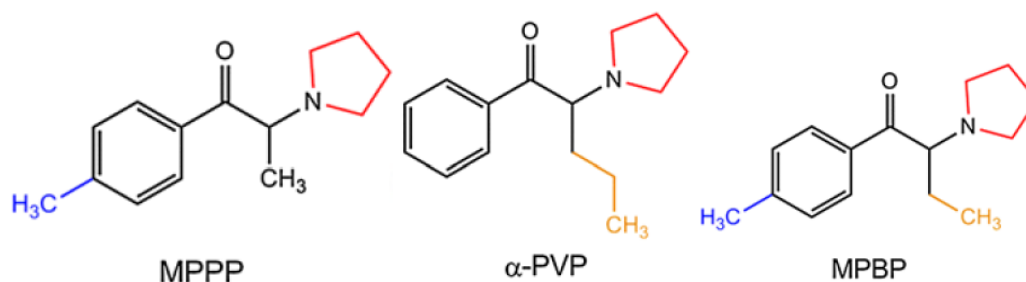


Figure 9: Example chemical structures of N-pyrrolidine cathinone derivatives. Chemical modifications to cathinone structure highlighted in red, blue and yellow. Image adapted from Valente et al., 2013

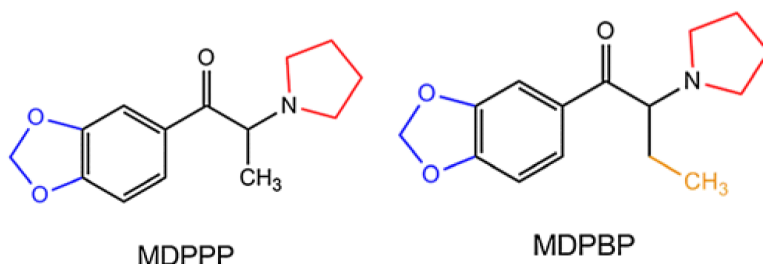


Figure 10: Example chemical structures of 3,4-methylenedioxy-N-pyrrolidine cathinone derivatives. Chemical modifications to cathinone backbone highlighted in red, blue and yellow. Image adapted from Valente et al., 2013

1.5.5 Stereochemistry and Metabolism

The class of phenylalkylamines, including amphetamines and cathinones, are chiral compounds.⁶⁵ Due to their asymmetric carbon atom in the side chain, two enantiomers exist: (S)- and (R)-form. Based on their configuration, (S)- and (R)-enantiomers differ in terms of pharmacological activity and metabolism.

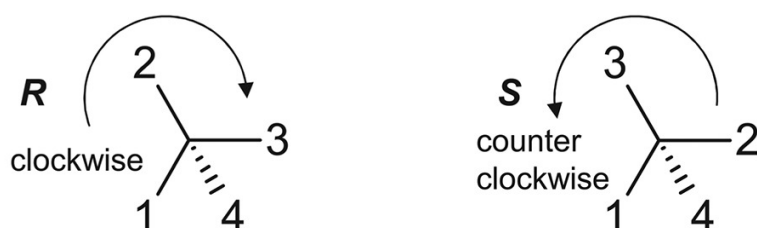
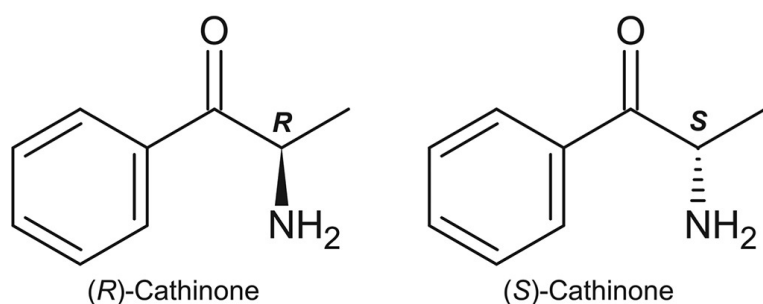


Figure 11: Chemical formula of (R)- and (S)-Cathinone together with an exemplary carbon-atom in either (R)- or (S)-configuration. Based on the priority of the substituents (1 being the highest and 4 being the lowest) and their arrangement in a three-dimensional space, it can be determined as either R- or S-configured. Image adapted from Schwelm et al., 2020

(S)-Amphetamine for instance is three to five times as potent as its (R)-enantiomer in terms of psychostimulatory effects.⁶⁵ Users injecting (R)-methamphetamine describe its effects as less desirable in comparison to the (S)-variant. This leaves the (S)-enantiomer of amphetamine to be more frequently abused and associated with higher potency in regards of causing central nervous effects, whereas the (R)-enantiomer shows no signs of abuse liability.⁶⁷ Furthermore, formulations sold for medical usage differ in terms of use case and effects based on whether (R)- or (S)-enantiomers are prescribed. Examples would include (S)-methamphetamine which is prescribed as a prescription-only medication in the United States whereas (R)-methamphetamine is given as an over-the-counter inhalable decongestant.

Amphetamines and cathinones were reported to undergo stereoselective metabolism.³⁸ The natural cathinone for example undergoes phase I metabolism in the liver. The reduction of the β -keto group is producing two different molecules, cathine and norephedrine.

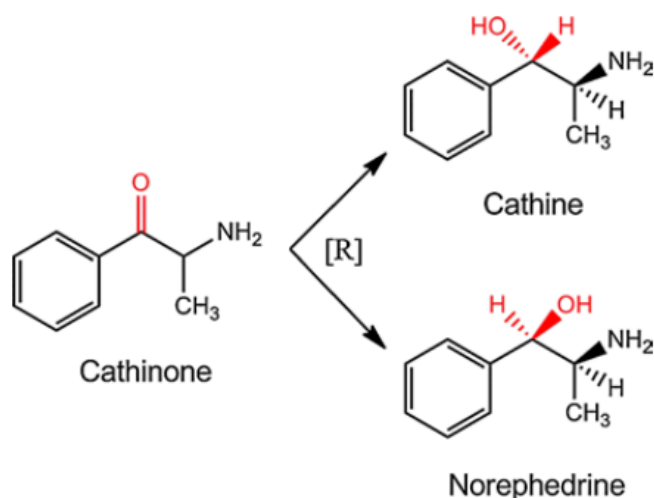


Figure 12: Depiction of phase I metabolism of cathinone. Shown is the reduction "[R]" of the keto group, resulting in metabolites Cathine and Norephedrine. Image adapted from Valente et al., 2014

Mephedrone is observed to undergo three distinct phase I metabolic pathways with 8 different metabolites identified in either rat or human urine samples, some detected only in vivo.⁶⁶ CYP450 2D6 was identified to be the main responsible enzyme for metabolising mephedrone in human bodies. The metabolism is more extensive and includes N-demethylation, oxidation of the ring methyl-group which can be further oxidized into a carboxylic acid and reduction at the beta-keto group. This leads to a variety of different enantiomers, as reduction of the keto group provides a second chiral centre and may therefore result in 4 different enantiomers.

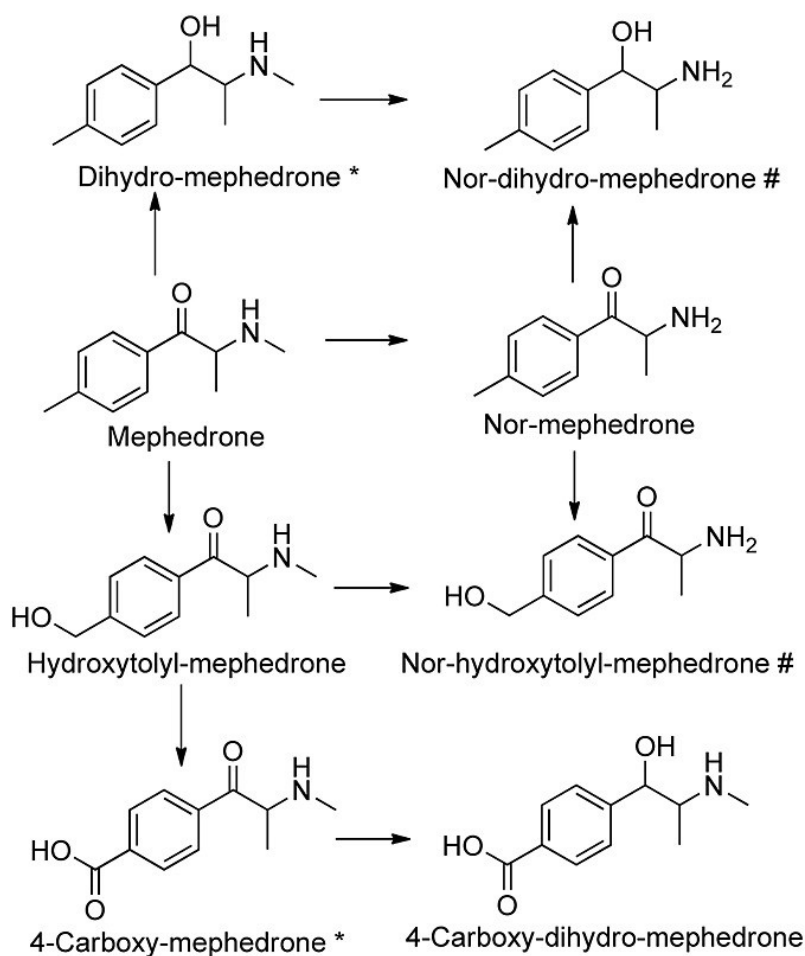


Figure 13: Suggested phase I metabolites of Mephedrone. No emphasis on stereochemistry is displayed in this figure. Please note that there are at least two enantiomers for each substance, four for the ones that have undergone reduction of the keto group. Image adapted from Pedersen et al., 2012

1.5.6 Mechanism of Action

As structural semblance and similar stimulatory effects between the synthetic derivatives exist, amphetamine and naturally occurring compounds like cathinone are expected to show similarities in pharmacological effect and mechanism of action.³⁸ Based on that assumption it was predicted that synthetic cathinones would act as stimulants in the central monoamine system, allowing for more neurotransmitters to be released or to remain in the synaptic cleft.⁴⁷ Following the widespread arrival of cathinone derivatives on the market and the subsequent preclinical research, this prediction has been proven. The monoamine transporters SERT, NET and DAT seem to be affected.

There are two known mechanisms that explain stimulant-induced increase in extracellular monoamines: blockage of transporter mediated uptake and elevated presynaptic release. Blockage of transporter mediated uptake is generally achieved by stabilizing the corresponding transporter in its conformation.⁶ Antidepressant drugs like selective serotonin reuptake inhibitors (SSRIs), tricyclic antidepressants (TCAs) or recreational ones like cocaine function this way and are considered “blockers”.⁵⁵ They are not transported into the cell.

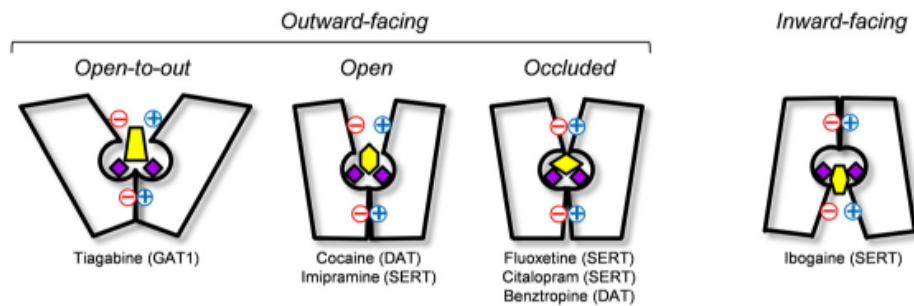


Figure 14: Schematic illustration of different inhibitors (yellow squares or hexagons) stabilizing monoamine transporters in various conformations. Image adapted from Kristensen et al., 2011

The other way of increasing extracellular monoamine levels is by stimulating neurotransmitter release. Synthetic cathinones are known substrates of NET, DAT and SERT.⁵⁵ They are transported into the cell by the transporters and are suggested to disrupt vesicular storage and stimulate non exocytotic release by initiating transporter-mediated efflux.⁵⁶ Mephedrone, methyline and MDMA are considered non-specific transporter substrates (meaning that they bind and are transported by SERT, NET and DAT in a similar manner) whereas amphetamine is more selective for NET and DAT.

There are many models that try to explain the exact modalities of reverse transport. Sitte et al.⁵⁶ provide an in-depth review: They suggest two types of reverse transport. One is tied to changes to ion gradients between the extra- and intracellular fluid and the other one is tied to the binding of releasers to the transport protein.

Normal transport under physiological conditions begins when substrate is bound to the outward facing conformation of the transporter, together with its co-substrates sodium and chloride. As the extracellular gradient of sodium is a lot higher compared to the intracellular one, sodium acts as the driving force for substrate transport into the cell, thus making inward transport more favourable. As substrate and co-ions are released into the presynaptic cell, the inward-open conformation of the transporter should in theory be reaccepting the substrate and transport it outwards again. This however does not happen as intracellular substrate is re-stored in presynaptic vesicles, mediated by vesicular transporters. Therefore, the intracellular levels of substrates are low in comparison to the extracellular levels and transporter-mediated efflux does not happen. This would explain why endogenous substrate is not reverse transported out of the cell. Intracellular sodium is removed via a Na^+/K^+ -ATPase that exchanges sodium for potassium ions, maintaining the ion gradient.

The research conducted by Sitte et al. suggest that changes to the sodium gradients could change the direction of substrate transport. A sodium gradient that is higher in the intracellular side would cause the transporter to rather accommodate the inward-open conformation and would allow for substrate to be released out of the cell. Even if the electrical currents conducted by transporter proteins are significantly lower in comparison to ion channels, changes to that mechanism could have significant effects on the equilibrium of substrate intake and release. Amphetamines are suggested

to trigger an increased influx of Na^+ -ions by making the transporter change into a “channel-mode”. This would allow for a influx of sodium, causing membrane depolarization and the transporter to favour an inward-open conformation, therefore allowing even more substrate to be released. This notion is reinforced by research of Sitte et al.⁶⁴, showing that dopamine reuptake transporters have a 25% higher conductivity when bound to amphetamine than their usual substrate dopamine. This increased influx of sodium, the concluding changes in ion gradients and the subsequent reverse transport could be one way that amphetamines cause reverse transport.

The next model mentioned by Sitte et al. is an oligomer-based counter transport model.⁵⁶

As mentioned before, SERT and other members of the monoamine transporter family have shown the ability to form oligomers. Here, an important role of transporter oligomerization in conjunction with the mechanism of action of amphetamines is explored: in this model, one releaser (e.g. the amphetamine molecule) is transported into the cell by one single transporter whereas the resulting efflux of substrate is performed by the remaining transporters in its vicinity.

This is highlighted as a highly plausible solution due to the following observations:

a) in past experiments, bell-shaped concentration-response curves have been observed with amphetamines.⁶³ As one transporter alone cannot perform substrate efflux and releaser influx simultaneously, multiple individual transporters must be involved. At 50% occupation, the maximum substrate release should be achieved. In this state, half of the transporters move amphetamine inwards as the other half moves substrate outwards. Higher levels of amphetamines should decrease substrate efflux as it would cause more transporters to be facilitating releaser uptake than substrate efflux. As bell shaped curves for the concentration-response have repeatedly been reported, strong evidence for this theorem is suggested.

b) Past research conducted by Seidel et al.⁶³ shows that a concatemer of serotonin- and amphetamine-resistant GABA-transporters (GAT-1) is affected in a similar way as SERT-oligomers. Amphetamines were able to promote GABA efflux even if they were not binding to the GABA-transporters themselves. This indicates that the SERT-mediated Na^+ -influx might be picked up by GABA-transporters GAT-1 in its vicinity, thus coercing it to take on its inward-open conformation.

In addition to amphetamines' effects on monoamine transporters, they are observed to affect vesicular monoamine transporters 1 and 2 (VMAT1 & VMAT2) as well as MAO-A and -B.⁵⁶

VMAT-1 & -2 are responsible for transporting monoamines into synaptic vesicles.⁵⁷

MAO-A and MAO-B are located within the presynaptic cell and are responsible for degrading any neurotransmitters that are not re-stored into vesicles. Both VMAT- and MAO-systems cause intracellular levels of neurotransmitters to be low. Amphetamines happen to compete with endogenous substrate for VMAT and appear to be inhibitors of MAO-A and -B.⁵⁸

This would mean that:

a) the rate of endogenous substrate re-stored into vesicles will be lowered and b) any endogenous substrate left over is less likely to be metabolized. This substrate will then be available for reverse transport.⁵⁸

Other research conducted by Zestos et al. suggests an involvement of protein kinase-C (PKC) in amphetamine stimulated dopamine efflux.⁵⁹

PKC phosphorylates serine residues near the N-terminus of DAT, and it is believed that said phosphorylation is contributing to the amphetamine-induced efflux of dopamine. Especially the β -isoform of PKC (PKC- β) debilitates the efflux when inhibited. However, the impact of PKC is not well understood as they are not the only kinases phosphorylating DAT (calmodulin dependent kinase 2 α also modulates DAT for example).⁵⁶ Neither the role of different PKC isoforms nor their respective affiliations to different transporters are fully clarified.

To summarize this chapter, it suffices to say that the exact mechanism of action of amphetamine-like substances is complex not fully understood. Amphetamine-like substances are suggested to interact with many components of synaptic neurotransmission and much is yet to be explored.

It is thus important to take away the following key facts:

#1) amphetamines and their synthetic counterparts act as substrates of the monoamine reuptake transporters,

#2) amphetamines also interact with monoamine-oxidases-A and -B and also with VMAT-1 and -2, thus interfering with storage and oxidation of neurotransmitters,

#3) the effects of amphetamine-mediated substrate efflux can be altered by protein kinase-C inhibitors,

#5) amphetamines are suggested to interfere with the stoichiometry of ion transport, changing sodium gradients and thus causing reverse transport and

#4) it is assumed that oligomerization plays a key role in understanding the mechanism of amphetamine-induced substrate efflux.

2. Motivation and aim for the thesis

2.1 Background

This thesis was initiated by a collaboration between the Pharmacoinformatics Research Group of the University of Vienna, led by Gerhard F. Ecker, and a division of the neuroscience research laboratory led by Harald Sitte of the Medicinal University of Vienna.

The research conducted in this thesis was intended as a follow-up of the work published by Felix P. Mayer, Daniela Cintulova, Harald H. Sitte et al., published in "Neuropharmacology Volume 148", in April 2019.⁶⁸ This research focused on a set of phase-I mephedrone metabolites and their effects on either the substrate flux on SERT, NET and DAT, the binding affinities on monoamine receptors and the abundance of the enantiomers in human urine. For this, their research team performed in vitro assays, namely uptake inhibition & release experiments as well as receptor and transporter binding and activation assays on human embryonic kidney 293 cells (HEK293 cells). Furthermore, they did enantiomeric profiling of nor- and 4-OH-mephedrone in human urine samples.

