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# Drugs, structures, fragments : substructure-based approaches to GPCR drug discovery and design

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CHAPTER 3

# Substructure Mining of GPCR Ligands Reveals Activity-Class Specific Functional Groups in an Unbiased Manner

This chapter is based upon:

van der Horst, E.; Okuno, Y; Bender, A; IJzerman, A. P. Substructure Mining of GPCR Ligands Reveals Activity-Class Specific Functional Groups in an Unbiased Manner. *J. Chem. Inf. Model.* **2009**, *49 (2)*, 348-360.

## 3.1 Abstract

In this study, we conducted frequent substructure mining to identify structural features that discriminate between ligands that do bind to G protein-coupled receptors (GPCRs) and those that do not. In most cases, special chemical representations resulted in the most significant substructures. Substructures found to be characteristic for the background control set reflected reactions that may have been used to construct this library, e.g. for the ChemBridge DIVERSet library employed here ester and carboxamide moieties. Alkane amine substructures were identified as most important for GPCR ligands, e.g. the butylamine substructure, often linked to an aromatic system. Hierarchical analysis of targeted GPCRs revealed well-known motifs and new substructural features. One example is the imidazole-like substructure common for the histamine binding receptor ligands. Another example is the planar ring system consisting of a fused five- and six-membered ring (indole-like substucture) common for the serotonin binding receptors ligands.

## 3.2 Introduction

Chemical structure mining has a long tradition in the prediction of molecular properties. Methods for analyzing the structural features of molecules can be broadly divided into two categories: methods that focus on predefined structural parts (fragments) and methods that consider the complete set of possible substructures of a molecule. Methods of the first category apply a set of fragmentation rules to partition the molecular structure into discrete fragments, which are then analyzed. Examples of such fragments are ring systems, linkers, and side chains,[1-5] synthetic building blocks,[6, 7] or algorithmically defined molecular fingerprints.[8, 9] Analysis of fragment frequencies has proven useful for the description and comparison of molecular databases and for the identification of 'chemical clichés'.[10, 11] For instance, unexplored parts of chemical space, with only a few fragments, become apparent, as well as the preferences of chemists for certain reaction types or starting materials, yielding a more densely populated chemical space. Analyzing the co-occurrence of fragment may further yield valuable information, i.e. on pairs that seem to avoid each other or pairs that constitute a common template. Analyzing fragment occurrences may also aid the design of new ligands in (chemical) fragment-based drug discovery[12] and is a prerequisite for similarity searching, an approach in which predefined structural parts are utilized to construct molecular fingerprints. A fingerprint is a reduced representation of the molecule that holds information on the presence or absence of certain features.[13] Features included in the fingerprint may be aforementioned (discrete) fragments, such as rings and functional groups (so-called structural keys, e.g. MDL keys[14]), but also algorithmically defined, such as arbitrary structural elements of fixed size, of which the circular fingerprint has gained some popularity.[15] Since predefined fragmentation rules are dependent on the choices of the chemist, analyses and predictive models are inherently biased.

Complementary to analysis using predefined structural fragments are methods that consider all possible substructures that are found in the 2D structure of a molecule. These substructure-based methods thus avoid the bias that is intrinsic to the use of predefined fragments. However, these approaches come at the price of computational expense. In a simple structure as for the amino acid alanine without explicit hydrogens, the number of substructures amounts to 20 already. Adding two methyl groups, i.e. the amino acid valine without explicit hydrogens, will yield 39 substructures. Because of the exponential growth of substructure count with increasing molecule size, most substructure methods seek ways to limit the number of substructures to be evaluated. Batista *et al*. described a method that uses repeated random fragmentation of molecules to generate profiles that served as a measure of molecular similarity,[16] and later applied this to database screening.[17] Although this work represents substructure analysis in an unbiased manner, the success of the method depends on the choice of parameters (iterations, number of bonds cut). Besides that, the set of evaluated substructures is not complete, i.e. the evaluation of a substructure depends on chance and not on occurrence in the molecules. Two other methods that are substructure-based are maximal common substructure analysis and frequent substructure mining. Maximal common substructure analysis finds the largest connected substructure that a certain number of molecules have in common.[18, 19] It is used for similarity and SAR analysis, for instance as implemented in commercial tools such as Pipeline Pilot (Scitegic) and ClassPharmer (Simulations Plus).[20, 21] Frequent substructure mining finds the most common substructures in one or more sets of molecules by considering all substructures that occur in the molecules. It uses a minimum-frequency constraint to control the amount of substructures that are evaluated. It is an application of frequent subgraph mining, which finds all frequently occurring connection patterns from a set of graphs. Recent advances in graph-mining algorithms, together with the steady growth of computing power have made it possible to mine datasets as large as 200,000 molecules on a standard PC.[22] Frequent substructure 'miners' have been successfully applied for prediction of CNS activity, bioactivity, and toxicity.[23-25] The SUBSTRUCT program developed by Engkvist *et al.* distinguished CNS active compounds with

approximately 80% accuracy. However, their approach was bound to a maximum size of the generated substructures, which was between 1 and 4 atoms by default. In contrast, Borgelt *et al.* identified substructures that model the activity classes for HIV-1 antiviral screening data, using an algorithm that was developed by these authors.[24] Similarly, Kazius *et al*. applied the frequent subgraph miner Gaston[26, 27] to mutagenic compounds and extracted a decision list of six discriminative structural features associated with mutagenicity.[28] For this, the authors adopted several different types of chemical representation. So-called elaborate chemical representation adds extra information to a molecule, for instance by adding extra labels to atoms or by replacing certain atoms with wildcards (abstractions). The authors obtained the most significant results when elaborate chemical representation was used. Similarly, others also reported improved findings when using, for instance, abstractions for rings and chains (reduced graphs).[29, 30]

In the context of GPCR (