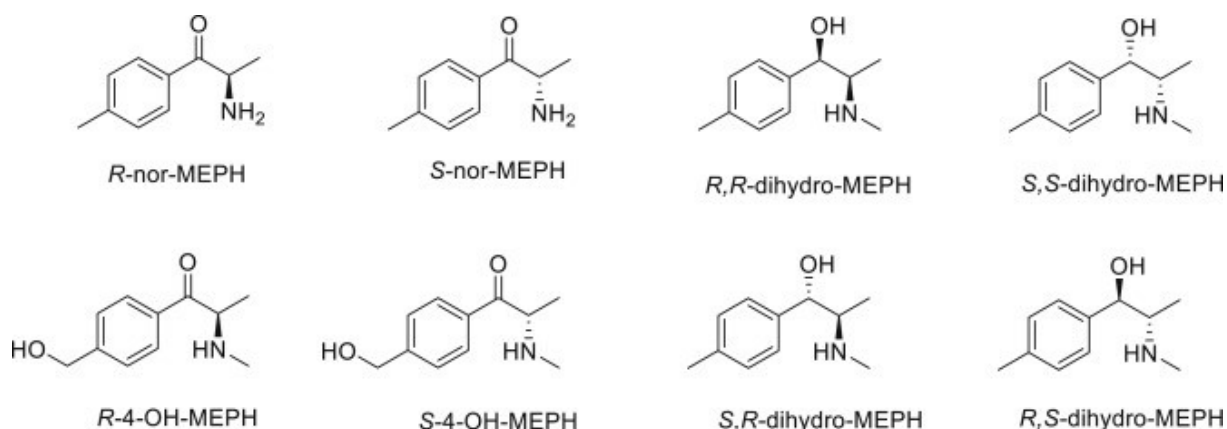


Figure 15: Chemical structures of phase-I metabolites. Emphasis on the stereoisomeric properties. Image adapted from Mayer et al., 2019

The results that served as a baseline for this thesis will be summarized in the following section:

A: The stereoisomers of phase-I metabolites are reuptake-inhibitors.

All of the tested stereoisomers of nor-, 4-OH and dihydro-mephedrone were inhibitors of SERT, DAT and NET, respectively. The IC₅₀ values for the metabolites were ranging from 3 to 10 μ M at NET and DAT with similar reported potencies for the enantiomers. At SERT, however, (R)-nor-mephedrone was 18 times less potent than its S-counterpart (observed IC₅₀ values were 200.0 and 10.9 μ M, respectively). (S)-4-OH-mephedrone and R-4-OH-mephedrone behaved in a similar manner, showing 19-fold differences in observed IC₅₀ (32.1 μ M for (S)-4-OH-mephedrone and 625.4 μ M for (R)-4-OH-

mephedrone) at SERT. The remaining four stereoisomers of dihydro-mephedrone showed different potencies at each monoamine transporter with IC₅₀ values ranging between 12 to 216 μ M.

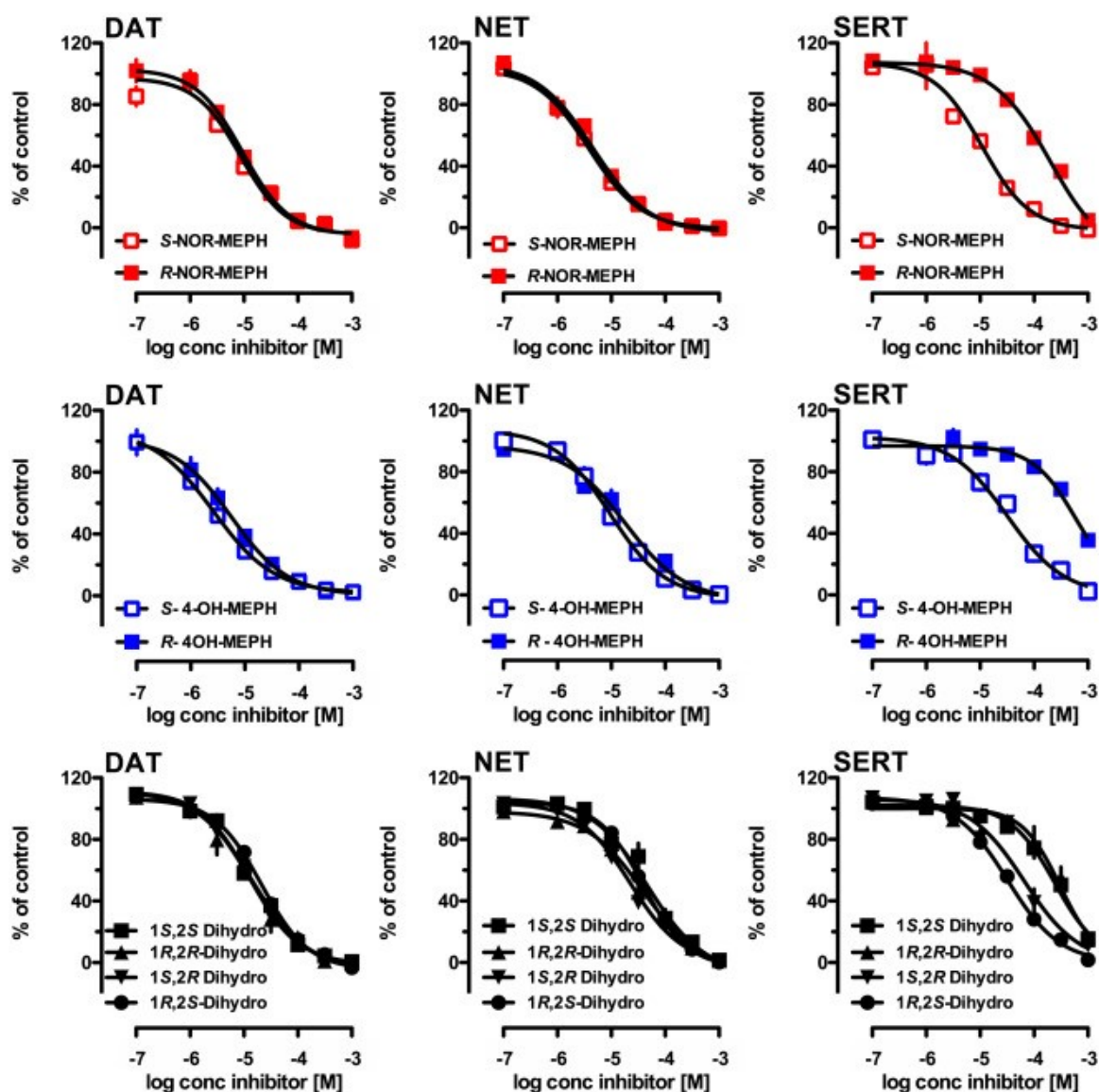


Figure 16: Effects of phase-I metabolites on transporter-mediated uptake into HEK293 cells. Uptake of [³H]MPP⁺ (NET and DAT expressing HEK293 cells) and [³H]5-HT (SERT expressing HEK293 cells) was inhibited with increasing concentrations of shown test substances. Shown are the differences in inhibition (y-axis) and the corresponding concentrations of the drugs (x-axis). Noteworthy are the differences between S- and R- diastereomers reported in SERT. Image adapted from Mayer et al., 2019

B: (S)-nor-mephedrone and (S)-4-OH-mephedrone affect SERT-mediated reverse transport differently than their (R)-counterparts.

Based on the results of the uptake-inhibition experiments, assays were performed to determine if reverse transport is also affected by the stereochemistry of the metabolites. Nor- and 4-OH-mephedrone were reported to induce reverse transport but in a manner different based on their stereochemistry.

For nor-mephedrone, (S)-nor-mephedrone showed no further increase in 5-HT release when

concentrations above 10 μM were applied. (R)-nor-mephedrone showed differences only at 30 μM and 100 μM when compared to the control. This indicates that S-nor-mephedrone might be a more potent releaser than R-nor-mephedrone, as lower concentrations of it initiate reverse transport.

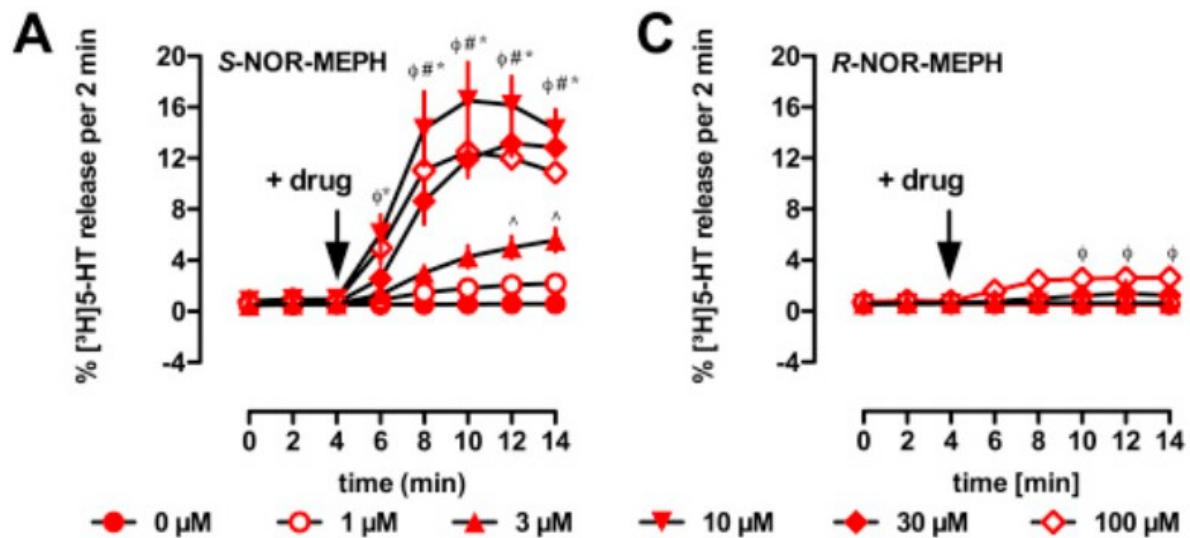


Figure 17: Effects of increasing concentrations of S-nor-mephedrone and R-nor-mephedrone on inducing reverse transport on human SERT. Plots show percentage of released $[^3\text{H}]5\text{-HT}$ (y-axis) against elapsed time (x-axis). The concentrations applied are shown as either full or hollowed circles, squares, or triangles. Test drugs were added after collection of three 2-minute fractions (at minute 0, 2 and 4 respectively) and 5 more fractions were taken after that, again in 2-minute intervals. (S)-nor-mephedrone shows higher potency to induce reverse transport more effectively and at lower concentrations than its (R)-counterpart. Image adapted from Mayer et al., 2019

4-OH-mephedrone showed a similar trend. (S)-4-OH-mephedrone showed significant effects on substrate-induced reverse transport with significant differences at concentrations of 10 μM or higher, whereas (R)-4-OH-mephedrone failed to show any effect at any concentration.

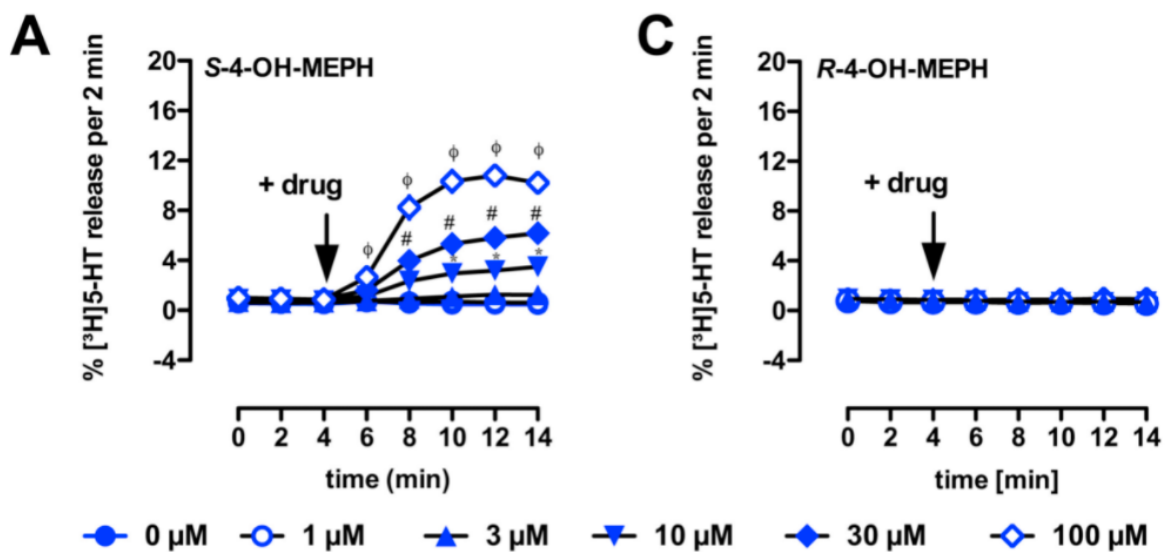


Figure 18: Effects of S- and R-4-OH-mephedrone on inducing reversed transport on human SERT. Percentage of released radiolabelled [3H]5-HT (y-axis) plotted against the elapsed time (x-axis). Test drugs were added after collection of three 2-minute fractions, five more fractions collected in subsequent 2-minute intervals. Different applied concentrations shown as either full or hollowed squares, triangles, or circles respectively. S-4-OH-mephedrones shows higher potency to induce reverse transport when compared to R-4-OH-mephedrone, as can be seen by flat shape of the (R)-4-OH-mephedrone curve. Image adapted from Mayer et al., 2019

In conclusion, the research shows that the pharmacodynamics of the phase-I metabolites at SERT are affected by stereochemistry, but not at NET and DAT. One of the assumptions resulting from this finding was that different interactions of S- and R-enantiomers within the binding pocket of SERT might cause this difference in potency. This was the initiator for this research thesis as Daniela Cintulova proposed a collaboration as a follow up for their findings.

2.2 Aim of this thesis

The findings described above raised questions about the stereoselective effects of substrates at the monoamine transporters NET, DAT and SERT. It is unclear why the stereochemistry of the phase-I metabolites caused no significant difference in potency at NET and DAT but showed significant divergences at SERT. We assumed that interactions at the SERT binding pocket must differ for (S)- and (R)-enantiomers and be the reason for the findings of Mayer et al. (2019). In addition, we hypothesized that interactions of non-conserved residues within the already highly conserved binding pockets of monoamine transporters might show differences in binding mode or positioning and therefore provide us with an idea of why SERT shows stereoselectivity different to its NET and DAT counterparts. We also checked if calculated affinities differ for (S)- and (R)-enantiomers, respectively, and used this thesis to assess scoring methods provided by different software solutions.

Stereoselectivity at SERT has already been observed when it comes to inhibition^{69,70,71}, with escitalopram - being the S-enantiomer of racemic citalopram – showing stronger binding to the s1 site than its enantiomeric counterpart, the latter even showing signs of antagonism.⁷²

Therefore, the aim of this thesis is to investigate the hypothesis of stereoselective binding of phase-I mephedrone metabolites at SERT and to look for differences in their binding mode. SERT was chosen as the first target to study, as it was the transporter where differences in terms of stereoselectivity were most pronounced. We used methods of chemo-informatics, which will be described in more detail in the next chapter.

3. Methods & Materials

3.1 In-silico drug design

Drug discovery, development and clinical testing is a lengthy, intense, and interdisciplinary endeavor.⁷³ Traditionally, scientists have relied on *in vivo* and *in vitro* models to test their hypotheses and to test new drugs.⁷⁴ Over the last decades the area of *in silico* pharmacology has steadily grown and is now a major contributor for the discovery and development of new therapeutic agents. The term “*in silico*” is a derivative of the already existing and commonly used terms “*in vitro*” and “*in vivo*”, describing studies performed within living organisms (*in vivo* translates to “in the living”) or outside of living organisms (*in vitro* = in glass). *In silico* refers to any methods done using computers or computer simulations and is a term first used in the early nineties.⁷⁴

The main advantages of *in silico* methods lie in their ability to allow for the processing of datasets of continuously increasing size and complexity in a time and cost-efficient manner. Nowadays, *in silico* methods are utilized in a plethora of different studies, including studying interactions of small molecules and their respective targets, pharmacophore-based modelling and screening, homology modelling or molecular dynamic simulations, just to name a few.⁷⁵ Beyond that, these tools have also allowed researchers to work with entire databases, thus allowing to access and process a wealth of experimental data. *In silico* methods allow for a simulation of biological systems and let us predict the pharmacodynamics or pharmacokinetics of potential drug candidates. All this simplifies the preselection of drug candidates with the prospect of finding more efficacious substances while decreasing risks of adverse effects. In the long run this decreases the risk of financial losses as new drugs should be less likely to be withdrawn from the market, neither due to insufficient efficacy nor due to adverse side effects. In addition, material and reagent usage is reduced as well as the total number of molecules produced, which further saves costs.

However, as each method has its shortcomings, *in silico* drug design has its own limitations⁷⁵ as well, and some predictions do not account for protein flexibility or molecule conformation, thus producing results varying in their predictive accuracy.⁷⁷

3.2 Molecular Docking

Docking is a technique in the field of molecular modelling which visualizes the favoured orientation of one molecule to another, in this case ligands to their target receptor protein. Different conformations of the ligand(s) are prepared and subsequently placed within a predefined binding pocket, a three-dimensional region located within the target protein. Scoring functions aid in distinguishing the more favourable poses, as they calculate binding energies or interaction patterns and rank poses based on their results.

In this research, the QPLD package (“*Quantum Polarized Ligand Docking*”) was utilized. It is part of Schrödingers Maestro Software version 2020-1⁷⁷, the GUI combining all Schrödinger products. QPLD combines Schrödingers Glide⁷⁸ docking algorithm with QSite⁷⁹, their in-house quantum mechanics/molecular mechanics solution. The algorithm begins with Glide docking, generating several protein-ligand complexes. After this step, QSite calculates atomic charges using “electrostatic potential fitting”. After this step has been completed, Glide re-docks the ligands and returns the energetically favourable poses. As for standard Glide Docking, a receptor-grid has to be generated first for the ligand conformations to be placed in.

3.2.1 Settings

Prior to performing the actual docking run, settings for ligand and protein preparation have to be determined, a receptor grid as well as the final settings for the docking run have to be set.

3.2.1.1 Protein Preparation

3.2.1.1.1 Choice of receptor .pdb-file

The receptor chosen for this research is a homology model of human SERT in its outward occluded conformation. The model was provided by Eva Hellsberg²⁸ and we would like to thank her on this occasion. At the time being, there is no model on the Protein Database that shows SERT bound to any substrate. As inhibitors on monoamine transporters tend to be rather large and bulky molecules, the actual binding pocket of transporters crystallized while bound to inhibitors remains large and doesn’t accommodate the smaller substrates properly. In addition, side chain orientations differ between the outward-occluded and outward-open state. As we are investigating substrate-like substances in this research, Hellsberg et al.’s model was chosen.

3.2.1.1.2 Protein Preparation Wizard

The .pdb-file needs to be uploaded in order to prepare the protein for further usage. The so-called Protein Preparation Wizard opens up a panel, offering the user the choice of which information the algorithm should add to said protein. In this case, the standard settings were chosen, and pre-processing was initiated. Afterwards, the hydrogen bonding was optimized, and the protein was energy minimized. The following “view problems” analysis registered no errors.

3.2.1.2 Ligand Preparation

LigPrep,⁸⁰ Schrödingers ligand preparation suite, allows the user to generate variations and energy minimized 3D structures of their ligand(s). It is a necessary step that adds hydrogens and takes into account ionisation states based on pH and stereochemistry.

In this research, ionization ranges were set for $\text{pH } 7.0 \pm 0.5$ in order to simulate physiological pH levels. Ionization and tautomerization were performed using Epik. Chiralities were retained from the input structure as specific ligands for (S)- and (R)- configurations were created.

3.2.1.3 Receptor Grid Generation

Ligands are docked into a predefined receptor grid, a three-dimensional space describing the active site of the protein. To set this up, the Receptor Grid Generation panel was used. After uploading the prepared receptor protein, the position and size can be defined. Furthermore, positional, hydrogen-bond or metal constraints can be applied.

For these docking runs, the .pdb-file of hSERT in its outward occluded state (now referred to by its filename "hsert_ceo.pdb"), was selected after protein preparation. The ligand in place, a docked molecule of serotonin in the s1 site, was picked to determine the centre of the grid. This is part of the Receptor Grid Generation panel, as it offers the user to pick the ligand in place by clicking on it in the GUI and using it to "define the receptor". No constraints were set.

3.2.1.4 Ligand Docking

As mentioned before, the QPLD algorithm is used to dock ligands within the receptor grid generated beforehand. As it is part of the maestro suite, it has its own panel. The panel consists of 5 different tabs, "Receptor", "Initial Docking", "QM Charges", "Redocking" and "Final Selection". In the "Receptor" tab, we selected our previously generated receptor grid (saved as a .zip file). For "Initial Docking", we checked the XP pre-set for docking precision and set the number of maximum poses per ligand at 25. In the "QM Charges" tab, we chose Jaguar and set the QM level to accurate. For "Redocking", the precision was set on XP again with a maximum of poses set to 20. For "Final Selection", we chose Glide-Score. All other settings not mentioned here were left at default.

After the docking run was completed, every pose of the LigPrep-file is generated in .sdf-format. Each pose has its docking score calculated, listed in kcal/mol and serves as a reference for binding free energies.

3.2.2 Redocking serotonin

Before docking molecules of interest, our docking method needs to be validated. This was done by docking a serotonin molecule into the binding pocket of our hSERT model. However, at the time being, we used a Glide docking protocol to redock serotonin, as the idea of using QPLD came up later. The goal was to replicate the position that Hellsberg et al.²⁸ have achieved during their research. The later docking runs utilized the QPLD protocol mentioned above.

Protein preparation and ligand preparation procedures are the same as for the QPDL docking runs. A .sdf-file containing one molecule of serotonin was used as the ligand. The settings were left at default with “use input partial charges” checked in the “Ligands” tab of the Glide Docking panel. No constraints are set, and 20 poses are set to be generated. The standard precision preset is chosen.

3.2.3 Docking phase-I mephedrone metabolites

Since the goal is to determine stereospecific binding patterns for the diastereomers of phase I mephedrone metabolites, they were docked into the s1 binding site of our hSERT homology model. For this, each enantiomer was treated as a separate entity and was added to the maestro workspace using the 2D-Sketcher, a tool that lets the user draw a chemical structure in 2D to then be imported as a three-dimensional molecule.

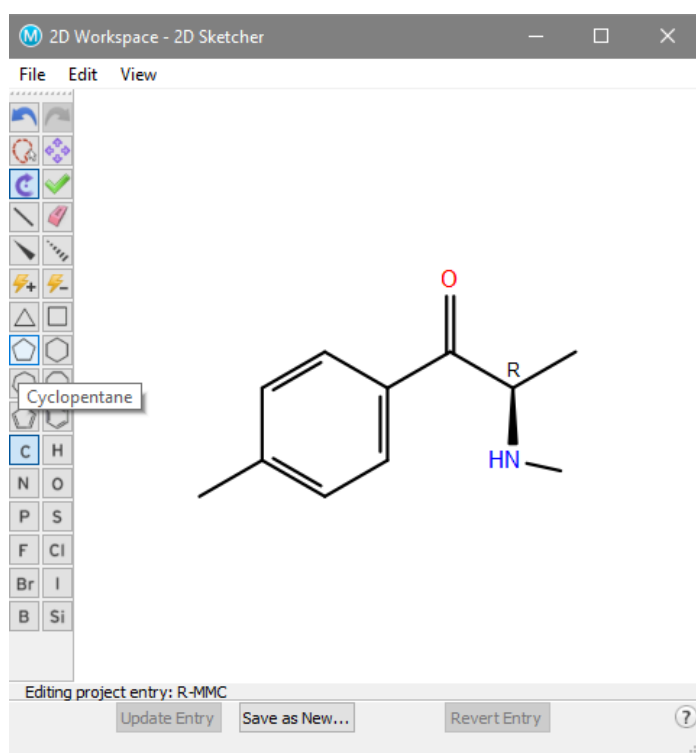


Figure 19: Screenshot of the 2D Sketcher panel. Molecules drawn can be imported as new entries to the workspace and then be worked with. Image taken from Maestro 2021-3

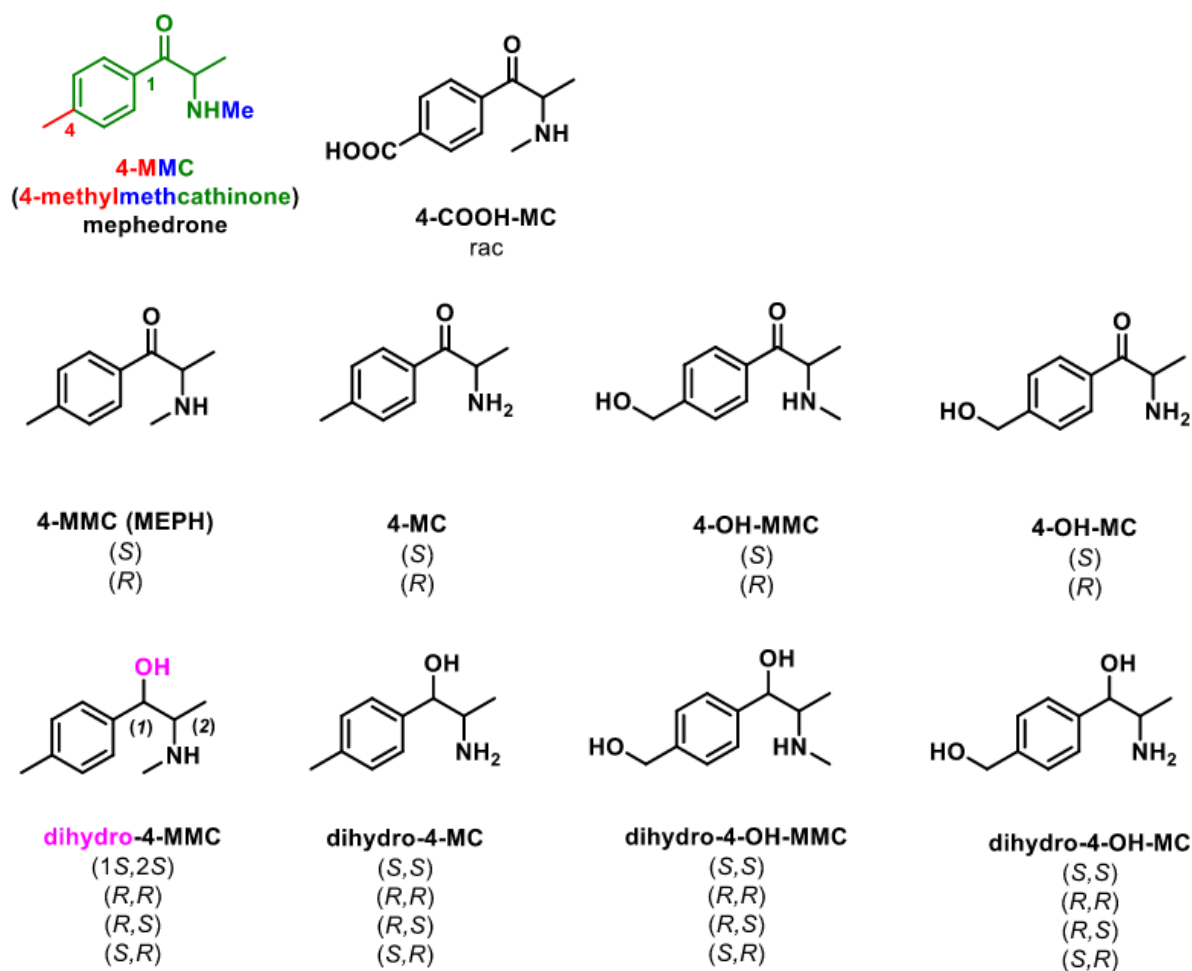


Figure 20: The nomenclature and chemical structures of phase-I mephedrone metabolites of interest. The letters in brackets depict different enantiomers of each molecule. The parent structure 4-MMC (top left) exemplifies the cathinone backbone (green) and the two methylation sites (red and blue). Dihydro-4-MMC (bottom left) exemplifies the reduction of the keto group (pink) and the resulting second chiral atom (1 and 2 in brackets).

Images provided by Dr. Daniela Cintulova, 2019

Each molecule in every available configuration shown in Figure 16 was added as a separate entry to the Maestro workspace, resulting in a total of 26 different molecules. Using the settings described in chapter 3.2.1.4, two separate docking runs were performed – one for the metabolites with one chiral centre and one for the dihydro-metabolites, with two chiral centres each. The poses were saved as separate .sdf-files.

3.3 Binding Affinity Prediction

Affinity predictions are functions frequently used in the field of computational chemistry and molecular modelling. In this project, affinity prediction programs are utilized to score the docking poses. Reason behind this was to investigate if the lower IC₅₀-concentrations and thus higher potencies of some substrates also translate to a better scoring. Therefore, a variety of scoring functions were used and will be described in the following section.

Different software solutions utilize one or more algorithms to calculate binding affinities based on a set of different parameters. For this thesis, Schrödingers Maestro, SeeSAR, MM-GBSA, LigandScout and MOE were used. Their respective algorithms for affinity prediction are described in the following chapter. The docking poses created in Schrödingers Maestro are loaded into the aforementioned software solutions and re-scored.

As calculated scores cannot be directly compared across different programs, Spearman Rank and Pearson correlation coefficients were calculated afterwards to determine if and how predicted affinities correlate to in vitro results.

The Pearson correlation coefficient is a measure of relationship between two variables.⁸¹ If data is correlated, the change of magnitude in variable 1 is associated with the change of variable 2. This might either be a positive correlation (variable 1 increases, so does variable 2) or negative correlation (as variable 1 increases, variable 2 decreases). Correlation is defined as a coefficient ranging from -1 to 1. A coefficient of 0 stands for a non-existing correlation between two variables whereas -1 or +1 describe a perfect negative or positive correlation. However, such absolute relationships are not typical in biological or medical research. Values between 0 and ± 1 are subject to interpretation. As a rule of thumb, values ranging from 0.00 - 0.09 can be considered negligible, 0.1 – 0.39 weak, 0.4 – 0.69 moderate, 0.70 – 0.89 strong and 0.90 - 1.00 as very strong.

In contrast, the Spearman Rank correlation coefficient does not consider individual data points but their respective ranks. It can analyse relationships for any nonlinear, monotonic data. Like the Pearson correlation coefficient, it also ranges from -1 to 1 and can be interpreted accordingly.

As predicted binding affinities are displayed as negative values, and experimental tested affinities such as IC₅₀ concentrations are reported positive, a negative correlation and negative Spearman coefficients close to -1 would be ideal. Pearson and Spearman Rank correlation coefficients were all calculated in Microsoft Excel.

3.3.1 Docking Score

The Glide Docking Score is one of the scoring functions that Schrödingers Glide docking algorithm has to offer. It calculates binding free energies and utilizes the GlideScore scoring function, after energy penalties from Epik⁸² have been included. These energy penalties stem from specific tautomeric forms, which are considered unlikely to occur under biological conditions and are therefore deemed unusable to properly predict virtual screening experiments. The GlideScore is an empirical scoring function, integrating terms like lipophilic-lipophilic interactions, rotatable bonds, hydrogen bonds, coulomb-van der Waals energies and water displacement. Its main goal is to separate and rank

compounds that bind strongly from those with little binding affinity. Lower scoring compounds are considered better binders.

3.3.2 Glide G Score

“Glide G Score” is a term that stands synonymous for the GlideScore.⁸³ It basically depicts the Docking Score but without the Epik penalties applied. Schrödinger suggests using the docking score to assess ligand docking but as it was automatically written to the files after the docking run, it was considered as well.

3.3.3 Prime MM-GBSA

Prime is another software package included in the Maestro suite. It can be used to predict protein structures and to refine already existing X-ray or NMR structures.⁹⁴ This becomes especially important for active sites, where an accurate model is of utmost importance for accurate drug-design. Prime also offers comparative modelling and fold recognition to create homology models of higher quality. Furthermore, Prime is part of the induced-fit docking method, where it refines ligand binding modes and corresponding structural changes in the receptor. Lastly, it offers MM-GBSA calculations which were utilized in this project.

MM-GBSA stands for “molecular mechanics generalized born and surface area”.⁹⁵ It uses the continuum solvation model. As input, either the separate ligand and protein files or a complex including both entities can be used. For this thesis, the docked poses of all 26 separate ligands were examined.

3.3.4 LigandScout

LigandScout is a software solution designed by Inte:Ligand. It is most commonly known for its ability to form pharmacophore models, highlighting features for possible interactions of ligands with their target protein. In addition, the software can also be used to perform docking runs, screening operations and binding affinity calculations. The latter option is of interest for this project and has been utilized. LigandScout uses the MMFF94 force field algorithm, initially published by Merck.⁸⁴ LigandScout allows for binding affinities to be calculated in a similar manner to Schrödingers Glide or MM-GMSA algorithms.

After each docking run, the “Binding Affinity Score” is displayed in kcal/mol, where a lower value indicates higher binding affinity. For this project, the docking poses together with the prepared target protein were loaded into the “Structure-Based” workspace. The binding affinities and more variables can now be calculated by simply clicking on the “Library”-tab in the top left of the screen and then selecting “Calculate MMFF94 Binding Enthalpy” and “Calculate Binding Affinity”. The resulting scores are then written to the .sdf-file of the poses.

3.3.5 SeeSAR

As a software suite developed by BioSolveIT, SeeSAR⁸⁵ serves as a solution for structure-based and ligand-based drug design. SeeSAR provides tools to assess physico-chemical attributes of molecules, predict their respective binding affinities and to visualize torsion-angles of covalent bonds. It utilizes its own HYDE scoring function, estimating ΔG -values for each individual atom, considering hydration, dehydration and protein-ligand interactions.^{86,88} This makes HYDE somewhat unique in its approach as it allows for changes in hydrogen bond energies and dehydration energies to be calculated per individual atom rather than considering any ligand as one entity, where solvent accessible area is used to estimate dehydration.

This scoring function is utilized here to re-score the docking poses created in Schrödingers Maestro suite. The target protein and the docked poses are loaded as .pdb and .sdf-files respectively, and affinity calculations as well as HYDE-based binding affinities per atom are then calculated. As HYDE reports scores as ranges and not exact values, estimated affinities cannot be included in any statistical evaluation. A pose-per-pose visual evaluation is thus included, as SeeSARs per-atom visualization scheme is a useful complement for this task.

3.3.6 Molecular Operating Environment (MOE)

Molecular Operating Environment (or short “MOE”) is a platform for computer-aided molecular design.⁸⁷ It offers software kits for three-dimensional visualization, structure- or ligand-based design, virtual screenings, antibody or peptide modelling and many more. Developed by the Chemical Computing Group, it serves by providing an additional scoring function for this project, where we used version 2019.0102. The scoring function utilized is the “Affinity dG score”. In order to perform the re-scoring, the .sdf-files containing the docked ligands and their corresponding information are converted to .mbd-files (*molecular data base*, a proprietary file format), loaded into the MOE workspace together with the receptor hSERT model. Then, the docking panel is opened, atoms around the ligands are defined as “binding pocket”, no “refinement” or “additional placement” are selected and the scores for the docked ligands can then be calculated.

3.4 Qualitative Approach

3.4.1 Interaction Fingerprints

Interaction fingerprints offer an easy way of visualizing trends in binding patterns and protein-ligand interactions.⁹⁶ They can be calculated using Canvas, a software package also included in Schrödingers Maestro suite. It offers the user tools to screen large amounts of compounds in little time. Canvas unifies tools for similarity searching and substructure matching, clustering and self-organizing maps, thus making large datasets more approachable and interpretable.

For this Project, the Interaction Fingerprint generator was used. When selected, it opens up a panel with three different tabs. The first tab “Fingerprint Generation” lets the user choose the source for fingerprint generation and the kinds of interactions that should be included. Docked poses are chosen as input and every kind of interaction is included in the fingerprint generation. After that, the fingerprints can be generated and displayed as an interaction matrix. In the second tab “Similarity” allows for similarity calculation and sorting. A choice of 24 similarity metrics is available. The last tab “Cluster” allows the user to calculate clusters for their docked poses, based on 9 different linkage methods. After the clusters have been calculated, their respective numbers are displayed, together with the clustering strain. After that, the clustering can be applied to the entries in the workspace. In this project, the default “Tanimoto” linkage method is used and the clustering subsequently applied to the docked poses.

3.4.2 RMSD clustering using KNIME

Root-Mean Square Deviation or common scaffold clustering is a method to identify groups within a set of conformations. In this case, the deviation between the individual docking poses is calculated and the poses are then sorted into specific clusters, based on the similarity to each other. Poses oriented in a similar manner and positioned more closely to each other are clustered together. Grouped poses can then be analysed based on their interactions, scores or three-dimensional orientation.

In this research project, a KNIME workflow provided by Florentina Troger is utilized to perform the RMSD clustering.

3.4.2.1 KNIME

KNIME, the Konstanz Information Miner, is a free data analysis software that incorporates methods from Data Mining, Analysis and Machine Learning.⁸⁹ It offers the user to create modular “step-by-step” workflows, offering a set of different nodes to modify, refine or filter data within a pipeline or data flow. Version 4.1.1 was used in this project.

To start a KNIME workflow, a Node that reads input data is required. After that, every kind of Node can be added after that, depending on what kind of further use one has for their data. The GUI of KNIME has a section called “Node Repository”, located in the bottom left by default. Here, the user can search for different Nodes and add them to their workflow by drag & drop. Furthermore, different software solutions offer their own KNIME extension, for example Schrödinger⁹⁰ or MOE.⁹¹ This allows for further customization and specialization as these extensions provide access to additional Nodes, expanding the spectrum for analyses of different data sets and/or types.

Each Node has an input and output port which need to be connected by simply drawing a line using the mouse cursor. One nodes output port should be connected to the input port of the following node in order to insure a proper data flow. Each node needs to be configured prior to execution. Simply clicking on it and checking the code inserted or the settings applied suffices, the node is then labelled “configured” and can be executed. Additionally, each node can be individually executed, and their respective output can be examined.

The “KNIME console”, located in the bottom right by default, indicates if any errors or warnings occur during execution and usually specifies the problem, making troubleshooting easier.

The overall advantage of using KNIME lies in the software’s capability to create specially designed workflows suited for one’s distinctive needs. In addition, KNIME allows for process automatization and workflows can also be reused for different purposes.

3.4.2.2 Preparation

Before using the KNIME workflow, the .sdf-file containing all the information regarding the docked poses needs to be converted to .mdb, a proprietary file format used by MOE. This can be done by opening a .sdf-file in MOE and then choosing to add it to a molecular data base. After that, it can be exported as .mdb-file by using the “save as” prompt. This is done twice, as the docked poses are created in two separate runs and are therefore saved as two different files.

In addition, a “SMARTS” string is required to act as a common scaffold. Similar to a SMILES string, SMARTS are used to give computers a language to interpret atoms and their corresponding bonds.⁹² Their functionality extends SMILES, as SMARTS can give information about atomic properties, chirality, charge and different types of atom bonds. Those can be used to either visualize molecules or scan libraries for similar structures. SMARTS strings were designed by Daylight Chemical Information Systems, Inc.⁹³

To create our own, the “SMARTS Editor” software was utilized. The SMARTS Editor⁹² is a software designed and published by BioSolveIT in cooperation with the Centre of Bioinformatics Hamburg. Its

GUI allows the user to draw chemical structures. Their respective strings are displayed in a field above and from there can be copied as text for further usage.

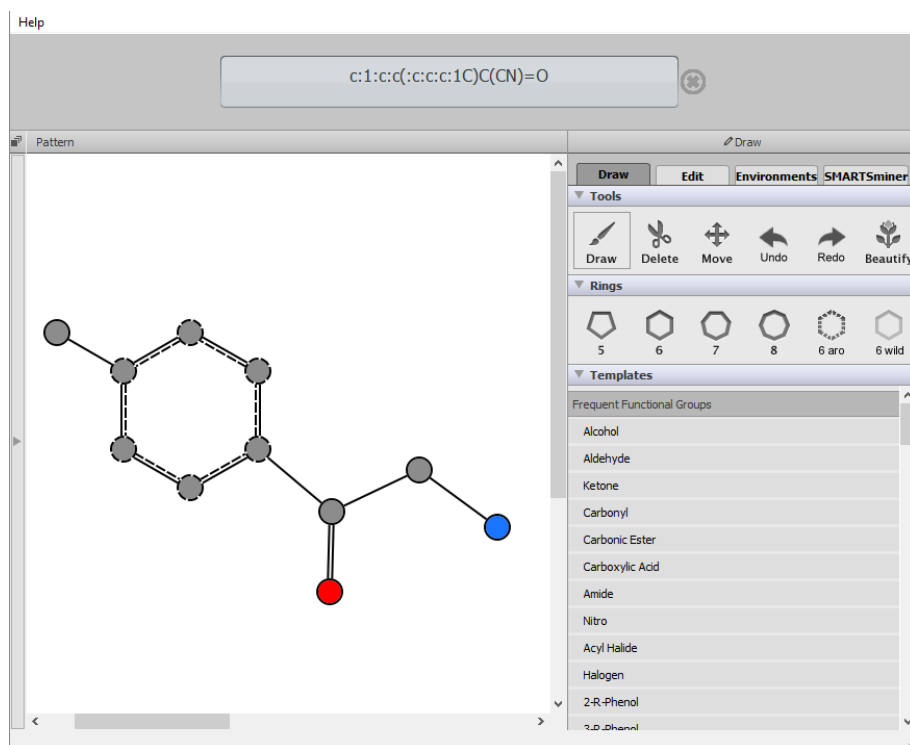


Figure 21: Screenshot of SMARTS Editor, Version 2.1.3. The GUI allows the user to either draw molecules from scratch, using the tools displayed in the right field and also allows SMARTS to be directly added into the text field in the upper middle to be visualized. Image adapted from Schomburg et al, 2013

Two separate SMARTS strings were initially created for the RMSD clustering: one for the dihydro-variants of the phase-I mephedrone metabolites and one for the metabolites that have the β -keto group as part of their structure. However, as one of the nodes misinterpreted the keto groups double bond, thus ultimately causing the workflow to crash, one string was selected for all ligands. SMARTS string “c:1:c:c(:c:c:c:1C)C(CN)O” serves as a common scaffold for the clustering process.

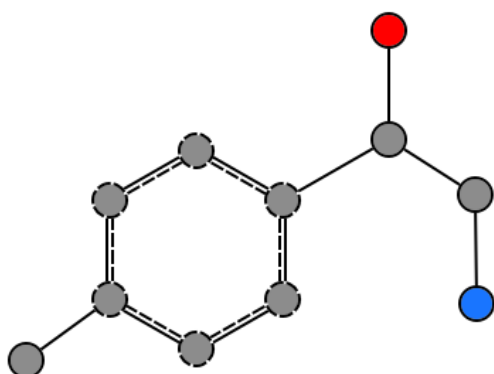


Figure 22: Depiction of SMARTS string “c:1:c:c(:c:c:c:1C)C(CN)O”, that ultimately serves as scaffold for the common scaffold clustering. Oxygen atoms (red), nitrogen (blue) and carbon (grey) are depicted as spheres. Solid lines between spheres visualize covalent bonds, solid lines combined with dashed lines depict aromatic bonds. Screenshot taken from SMARTS Editor, adapted from Schomburg et al., 2013

3.4.2.3 Workflow

The KNIME workflow used to cluster the docking poses is depicted below and the important nodes will be explained in the following section.

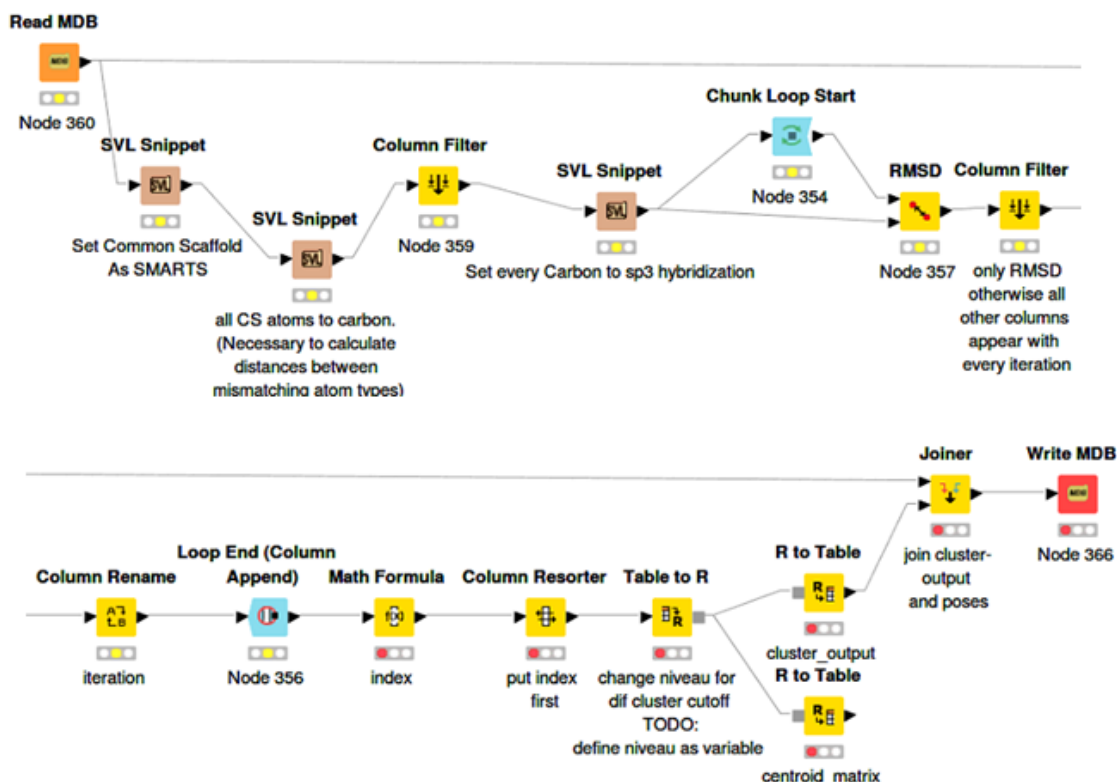


Figure 23: Depiction of KNIME workflow for common scaffold clustering. As the workflow is too wide to be fitted horizontally, the picture has been split in two. Read from top to bottom, left to right.
Screenshot taken from KNIME Version 4.1.1, adapted from Berthold et al., 2021

- Read MDB: Opens and reads a .mdb (**m**olecular**d**atabase)-file
- SVL Snippet #1: Sets a common scaffold for clustering. A SMARTS string can be added here
- SVL Snippet #2: Every heteroatom in the SMARTS string is converted to a carbon atom for the clustering to work
- Column Filter #1: Filters out unnecessary information of the .mdb file.
- SVL Snippet #3: Every carbon is set to sp3 hybridization.
- Chunk Loop Start: Iterates over the start of the input data table. Splits data into chunks to speed up execution.
- RMSD: calculates RMSD and atomic displacement between a reference structure and the rest of the conformers. Reference structure is set as the first in the table.
- Table to R: Here one can set the RMSD cut-off to determine cluster membership. A default setting of 3 is kept.
- R to Table: Information regarding the cluster allocation of each entry is written to the .mdb file

- Joiner: Joins information created in the workflow with the original file, so no information is lost
- Write MDB: a .mdb file is written as final output. Cluster allocation is added for every entry.

After the clustering process has completed, a new column named “common scaffold cluster” is created and the corresponding cluster number is allocated to each entry within the file.

4. Results

4.1 Docking & Qualitative Approach

4.1.1 Redocking Serotonin

Prior to this redock, different Glide docking runs including SP and XP precision pre-sets were utilized to find a docking protocol most suitable for this project. However, the docked molecule of serotonin is not suitable as a reference but rather as a guidance, as the hSERT model is not an X-ray structure. The binding mechanism of Serotonin is explained in literature and serves as a guidance for our own docked molecules.

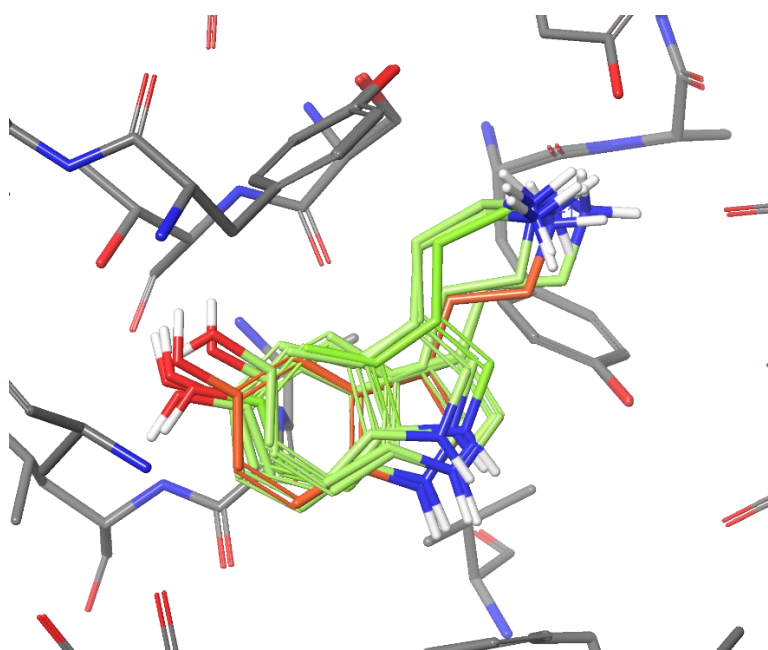


Figure 24: Redocked poses of Serotonin within the s1 site of our hSERT model. The “native” Serotonin is displayed in orange, the selection of docked poses in light green. The other sticks represent the residues of the s1 binding site. Picture rendered in Maestro version 2021-3.

In terms of interactions, Hellsberg et al. refer to an ionic interaction between the Asp98 (D98) and the cationic nitrogen of serotonin, an interaction between Thr438 (T438) and the 5-OH group and the indole ring of serotonin directed towards Phe341 (F341).²⁸ These interactions and the positioning were replicated and displayed by the Maestro. However, some poses had the aromatic ring positioned differently, placed within subsite C. As this position is not referred to in literature^{28,97} and does not seem to reflect the correct binding mode. Poses oriented in a similar matter will not be considered for further usage.

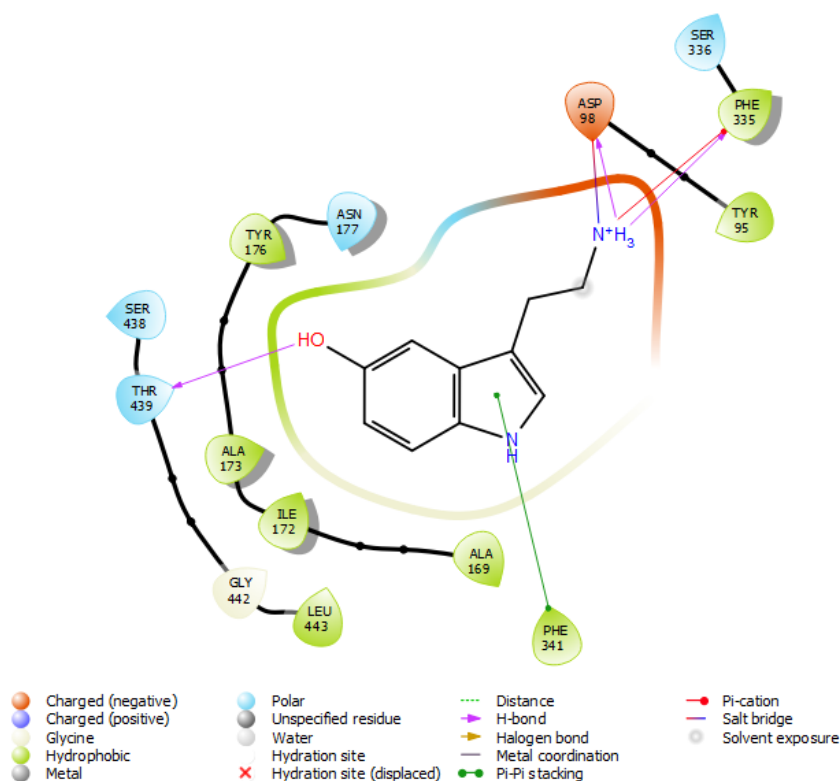


Figure 25: Interactions of the 5-HT molecule docked by Hellsberg et al.. The aforementioned interactions are displayed in the legend below. Screenshot taken from the ligand interaction panel of Maestro version 2021-3.

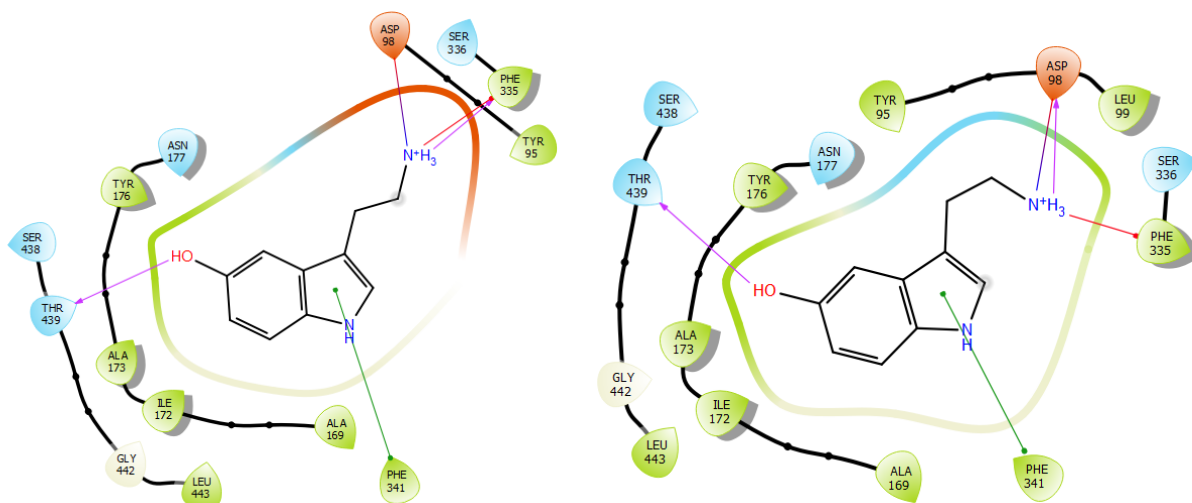


Figure 26: Two exemplary interaction diagrams of docked serotonin poses The salt-bridges to Asp98 were almost always present, as was the hydrogen-bond to Thr439. Images taken from Maestro 2021-3

4.1.2 Docking the phase-I mephedrone metabolites

The docking runs concluded in a total of 86 poses for the enantiomeric metabolites and 403 poses for all the dihydro-metabolites. As 403 poses were eventually considered too much to analyse individually, a number of poses were sought out and deleted from the file. The criteria for deletion were:

- a) identical interaction partners,
- b) visual superposition of stick models when observed in SeeSAR and
- c) positioning of the aromatic moiety in subsite C

This allowed us to reduce the number to 83 individual poses for the dihydro-metabolites.

4.1.3 Examination of poses and binding modes

Initial observations show that the docking protocol positioned the aromatic moieties of the mephedrone metabolites in either subsite B or subsite C, all while orienting the cationic nitrogen towards Asp-98. As the transport mechanism of serotonin requires its aromatic moiety to be placed within subsite B, we assumed that similar positioning is required for our mephedrone metabolites to be treated similarly.

The emphasis was set on the metabolites with only one chiral atom, as those 8 were showing differing levels of activity at SERT. As the overall trend showed a preference of (S)-enantiomers over (R)-enantiomers, we looked for reasons that could explain this phenomenon.

(rac)-4-COOH-MC:

Instances of (R/S)-4-COOH-MC showed a trend of positioning their aromatic rings within subsite C rather than B. As subsite B tends to be more hydrophobic, the negatively charged and hydrophilic carboxyl-group at position 4 of the aromatic ring might not interact favourably. This could be one reason why they effectively do not bind ((rac)-4-COOH-MC, which has a reported IC_{50} -concentration of 11432 μ M at SERT). One other reason why (rac)-4-COOH-MC doesn't bind could be its predicted negative charge: LigPrep predicted, that the nitrogen as well as the carboxyl-group would be dissociated at physiological pH. This would render the net charge of (rac)-4-COOH-MC zero, opening the possibility of interference with the stoichiometry of transported charges within SERT and could therefore be one cause for the lacking activity of (rac)-4-COOH-MC.

(R/S)-4-MMC (MEPH):

The docking run created three poses for (S)- and just two for (R)-4-MMC. The aromatic rings and the nitrogen-atoms overlap in all the poses. There is a preference to aim the keto-group towards Ser-438 and Tyr-176 for both enantiomers. However, the poses did not help in discovering any trends as interaction partners were practically the same. Pi-pi-stacks with Tyr-176 or Phe-341, salt-bridges to Asp-98 and pi-cation interactions between the cationic nitrogen and Phe-335 are reported for (S)- and (R)-enantiomers. The chiral methyl-group is favourably oriented towards Phe-335. However, we cannot explain why and assume that the docking algorithm preferred it as this orientation offered more room.

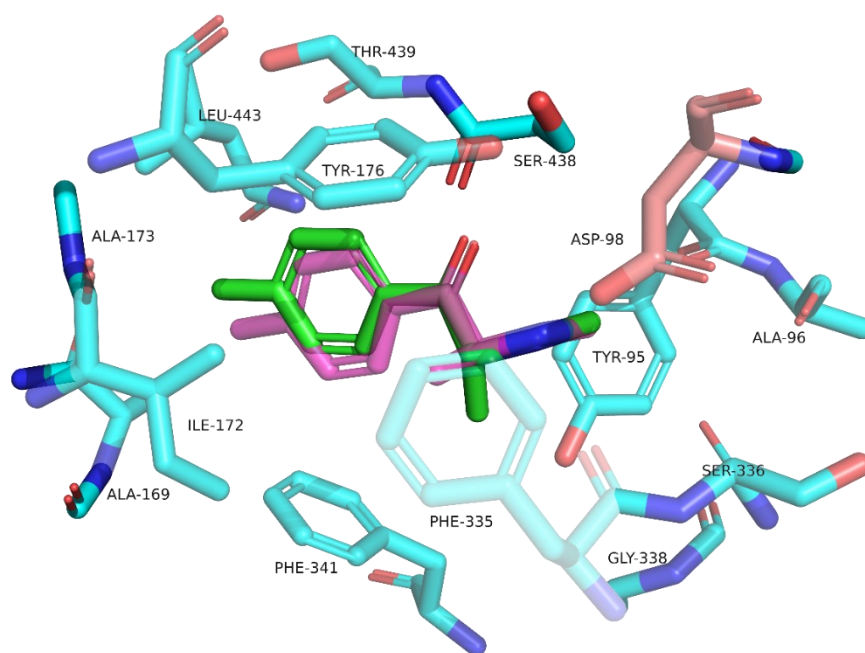


Figure 27: Exemplary poses of R-4-MMC (green sticks) and S-4-MMC (pink transparent sticks) in *s1* site of hSERT model (light blue sticks). Asp-98 highlighted in orange as an orientation. Picture rendered with PyMOL, version 2.6.

R/S-4-MC:

Here, the positioning becomes more ambiguous: More poses have been created for S-4-MC and the binding pattern switches between pointing the keto-group towards Ser-438 and Tyr-176 again or tilting the whole molecule by 180° along its longitudinal axis, pointing in the opposite direction. As a result of this flip, the chiral methyl-group is either positioned more towards Phe-335 or Tyr-95. In terms of interactions, similar interactions like (R/S)-4-MMC are reported by Maestro. Asp-98, Tyr-176 and Phe-335 act as main interaction partners for (S)-4-MC.

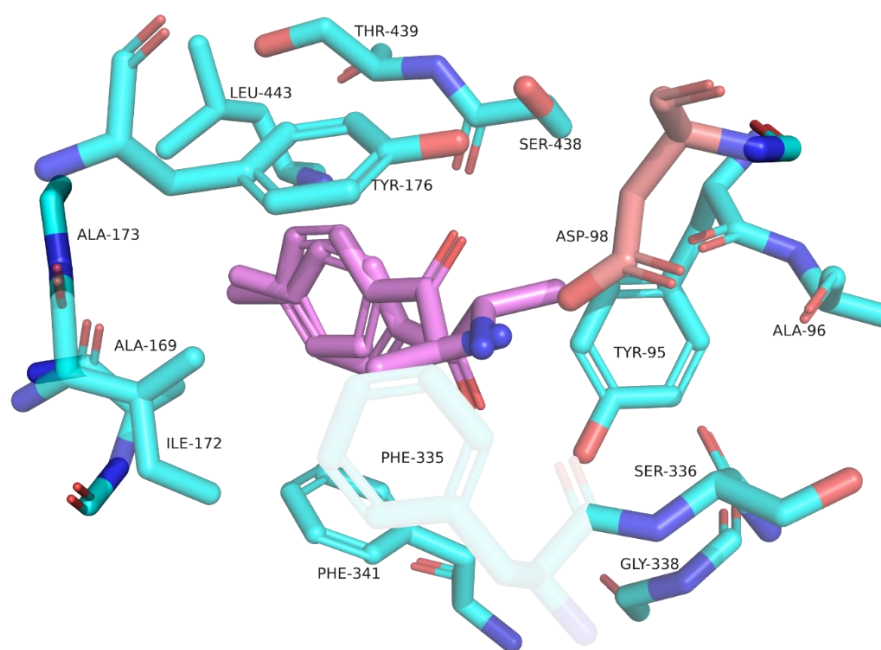


Figure 28: Two poses of S-4-MC (pink sticks) in the s1 binding site of a hSERT model (light blue sticks) with Asp-98 highlighted for orientation. Emphasis on the different orientation of the keto-groups of the ligands, diverging by almost 180°. Picture rendered in PyMOL Version 2.6

A similar trend is shown for (R)-4-MC: Only two poses were created, both of them oriented in a similar manner to its (S)- counterpart. No significant conclusion on its binding pattern can be drawn. A distinct binding and interaction pattern for either enantiomer is not visible.

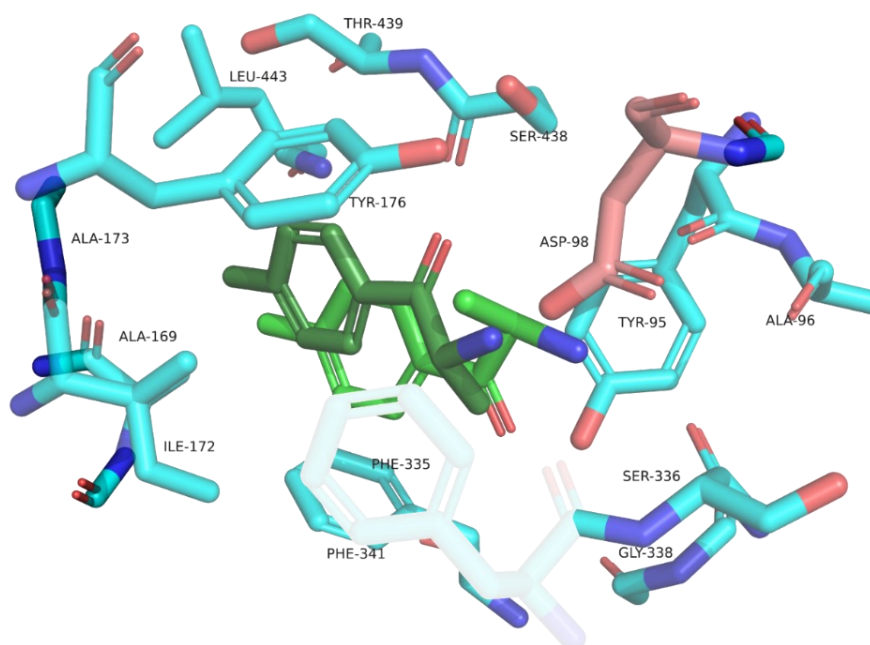


Figure 29: Positioning of two R-4-MC entities (light and dark green sticks) within the s1 binding site (light blue sticks) of hSERT model with Asp-98 highlighted in orange as orientation. The two different poses are oriented in a different manner. Picture rendered in PyMOL Version 2.6

R/S-4-OH-MMC:

R-4-OH-MMC showed a similar trend like the preceding molecules: The poses can be split up in either having the keto-group pointed towards Ser-438 and Tyr-176 or in the opposite direction. S-4-OH-MMC however, showed just one positioning. Having the keto-moiety pointing “up” towards Ser-438 and Tyr-176 seemed to be the favourable positioning for this metabolite. The added 4-OH-group shows interactions with the OH-group of Thr-439 or with the backbone oxygen of Ala-169. Pi-pi-stacking with Tyr-176, a salt-bridge with Asp-98 and pi-cation interactions with Phe-335 are present. However, no interaction pattern seemed to be exclusive for either the (S)- or the (R)-enantiomer.

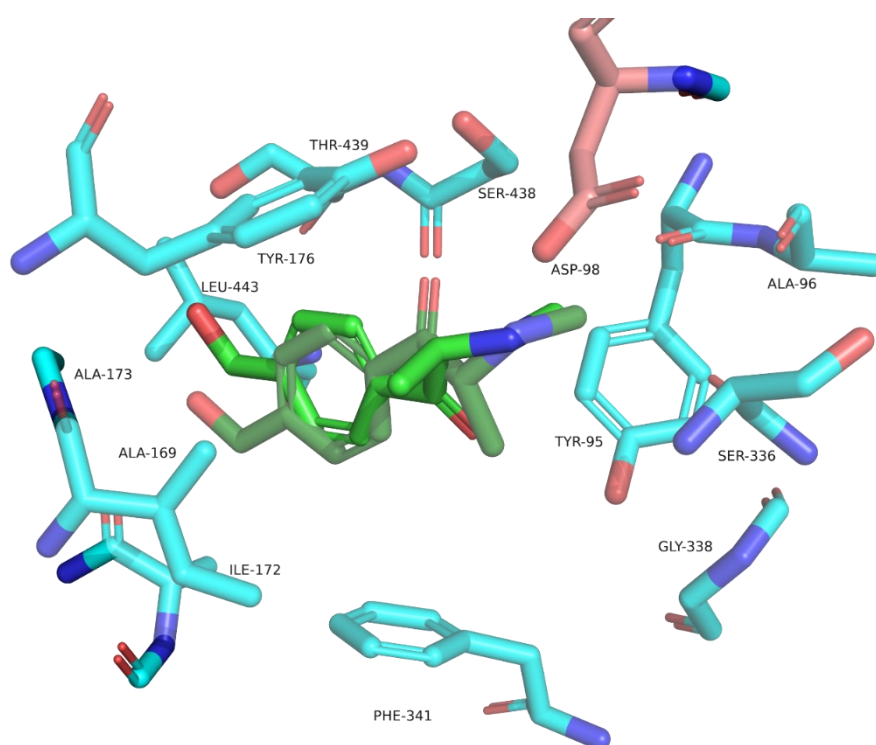


Figure 30: Two poses of R-4-OH-MMC (light and dark green sticks) in s1 site of hSERT model (light blue sticks). Asp-98 highlighted as orange sticks for orientation. Phe-335 was removed for better visibility. Picture rendered in PyMOL 2.6

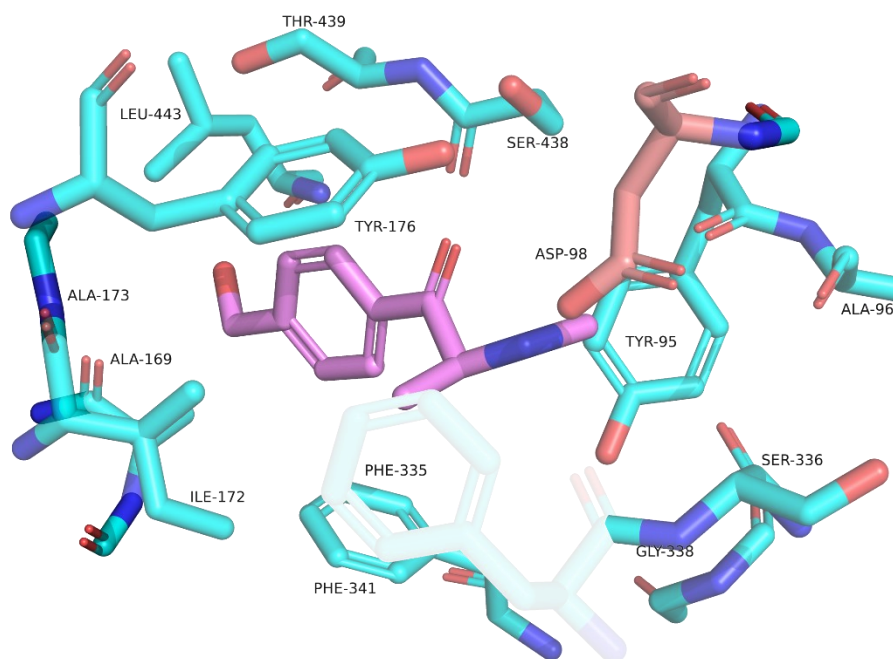


Figure 31: S-4-OH-MMC (pink sticks) in s1 site of hSERT model (teal sticks) with Asp-98 highlighted in orange. Picture rendered in PyMOL version 2.6.

(R/S)-4-OH-MC:

(R)-4-OH-MC is exclusively positioned with the keto-group pointed “down”, away from Tyr-176 and Ser-438. All the poses have the cationic nitrogen directly overlapping, with little variety showing in the way the rest of the poses are angled within subsite B. The 4-OH-group forms hydrogen bonds with either Ser-438 or the backbone of Ala-169 whereas pi-pi-stacking is reported between the aromatic rings and Tyr-176. Pi-cation interactions between the phenyl-ring of Phe-335 as well as the salt-bridge between Asp-98 and the cationic nitrogen are also reported by Maestro. The interactions are the same for (S)-4-OH-MC, with only some poses forming pi-pi-stacks with Phe-341 rather than Tyr-176. Furthermore, S-4-OH-MC was docked with a lower level of overlapping. A higher degree of flexibility was given when it comes to the positioning of the aromatic moiety, as the aromatic rings are not completely superposed on top of each other. Again, there is no binding pattern that is exclusive to either enantiomer.

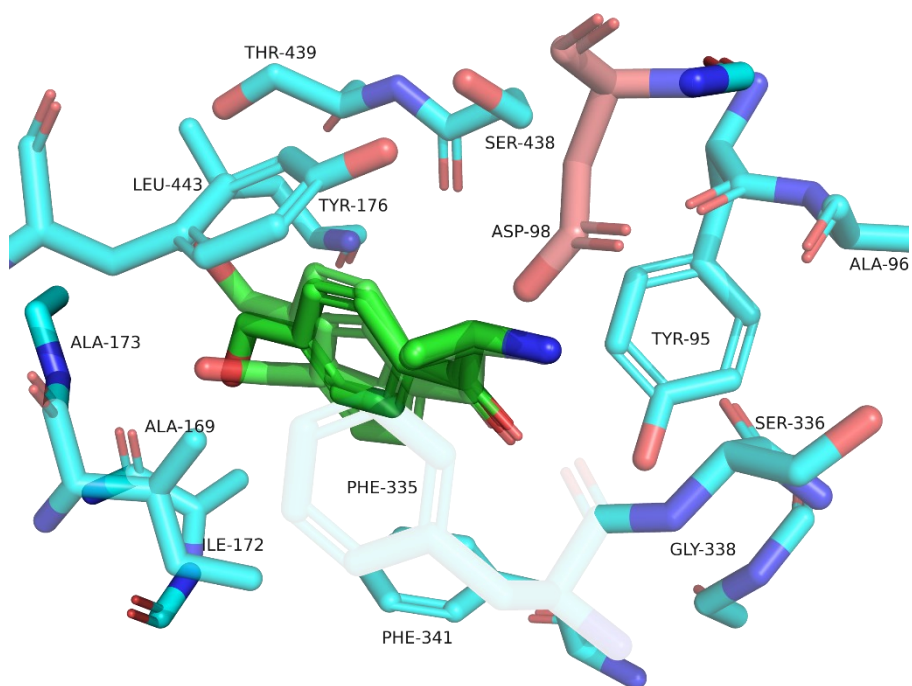


Figure 32: Poses of R-4-OH-MC (green sticks) in s1 site of hSERT model (teal sticks) with Asp-98 highlighted in orange. Picture rendered in PyMOL 2.6.

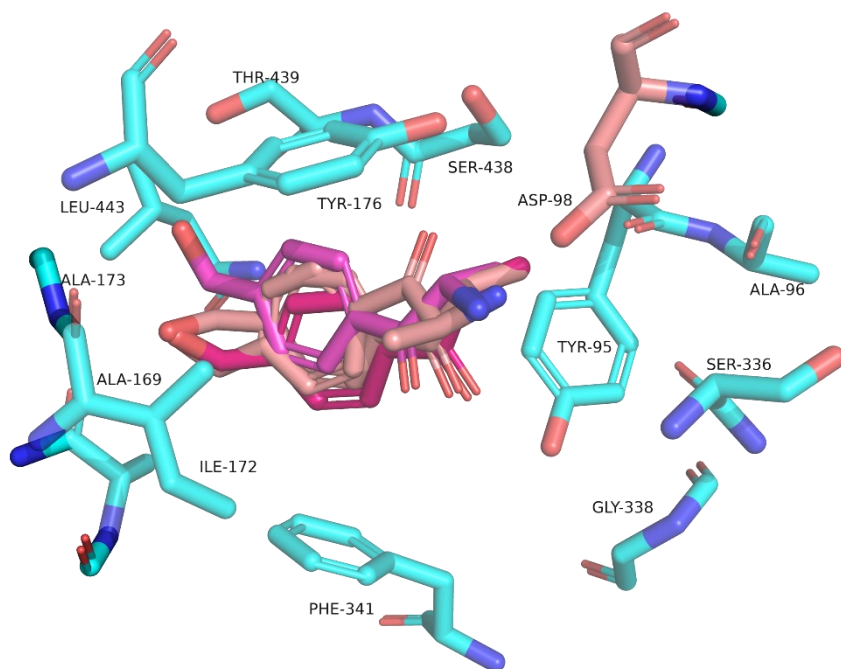


Figure 33: Poses of S-4-OH-MC (sticks in various shades of rose) within s1 site of hSERT model (teal sticks) with Asp-98 highlighted in orange. Emphasis on the ambiguous positioning of the keto-group and the resulting different tilts of the molecules. Picture rendered in PyMOL 2.6

Dihydro-metabolites:

Analysing the poses of the dihydro-variants gives no insight in a specific binding pattern that is exclusive to one of the four diastereomers. The added hydroxy-group allows for the formation of hydrogen-bonds with the backbone carbonyl of Phe-335. However, this is something that has been shown for all 4 types of configurations. In addition, as the sp³ hybridized carbon atom attached to the hydroxy-group is less planar than the sp² hybridized keto-carbon, poses showing different tilt angles along the longitudinal axis of the ligands have been created by the docking algorithm. There is too much flexibility within the sidechains of the dihydro-metabolites and too many observed interaction patterns for any trend to be visible.

Initial verdict:

The first analysis of the docking poses shows no visible trend for neither the positioning of the different ligands nor for any kind of binding pattern that is specific to (S)- or (R)-enantiomers. This raises doubts in the methodology as this docking algorithm might not be precise enough to take these small differences between the metabolites into account. This leaves little to say about the dihydro-metabolites: With no trend regarding stereoselectivity visible among the reported IC₅₀-concentrations and overall activity lower in comparison to the metabolites with the keto-group in place, finding a specific binding mechanism that explains this was not possible.

4.1.4 Interaction Fingerprints & Clustering

Interaction fingerprints for the 86 poses of (R/S)-enantiomers and for the 83 poses of dihydro-diastereomers were calculated in their respective runs. Clustering was applied with the settings mentioned in chapter 3.4.1. The 86 poses were clustered in 13 groups whereas the 83 poses were clustered in 11 groups.

4.1.4.1 Cluster of (R/S)-Enantiomers

Cluster	Population	Driving Interactions
1	4 (4 R)	Tyr-95, Phe-335, Tyr-176
2	2 (1 S, 1 R)	Tyr-176 & Phe-341
3	24 (19 S, 5 R)	Tyr-176 Phe-335, Tyr-95
4	5 (5 S)	Phe-335 & Tyr-176
5	3 (3 S)	Ala-169
6	23 (6 S, 17 R)	Tyr-176, Phe-335, Ala-169
7	1 (1 S)	Thr-439, Ala-173
8	10 (4 S, 6 R)	Phe-335, Thr-439
9	4 (4 R)	Phe-335, Thr-439
10	7 (3 S, 4 R)	Phe-335, Tyr-176
11	1 (1 R)	Phe-335
12	1 (1 S)	Phe-335
13	1 (1 S)	Phe-335, Phe-341

Figure 34: Table depicting Interaction Fingerprints of (R/S)-enantiomers. Number of clusters with corresponding populations and driving interaction partners that form the cluster.

Clustering based on the interaction fingerprints has shown no trend in terms of preferred interaction partners or population size. The clusters with bigger population sizes show no real preference for neither (R)- nor (S)-configured enantiomers. Metabolites that possess a OH-group at position 4 of the aromatic ring are shown to form hydrogen-bonds with either Thr-439 or Ala-169. Tyr-176 and Phe-341 are preferred partners for pi-pi-stacking interactions. Phe-335 either performs pi-cation interactions with the cationic nitrogen and/or performs hydrogen-bonds with its backbone carbonyl-group. Tyr-95 forms hydrogen-bonds with its hydroxy-group and the cationic nitrogen in some cases. All of these interactions, however, are non-specific, i.e. they are reported by (R)- and (S)-configured metabolites and happen regardless of N-demethylation.

4.1.4.2 Cluster of dihydro-diastereomers

Cluster	Population	Driving interactions
1	1	H-Bond Tyr-95 & Ala-169, no Asp-98
2	2	H-Bond Tyr-95, pi-pi stack Phe-341
3	47	Salt-bridge Asp-98
4	1	Pi-cation Phe-335, pi-pi-stack Tyr-176
5	15	Nitrogen H-Bond Phe-335 & Asp-98
6	1	Pi-cation Phe-335, pi-pi-stack Tyr-176
7	3	4-OH has H-Bond to Ala-169
8	1	H-Bond to Thr-439
9	1	2 H-Bonds to Phe-335, H-Bond Tyr-176
10	6	H-Bond Ala-169, pi-pi-Stack Tyr-176
11	5	H-Bond to Phe-335

Figure 35: Table depicting cluster results of dihydro-metabolites with their corresponding populations and driving interaction patterns.

Tyr-95, Ala-169's and Phe-335's backbone carbonyl form hydrogen-bonds in a manner identical to the metabolites with just one stereo-centre. Pi-pi stacks and pi-cation bonds are also reported to be formed with the same amino-acid residues, namely Ty-176 and Phe-341 as well as Phe-335. Nonetheless, the most populated cluster was formed based solely on the salt-bridge to Asp-98.

4.1.5 Evaluation in SeeSAR

The receptor .pdb-file and the .sdf-file containing all the poses were loaded into the workspace of SeeSAR. After completion of this step, estimated affinities, torsion qualities, intra- and intermolecular clashes were calculated. This then allows for a pose-by-pose evaluation in conjunction with the information that HYDE's per-atom affinity calculations provide. This evaluation was used as an extension of the pose-evaluation and interaction clustering that was completed beforehand in Schrödinger's Maestro. The emphasis in SeeSAR was to investigate if HYDE shows any differences in scores when addressing the orientation of chiral substituents. In addition, the differing orientation of keto-groups was investigated.

Keto-group orientation:

One trend which was unanimously present among all poses was the keto-group orientation: it could either point “upwards” towards Tyr-176 and Ser-438 or “downwards” into the binding pocket. HYDE suggests that the “downward” positioning of the keto-group is favourable. The contribution of desolvation energies are implied to be more beneficial for overall binding.

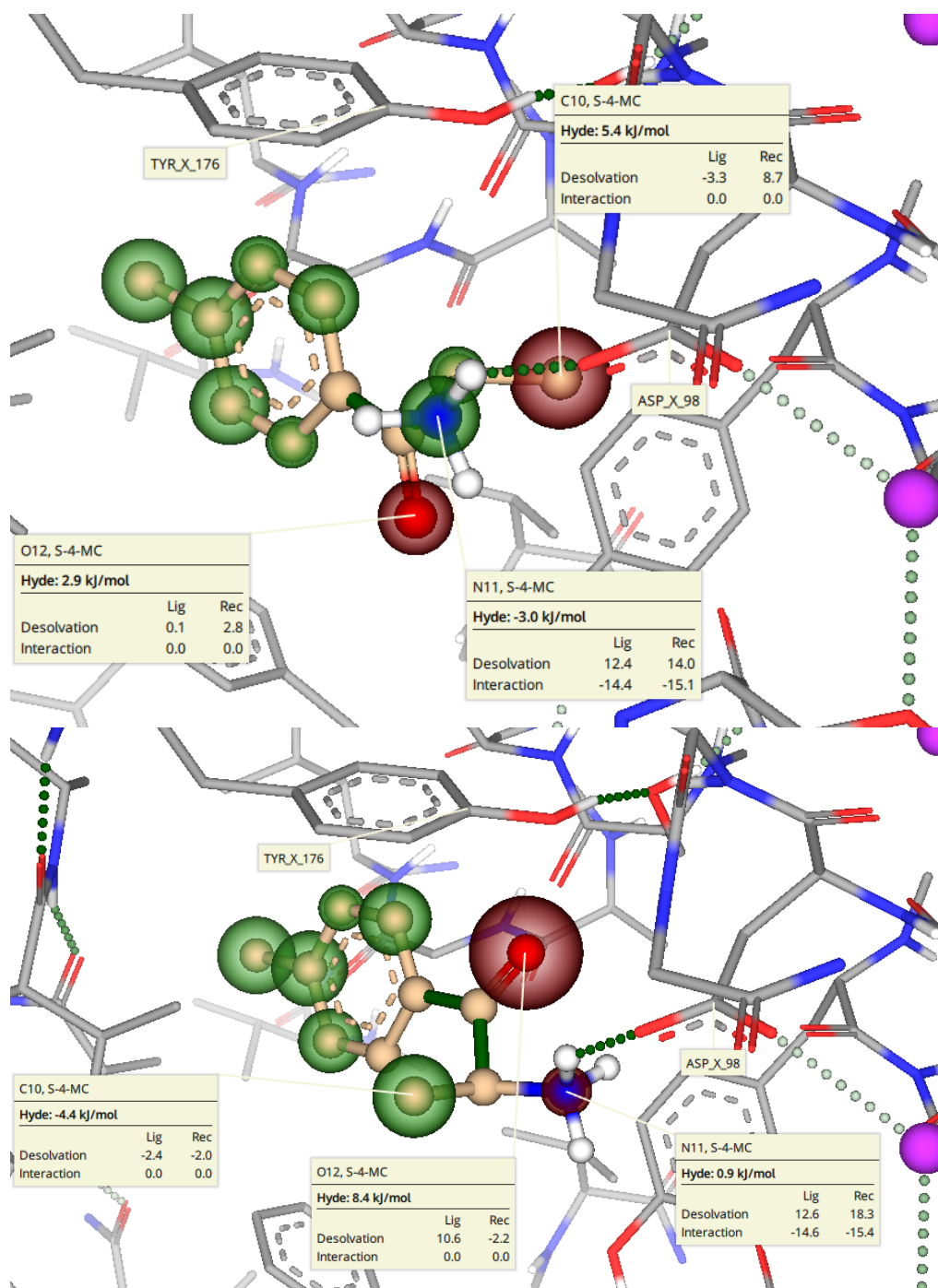


Figure 36: HYDE-scoring on two poses of (S)-4-MC. Contribution to calculated binding affinities per atom are depicted in red spheres (bad) or green spheres (good) surrounding the atoms. The bigger the sphere, the bigger it's contribution. Individual contribution in kJ/mol is shown for selected atoms. Lower values are deemed better. Screenshot taken in SeeSAR version 11.2

Position of chiral methyl-group:

The chiral methyl-group of the metabolites is positioned facing either Phe-335 or Tyr-95. The binding site offers more space facing towards Phe-335 and the docking algorithm utilizes this space by either positioning the nitrogen-atom or the methyl-group into this vicinity. Whatever functional group is positioned in this space is scored more favourably by HYDE. Desolvation energies are lower for either the methyl-group or the nitrogen when it is there and it is thus contributing positively to the calculated binding affinity. However, the functional group on the opposite site is always contributing negatively to the calculated binding affinity. Whether or not a more easily desolvated cationic nitrogen is better than a methyl-group cannot be said, as the calculated binding affinities between the (R)- and (S)-enantiomers are similar. Furthermore, the differences in positioning of chiral substituents can be applied to every metabolite tested, as their differences in stereochemistry can be negated by a 180° flip along the longitudinal axis. (S)- and (R)-metabolites would then have their cationic nitrogens and chiral methyl-groups superposed on top of each other. The docking algorithm shows ambiguity when it comes to the rotation of the metabolites and thus allows the chiral substituents to be placed either way. We cannot say that this small insight HYDE offers contributes to solving the questions asked in our hypothesis as the orientation of cathinones within the binding site is not well understood.

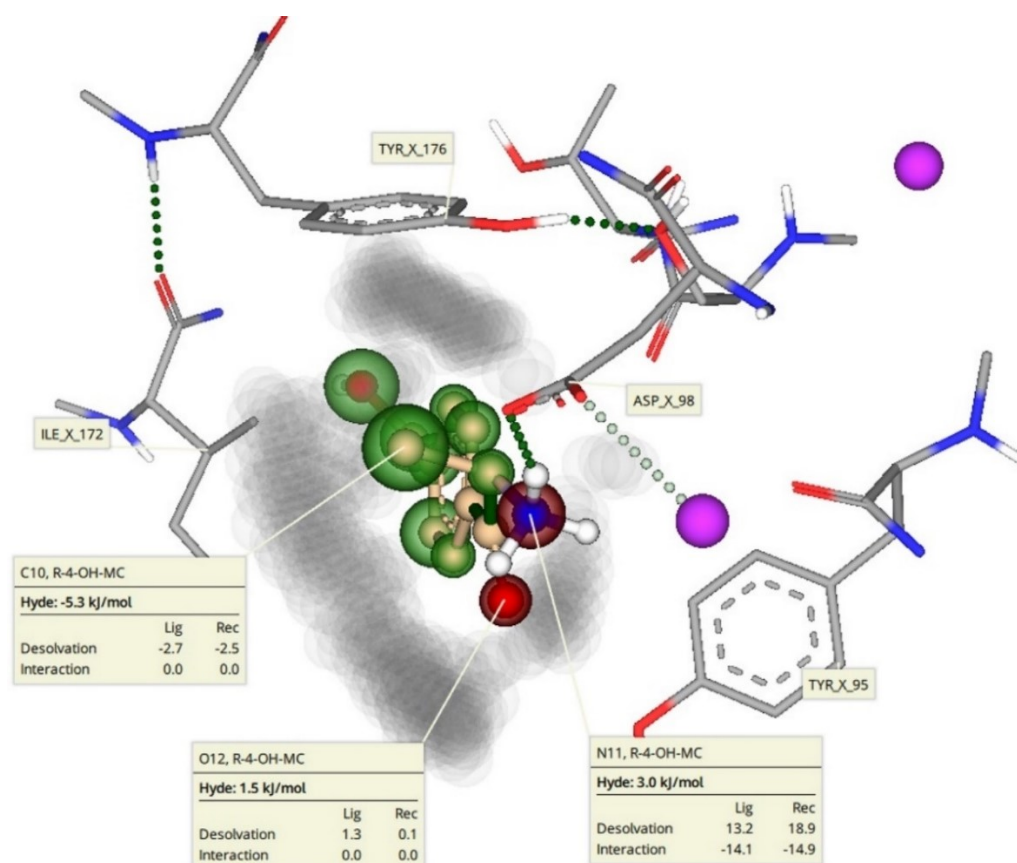


Figure 37: R-4-OH-MC in s1 binding site of hSERT model. Some amino acids have been removed for better visibility. Grey fog indicates unoccupied space in binding site. Emphasis on the chiral methyl-group (C10) and cationic nitrogen (N11), which contribute either positively or negatively to the estimated binding affinity. Screenshot taken in SeeSAR version 11.2

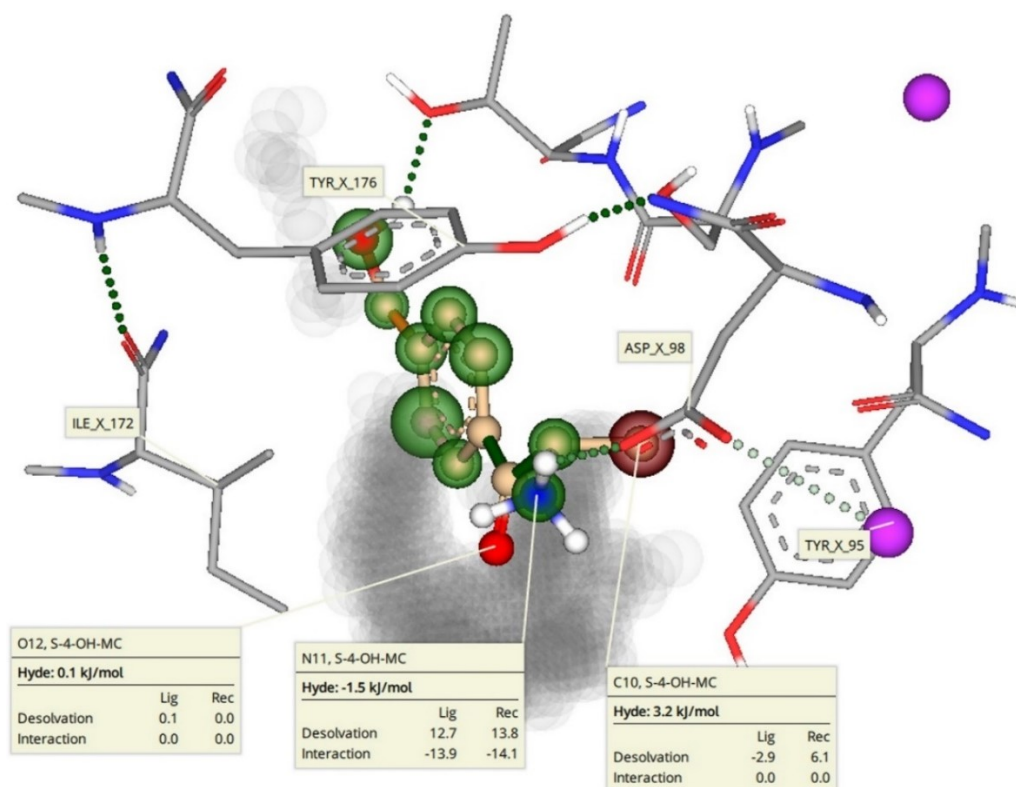


Figure 38: Pose of S-4-OH-MC in s1 binding site of hSERT model with some amino acids removed for better visibility. Grey fog symbolizes unoccupied space in the binding pocket. Emphasis on the chiral methyl-group (C10) and cationic nitrogen (N11) which contribute either negatively or positively to the estimated binding affinity contrary to R-4-OH-MC. Screenshot taken in SeeSAR version 11.2

4.1.6 Common Scaffold Clustering

The clustering was performed in two different runs, as two different files for either the metabolites with one or two chiral carbon atoms were available. Information gathered from this process was visualized in Schrödingers Maestro, as KNIME offers no option to visualize molecules in a three-dimensional space. Based on the cluster allocation, the entries were coloured differently, and the resulting clusters analysed.

With an RMSD cutoff of 3, we received two clusters for the metabolites with one chiral carbon and just one cluster for the metabolites with two chiral carbons.

The only difference between the first two clusters turned out to be the positioning of the aromatic ring, as 14 of the 86 poses have the aromatic ring positioned outside of subsite B, whereas the remaining 72 have it positioned inside subsite B. This turned out to be the only driving factor. Both clusters were populated with (S)- and (R)-enantiomers.

4.2 Quantitative approach & Affinity Prediction

Different software solutions have been utilized in this project in order to predict binding affinities for all 26 individual compounds. After the docking run has been concluded, an individual score for each pose has been calculated in kcal/mol and written to the .sdf file. As is for every following score, the more negative the value, the better the binding affinity. The results were written as numeric values which were then directly compared. To further test if the affinity calculations can correctly predict affinities that correlate to IC_{50} -values from in-vitro experiments, we first had to transform every IC_{50} -concentration to pIC_{50} . The pIC_{50} is the negative log of IC_{50} after it has been converted to molar. The higher the pIC_{50} -value, the more potent the compound binds. This provides not only a better sense of the potencies, but also allows for correlation coefficients to be calculated as potencies can now be displayed on a linear axis instead of a logarithmic one. Overall, we wanted to explore if (S)-enantiomers score significantly better than their (R)-counterparts, as (S)- is overall more potent at SERT than (R)-.

In the following sections, the results of the different scoring functions including their correlation coefficients will be presented to be assessed individually. For this, the best score of each metabolite has been selected and will then be compared to its reported potency, depicted as pIC_{50} -value. Every value is rounded to three decimal places for easy readability. An overall assesment of the scoring functions across all metabolites as well as across the different scoring functions will be provided. This should give an overview of how the functions perform when compared to in-vitro affinities and whether or not the results can contribute to our research question.

It has to be noted that no pose for 1R,2S-dihydro-4-OH-MC was generated during docking. This allowed no data to be collected for this metabolite and the resulting calculations will not take it into account. All calculations were performed in Microsoft Excel.

4.2.1 Maestro Docking Score and Glide G Score

Metabolite	pIC ₅₀	Docking Score	Glide G Score
S-4-MC	4.963	-6.289	-6.416
R-4-MC	3.699	-4.682	-5.312
S-4-MMC	4.971	-5.450	-5.925
R-4-MMC	4.126	-5.854	-6.329
S-4-OH-MC	4.398	-6.553	-7.183
R-4-OH-MC	3.169	-6.747	-7.377
S-4-OH-MMC	4.494	-5.827	-6.302
R-4-OH-MMC	3.204	-6.704	-7.179
S-4-COOH-MC	1.942	-1.109	-1.662
R-4-COOH-MC	1.942	-2.607	-3.161
1R,2R-dihydro-4-MC	4.127	-7.183	-7.193
1R,2S-dihydro-4-MC	4.175	-6.433	-6.442
1S,2R-dihydro-4-MC	3.749	-6.323	-6.332
1S,2S-dihydro-4-MC	4.492	-7.513	-7.522
1R,2R-dihydro-4-MMC	3.520	-6.885	-6.885
1R,2S-dihydro-4-MMC	4.492	-4.733	-4.733
1S,2R-dihydro-4-MMC	4.183	-7.607	-7.607
1S,2S-dihydro-4-MMC	3.605	-6.487	-6.487
1R,2R-dihydro-4-OH-MC	2.863	-7.428	-7.437
1R,2S-dihydro-4-OH-MC			
1S,2R-dihydro-4-OH-MC	2.937	-7.881	-7.890
1S,2S-dihydro-4-OH-MC	3.231	-7.856	-7.865
1R,2R-dihydro-4-OH-MMC	3.231	-8.175	-8.175
1R,2S-dihydro-4-OH-MMC	3.304	-7.075	-7.075
1S,2R-dihydro-4-OH-MMC	2.501	-8.334	-8.334
1S,2S-dihydro-4-OH-MMC	2.733	-7.884	-7.884

Figure 39: Table containing all the metabolites with their pIC₅₀-values and scores. A pose for 1R,2S-dihydro-4-OH-MC was not created for unknown reasons, thus providing no data. A higher pIC₅₀-value indicates higher potency, a lower score depicts higher estimated binding affinity.

Depicted are the best scored poses for each metabolite with their respective pIC₅₀ as a reference. The maestro Docking Score and Glide G Score fail to mimic the trends that were observed in vitro, as (R)-enantiomers score better than their (S)-counterparts with S-4-MC being the only exception. This shows that affinity predictions using the Glide-algorithm are unsuited to replicate in vitro data and can thus not be assessed to answer out hypothesis.

The dihydro-metabolites, showing lower pIC₅₀-values across the board, showed similar or significantly better scores than their counterparts with just one stereocentre. This stands in direct contradiction to their reported potencies, which are overall lower.

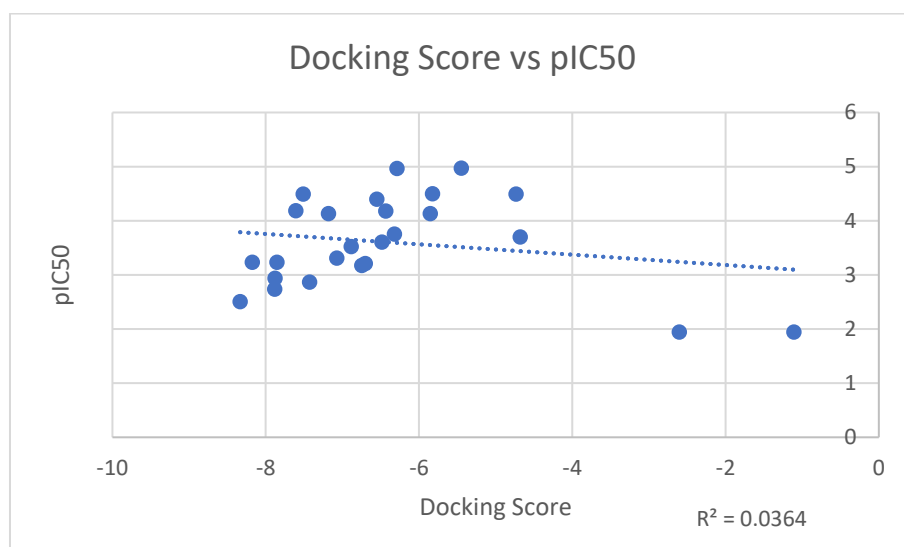


Figure 40: Graph showing Docking Score (kcal/mol) on the x-axis plotted against pIC₅₀ values of the metabolites on the y-axis with the Pearson correlation coefficient (R^2) in the bottom right corner.

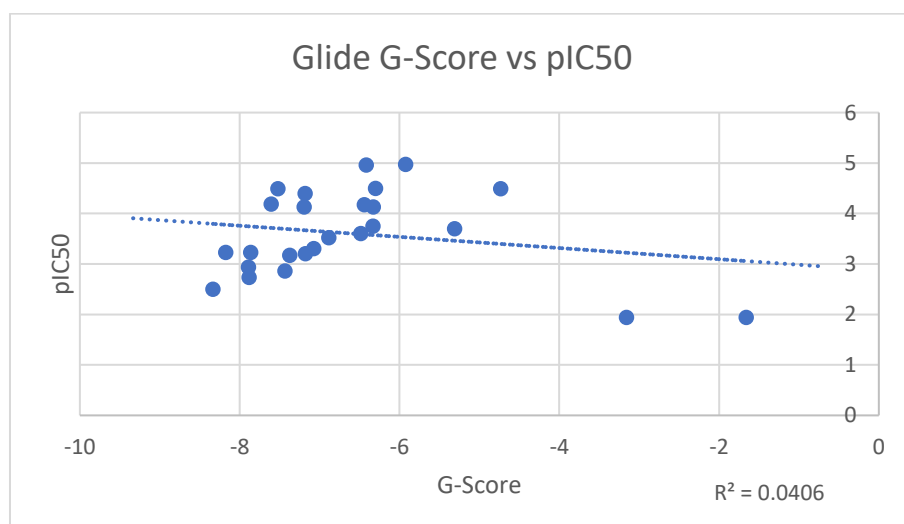


Figure 41: Graph depicting Glide G-Scores (x-axis) plotted against pIC₅₀-values (y-axis) with the Pearson correlation coefficient (R^2) in the bottom right corner.

4.2.2 Prime MM-GBSA

Metabolite	pIC ₅₀	MM-GBSA Binding dG
S-4-MC	4.963	-40.320
R-4-MC	3.699	-39.792
S-4-MMC	4.971	-31.847
R-4-MMC	4.126	-37.261
S-4-OH-MC	4.398	-43.142
R-4-OH-MC	3.169	-45.700
S-4-OH-MMC	4.494	-33.767
R-4-OH-MMC	3.204	-41.254
S-4-COOH-MC	1.942	-19.341
R-4-COOH-MC	1.942	-18.334
1R,2R-dihydro-4-MC	4.127	-34.507
1R,2S-dihydro-4-MC	4.175	-32.769
1S,2R-dihydro-4-MC	3.749	-38.981
1S,2S-dihydro-4-MC	4.492	-38.602
1R,2R-dihydro-4-MMC	3.520	-29.405
1R,2S-dihydro-4-MMC	4.492	-27.620
1S,2R-dihydro-4-MMC	4.183	-37.184
1S,2S-dihydro-4-MMC	3.605	-38.298
1R,2R-dihydro-4-OH-MC	2.863	-35.519
1R,2S-dihydro-4-OH-MC		
1S,2R-dihydro-4-OH-MC	2.937	-42.090
1S,2S-dihydro-4-OH-MC	3.231	-36.341
1R,2R-dihydro-4-OH-MMC	3.231	-47.153
1R,2S-dihydro-4-OH-MMC	3.304	-31.334
1S,2R-dihydro-4-OH-MMC	2.501	-36.863
1S,2S-dihydro-4-OH-MMC	2.733	-42.920

Figure 42: Table depicting pIC₅₀ values of metabolites and their calculated MM-GBSA Binding dG score.

The binding free energy is estimated in Prime MM-GBSA. It follows the principle of the Docking and Glide G Score, meaning that lower values translate to better binding affinity. The overall picture is similar to the aforementioned scoring options: (S)- does not show trends of performing better than (R)-, as can be seen by comparing the scores of (R/S)-4-OH-MC or (R/S)-4-MMC for example.

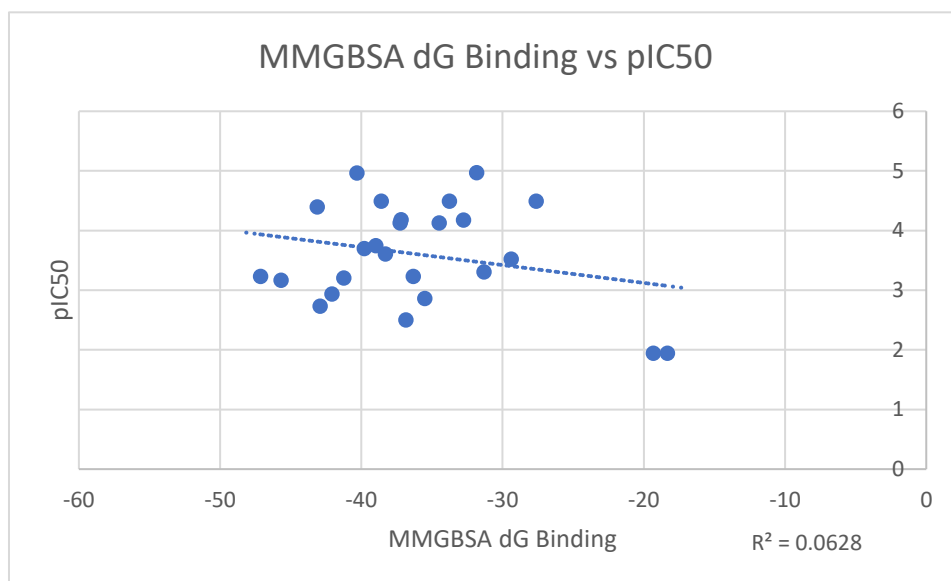


Figure 43: Graph depicting MM-GBSA dG Binding scores (x-axis) plotted against pIC50—values of mephedrone metabolites (y-axis). Pearson's R^2 shown in bottom right corner.

Pearson Correlation coefficients report an R^2 value of 0.06 with a p-value of 0.58 and $R^2 = 0.013$ and $p=0.53$ for the Spearman Ranked correlation coefficients.

4.2.3 LigandScout

Metabolite	pIC ₅₀	Binding Affinity Score	MMFF94 Binding Enthalpy
S-4-MC	4.963	-21.100	-63.587
R-4-MC	3.699	-18.200	-70.012
S-4-MMC	4.971	-17.000	-59.629
R-4-MMC	4.126	-18.680	-69.415
S-4-OH-MC	4.398	-20.940	-68.525
R-4-OH-MC	3.169	-20.440	-78.358
S-4-OH-MMC	4.494	-16.920	-62.680
R-4-OH-MMC	3.204	-18.030	-75.203
S-4-COOH-MC	1.942	-14.160	-69.841
R-4-COOH-MC	1.942	-19.200	-78.292
1R,2R-dihydro-4-MC	4.127	-19.840	-59.431
1R,2S-dihydro-4-MC	4.175	-17.020	-52.719
1S,2R-dihydro-4-MC	3.749	-21.470	-60.363
1S,2S-dihydro-4-MC	4.492	-24.250	-58.990
1R,2R-dihydro-4-MMC	3.520	-20.570	-58.141
1R,2S-dihydro-4-MMC	4.492	-14.710	-38.756
1S,2R-dihydro-4-MMC	4.183	-20.950	-53.409
1S,2S-dihydro-4-MMC	3.605	-23.940	-42.368
1R,2R-dihydro-4-OH-MC	2.863	-26.690	-65.756
1R,2S-dihydro-4-OH-MC			
1S,2R-dihydro-4-OH-MC	2.937	-23.910	-62.453
1S,2S-dihydro-4-OH-MC	3.231	-20.980	-50.987
1R,2R-dihydro-4-OH-MMC	3.231	-23.110	-66.872
1R,2S-dihydro-4-OH-MMC	3.304	-19.000	-57.348
1S,2R-dihydro-4-OH-MMC	2.501	-27.170	-68.202
1S,2S-dihydro-4-OH-MMC	2.733	-23.230	-50.641

Figure 44: Table presenting metabolites with corresponding pIC₅₀-values, binding affinity scores and estimated MMFF94 binding enthalpy.

The calculations performed by LigandScout report (R)-enantiomers showing better binding affinity scores than their (S)-counterparts in all instances but one. The MMFF94 binding enthalpies perform even less favourably for the (S)- enantiomers; (R)- is predicted to be the better binder across the

board, again in contrast to the in-vitro IC₅₀-concentrations. Estimated affinities and observed concentrations also do not correlate, as the following graphs will demonstrate.

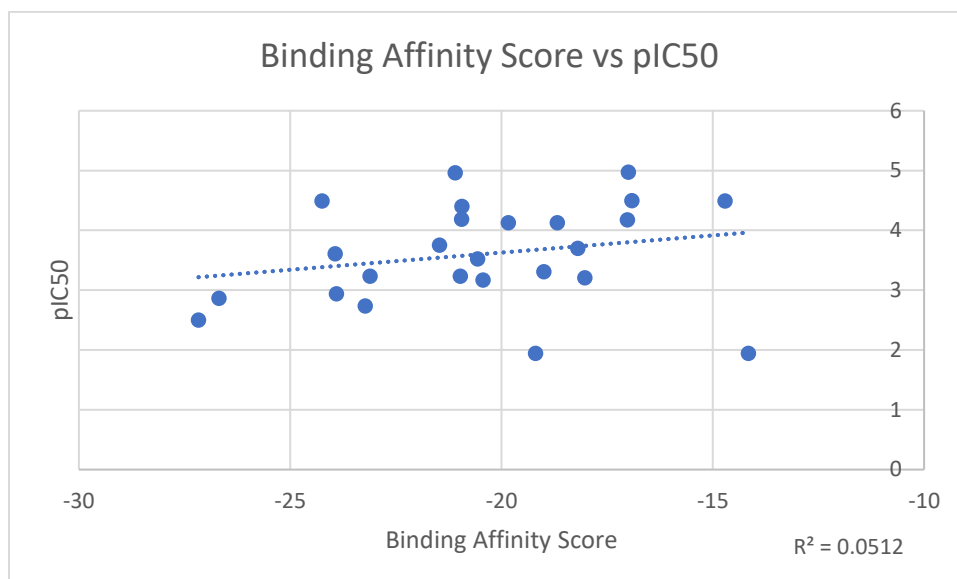


Figure 45: Scatterplot showing the LigandScout Binding Affinity Score (x-axis) plotted against pIC₅₀-values of corresponding mephedrone metabolites (y-axis). R² of the Pearson Correlation coefficient in bottom right.

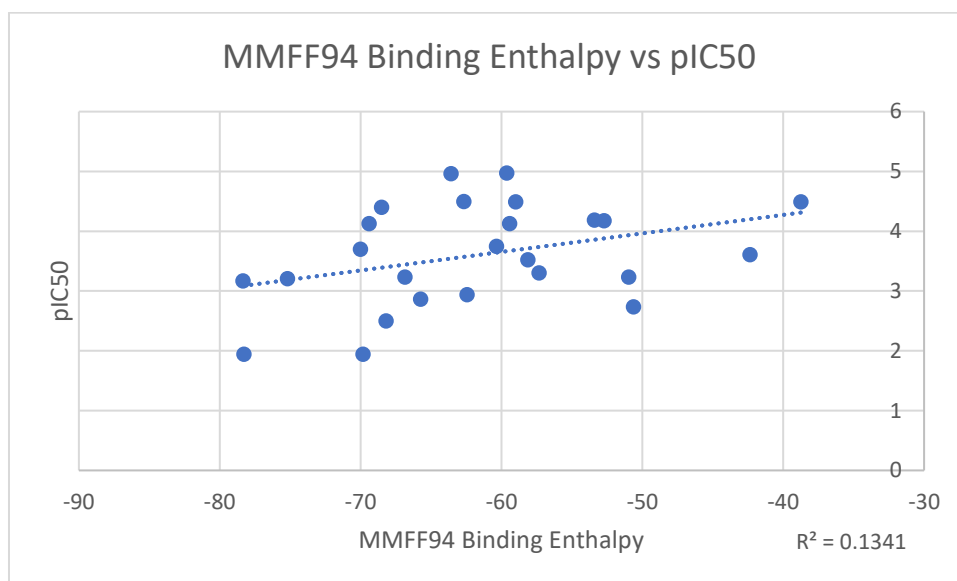


Figure 46: Scatterplot showing estimated MMFF94 Binding Enthalpy (x-Axis) plotted against pIC₅₀-values of corresponding mephedrome metabolites (y-axis). R² of the Pearson Correlation coefficient in bottom right.

The Pearson's Correlation coefficients, which are depicted in the plots above, show very weak correlation between the pIC₅₀-values and their respective estimated affinities. R² is very low in both cases and p-values come in at p=0.43 for the Binding Affinity Score and p=0.14 for estimated MMFF94 Binding enthalpy.

Spearman Ranked correlation coefficients are R²=0.077 and R²=0.1178 with p=0.31 and p=0.23 for the Binding Affinity scores and MMFF94 binding enthalpy estimations respectively.

4.2.4 MOE E-Score

Metabolite	pIC ₅₀	MOE E-Score
S-4-MC	4.963	-4.445
R-4-MC	3.699	-4.993
S-4-MMC	4.971	-4.670
R-4-MMC	4.126	-5.258
S-4-OH-MC	4.398	-5.221
R-4-OH-MC	3.169	-5.083
S-4-OH-MMC	4.494	-5.090
R-4-OH-MMC	3.204	-5.181
S-4-COOH-MC	1.942	-5.671
R-4-COOH-MC	1.942	-5.770
1R,2R-dihydro-4-MC	4.127	-5.214
1R,2S-dihydro-4-MC	4.175	-4.897
1S,2R-dihydro-4-MC	3.749	-4.729
1S,2S-dihydro-4-MC	4.492	-4.873
1R,2R-dihydro-4-MMC	3.520	-4.818
1R,2S-dihydro-4-MMC	4.492	-4.737
1S,2R-dihydro-4-MMC	4.183	-4.207
1S,2S-dihydro-4-MMC	3.605	-5.126
1R,2R-dihydro-4-OH-MC	2.863	-5.065
1R,2S-dihydro-4-OH-MC		
1S,2R-dihydro-4-OH-MC	2.937	-5.156
1S,2S-dihydro-4-OH-MC	3.231	-5.270
1R,2R-dihydro-4-OH-MMC	3.231	-6.288
1R,2S-dihydro-4-OH-MMC	3.304	-5.538
1S,2R-dihydro-4-OH-MMC	2.501	-5.146
1S,2S-dihydro-4-OH-MMC	2.733	-5.660

Figure 47: Table depicting mephedrone metabolites with their corresponding pIC₅₀-values and MOE Affinity dG scores.

The estimations performed by MOE follow the trend of the preceding software solutions. Again, (S)- does not show better affinities than (R)- with S-4-OH-MC being one exception with a marginal increase of roughly 3%. Furthermore, substances with higher pIC₅₀-values and therefore higher potency do not report to have scored better.

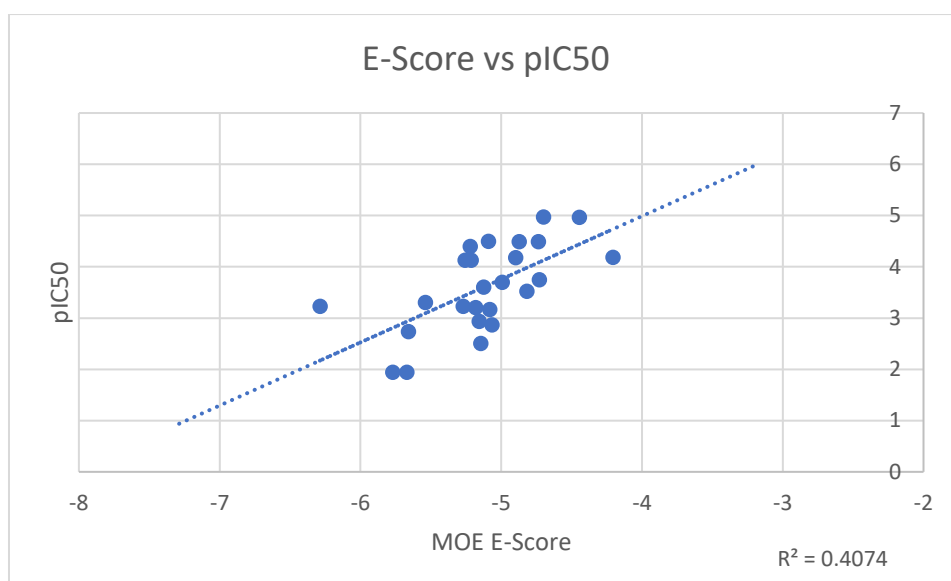


Figure 48: Scatterplot showing MOE E-Scores (x-axis) plotted against the pIC50-values of corresponding mephedrone metabolites. Pearsons R^2 depicted in the bottom right.

Pearson Correlation analyses report $R^2=0.4074$ and $p > 0.05$ whereas calculation of the Spearman Ranked correlation coefficients report $R^2=0.4001$ and $p=0.075$. This makes the affinity dG score the method that shows the highest statistical significance, but the R^2 of 0.40 shows only weak correlation.

4.2.5 Summary of Scoring Methods

	E-Score	Docking Score	G Score	MM GBSA Binding dG	Binding Affinity Score	MMFF94 Binding Enthalpy
Pearson R^2	0.4074	0.0364	0.0406	0.0628	0.0512	0.1341
p-value	0.0011	0.6624	0.6481	0.5865	0.4320	0.1408
Spearman R^2	0.4001	0.0869	0.0052	0.0136	0.0767	0.1178
p-value	0.0760	0.2863	0.7142	0.5331	0.3090	0.2328

Figure 49: Pearson and Spearman correlation coefficients of every prediction method utilized, calculated in Microsoft Excel.

All the correlation coefficients were calculated in one Excel, where every datapoint and rank were inserted into a table and Spearman and Pearson correlation coefficients were calculated. As mentioned before, Spearman correlation uses ranks whereas Pearson correlation uses real datapoints. The latter method is less robust but is considered more powerful. The most significant results were found for the Affinity dG score. This method shows the best correlation with a Pearson coefficient of 0.4074 and a p-value of 0.0011.

5. Conclusion and Outlook

In this diploma thesis, docking experiments of substrate-like molecules were performed. Based on these initial results, analyses from a quantitative and qualitative perspective were performed. Binding patterns and the positioning within the binding site of the docked poses were investigated and different scoring functions were utilized to assess the estimated binding affinities of the poses. Together with an analysis of the interaction patterns and common scaffold clustering, these methods were ultimately used to look for insights on the stereoselective interactions of phase I mephedrone metabolites with the human serotonin reuptake transporter. We wanted to see if changes in stereochemistry translated to differences in binding patterns and estimated affinities and could therefore explain why our colleagues observed (S)-enantiomers of mephedrone metabolites to be more potent inhibitors and releasers than their (R)-enantiomeric counterparts.

Starting with the qualitative assessment of the docking poses, we were unable to determine key differences in binding patterns between the enantiomers. This could have a few possible reasons that we will discuss in this section:

First, the differences between (S)- and (R)- enantiomers is just the orientation of the methyl-group and the nitrogen atom. These two substituents are similar in size and it can very well be that the docking algorithm we used is not precise enough to assess these small differences of the ligands. It would be interesting to see if other docking experiments using induced fit docking could present different results. Induced fit docking renders the ligand as well as the side chains of amino acid residues flexible and would allow the binding pocket to generate a more appropriate conformation for the ligands to bind than rigid docking like Glide. This could have offered more insight on if or how the binding pocket rearranges itself after different enantiomers have been docked. After the research part for this thesis had been completed, Schrödinger announced their new IFD-MD protocol.¹⁰¹ According to their website, it allows for IFD docking experiments to be coupled to molecular dynamic simulations. The top IFD-poses would be used to simulate ligand-receptor changes. Schrödinger also highlights that this protocol could allow for homology models to be used with higher confidence. However, molecular dynamic simulations are quite time and resource-intensive processes and would most likely go beyond the size of a diploma thesis. If time were not a constraint, this would have been an interesting approach for further research on this matter.

Second, we cannot rely on much past research as very few docking experiments with cathinones were performed. At the time, we found little research that performed docking experiments in monoamine transporters, yet alone in its the outward-occluded conformation. This left us in the dark on how exactly cathinones or mephedrones are likely to bind. One paper by Saha et al.¹⁰⁰ mentions experiments conducted with (S)-enantiomers of 4-MEC and 4-MePPP in the s1 site of a drosophila

melanogaster DAT model and a homology model of SERT. They mention aromatic moieties to be positioned in subsite B of the s1 site, something we observed with our models as well. The figures published in this paper also show poses of 4-MEC with the ligands keto-group pointed in direction of Tyr-176 and Ser-438. It could be that this orientation is somewhat favourable in comparison to the orientation that some of our poses report. One could assume that the “upwards” orientation of the keto-group might be the correct orientation that cathinones assume. However, this insight has to be taken with a grain of salt as only (S)-enantiomers were investigated, and we do not know how a (R)-enantiomer of 4-MEC would have docked on their experiments.

Third, the homology model that we used in this research needs to be addressed:

Homology models have the disadvantage of not being actual crystallized structures that show how a substrate or inhibitor actually bind within their respective binding sites. This doesn't allow us to use a bound molecule as reference and we had to rely on the docked serotonin molecule that our colleague Eva Hellsberg placed within the binding site. Even if the docking results with this model were more satisfactory than previous runs that were performed using a SERT structure in its outward-open conformation, we still do not know if the binding site is in its exact conformation that it would assume when bound to substrates *in vivo*. After all, it is still a model and any results we observed have to take that fact into consideration.

The quantitative assessment of the docking poses (i.e. the utilization of different scoring functions) was undertaken to assess whether or not scoring functions predict estimated affinities of the metabolites that correlate to their *in-vitro* potencies. If that were the case, (R)-enantiomers would score worse than (S)-enantiomers.

We used force-field based scoring functions (Prime MM-GBSA and MMFF94 Affinity calculations) as well as empirical scoring functions (Glide G-Score, Maestro Docking Score, HYDE and Affinity dG Score) to estimate our predicted binding affinities. Force-field based functions work by accumulating individual terms that quantify the van der Waals and electrostatic interaction energies of protein-ligand pairs.¹⁰³ Empirical Scoring functions on the other hand sum up factors like hydrogen bonds, steric clashes or hydrophobic effects.

The results that our scoring assessment show are not in conjunction to the IC₅₀-values that our colleagues assays have reported. Across the board, (S)- and (R)-enantiomers scored similarly, with no enantiomer significantly outscoring the other. Furthermore, the dihydro-metabolites, showing reduced potency *in-vitro*, were scoring better than their non-reduced counterparts, which is also contradictory. Correlation analyses of the scores in comparison to pIC₅₀-values confirmed the lack of correlation, as low R² values were reported for Spearman ranked as well as Pearson correlation coefficients. In summary, scoring methods were unable to help in the assessment of our hypothesis, as no differences between (S)- and (R)-enantiomers were found. In this section we try to explore why

this was the case.

First, the binding patterns of the metabolites were quite similar regardless of stereochemistry. Both enantiomers have the same number of functional groups and the effects of stereochemistry are quite small, given the fact that only the orientation of one cationic nitrogen and a methyl group was affected. As empirical scoring functions are taking hydrogen bonding and hydrophobic contact terms into account to assess binding affinities.¹⁰³ If (R)- and (S)-enantiomers report the same number of interactions with the same amino-acid residues at similar distances, it seems like a logical consequence that empirical scoring functions score the enantiomers similarly. The data the calculations rely on is too similar. In addition, the reduction of the keto group to a hydroxyl group on the mephedrone backbone now offers an additional functional group that can form hydrogen bonds with amino acid residues inside the binding pocket. As empirical scoring functions essentially sum up the contribution of individual terms, an additional hydroxy group that interacts could explain why the dihydro-metabolites showed better scores.

As force-field based scoring functions also failed to distinguish between (S)- and (R)-enantiomers, we also need to address why they assessed the enantiomers in a similar manner. The metabolites we investigated in this thesis are all stereoisomers of each other. Per definition, optical activities differ for the stereoisomers, yet physicochemical properties remain the same. This would mean that terms like solvation energies or accessible surface areas should also be the same for (S)- and (R)-enantiomers of metabolites with the same chemical structure. We thus assume that solvation models like MM-GBSA⁹⁵, which use terms for solvent accessible surface area and polar as well as non-polar solvation, cannot distinguish between (S)- and (R)- enantiomers as the terms affected are the same for both compounds. This together with the very similar binding interactions and positioning of the ligands could be one reason why MM-GBSA-based scoring functions reported similar affinities for both enantiomers. Calculations based on the MMFF94⁸⁴ force field also take van-der Waals and electrostatic interaction term into account. We assume that these terms differ too little between the docked poses of our chiral metabolites and are thus reason for our similar results. But again, this is just an assumption as a deeper insight into the exact modalities of MM-GBSA or MMFF94 calculations would be necessary to further explain our results.

The notion that scoring functions might not be ideal assessments to determine successful binding of ligands within protein targets has already been discussed in past research.¹⁰² It is likely that the small differences between enantiomers we observed are too complex for docking and scoring functions to consider. If stereoselective binding of phase I mephedrone metabolites happens in the s1 site of human SERT, we were unable to distinguish it.

In the end, this research could be further expanded by implementing other docking protocols like induced-fit docking or molecular dynamics. The docking we have performed is rigid docking, where the binding site is unable to adapt to the ligand. Induced-fit docking could be performed to see if and how the binding site changes when different mephedrone metabolites are docked within it. Furthermore, we have to remember that the exact mechanism of action of amphetamine- and cathinone-like substances is still not well understood. Assuming that it primarily interacts at the s1 site is not wrong, but we do not know the modalities of its binding kinetics, nor do we know how the receptor reacts when substrate-like substances are bound to it. For this, molecular dynamic simulations would be the next step but again, it is a very time consuming and resource-heavy process that would have gone beyond the scope and timely constraints of a diploma thesis. The allosteric effects of the s2 site are also worth mentioning. It is known that simultaneous inhibitor binding at the s2 site of SERT is affecting the dissociation rates of the second inhibitor molecule bound to s1.⁷¹ If and how mephedrone metabolites or substrate-like substances interact with the s2 site is something worth exploring.

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7.2 Abstract

Mephedrones are chemical derivatives of amphetamines and cathinones. Together they are members of the phenylalkylamine group of substances. Due to their stimulatory effects, amphetamine- and cathinone-like substances were used for medicinal purposes in the early 20th century. However, due to psychomotoric side-effects, prescription of these substances subsided. Nevertheless, recreational usage still remained, and it was during the early 2000s that cathinones and later on structural derivatives became prevalent on the recreational drug market. Due to their widespread abuse, they have become a topic for research.

This thesis is based on results of preceding research conducted by the group of Harald Sitte at the Medical University of Vienna. They investigated the effects of different phase-I mephedrone metabolites on the monoamine reuptake transporters SERT, NET and DAT. Their results suggest that inhibitory affinities are affected by the metabolites' stereochemistry at SERT. This thesis investigates the hypothesis that binding patterns within the central $s1$ binding site of SERT differ for (S)- and (R)-enantiomers and are thus reason for their differing potencies, which were reported as half maximal inhibitory concentrations (IC_{50}).

To investigate this hypothesis, the binding mode of a selection of phase I mephedrone metabolites needs to be analysed. To do this, docking these metabolites into the $s1$ site of SERT with the goal of finding binding interactions specific to enantiomers was performed. However, the docking experiments failed to report a binding pattern that was exclusive for either kind of enantiomer.

In addition to the docking experiments, affinity prediction programs were utilized. These affinity predictions give hints on which metabolites have higher estimated affinities for the binding site. In this diploma thesis, the Glide Score, Docking Score, MM-GBSA as well as scoring functions of MOE and LigandScout were used. The results of these affinity predictions were set in correlation to experimentally assessed potencies with the aim to test whether or not programs can accurately predict in-vitro results. This was done by calculating Spearman and Person correlation coefficients between experimental and predicted values, with the result that no scoring function showed statistically significant correlation.

These finding conclude that docking experiments together in conjunction with affinity prediction are unsuited to explain the potency differences that were observed in in-vitro experiments. The high level of similarity of docking results as well as the small differences between the enantiomers are assumed to be reason for this.

7.3 Zusammenfassung

Die Stoffgruppe der Mephedrone beinhaltet chemische Derivate der Amphetamine und Cathinone. Allesamt sind Teil der Familie der Phenylalkylamine, eine Stoffgruppe deren Derivate durch ihre stimulierende pharmakologische Wirkung in der ersten Hälfte des zwanzigsten Jahrhunderts zu medizinischen Zwecken eingesetzt wurde. Aufgrund von starken psychomotorischen Nebenwirkungen wurde das Verschreiben von Amphetaminen eingestellt. Sie wurden aber weiterhin zu missbräuchlichen Zwecken benutzt und sind heute durch verschiedene Derivate, welche auf der Amphetamin- bzw. Cathinon-Struktur basieren, global auf dem Schwarzmarkt vertreten. Durch ihr erneutes Aufkommen um die Jahrtausendwende und ihre weitere Verbreitung sind sie heute ein zentrales Forschungsobjekt.

Diese Diplomarbeit basiert auf den Forschungsergebnissen der Arbeitsgruppe von Harlad Sitte an der Medizinischen Universität Wien, welche verschiedene Phase-I Metaboliten des Cathinon-Derivats Mephedron untersucht haben. Dafür wurden verschiedene Metabolite an der Monoamin-Wiederaufnahmetransportern SERT, NET und DAT getestet. Ihre Ergebnisse deuten darauf hin, dass die Wirkstärke durch die Stereochemie der Metaboliten an SERT unterschiedlich stark ausfällt. In dieser Diplomarbeit wurde die Hypothese untersucht, dass die unterschiedlichen Wirkungsstärken der Metaboliten auf unterschiedliche Bindungsmodi in der zentralen S1 Bindungstasche des Serotonin-Wiederaufnahmetransporters zurückzuführen ist.

Dafür wurden Docking Experimente mit einer Auswahl von Phase-I Mephedron Metaboliten ausgeführt, für welche mittlere inhibitorische Konzentrationswerte (IC_{50}) aus Laborversuchen vorliegen. Die verschiedenen Wechselwirkungen der Metabolite mit dem Zielprotein wurden dabei untersucht. Es hat sich herausgestellt, dass die Docking Experimente keinen Bindungsmodus hervorgebracht haben, welcher sich für eines der Enantiomere spezifisch erwiesen hat.

Weiters wurden verschiedene Programme für Affinitätsberechnungen verwendet. Diese Programme können Ergebnisse eines Docking Experiments auswerten und erste Hinweise darauf geben, wie sich die Moleküle hinsichtlich ihrer Bindungsaffinität verhalten. In dieser Diplomarbeit wurden der Glide Score, Docking Score, MM-GBSA sowie Berechnungen von MOE und LigandScout verwendet. Die Ergebnisse dieser Vorhersagen wurden mit den IC_{50} -Werten in Korrelation gesetzt, um zu untersuchen, ob die Vorhersagen mit den experimentell ermittelten Werten übereinstimmen. Dafür wurden Spearman und Person Korrelationskoeffiziente berechnet.

Dabei hat sich gezeigt, dass für die Enantiomere welche unterschiedliche in-vitro Affinitäten vorgewiesen haben, keine signifikant unterschiedlichen Affinitäten berechnet wurden. Die

Korrelationen zwischen in-vitro Affinitäten und berechneten Affinitäten erwiesen sich als statistisch nicht signifikant.

Diese computergestützte Arbeit führt zum Schluss, dass Docking Experimente nicht in der Lage sind Bindungsmodi vorherzusagen, welche stereoselektives Binden der Metabolite in Betracht zieht.

Weiters hat sich gezeigt, dass Affinitätsberechnungen nicht mit experimentell ermittelten Affinitäten korrelieren. Sowohl die Ähnlichkeiten der Metaboliten als auch die sehr ähnlichen Docking-Resultate könnten dafür verantwortlich sein.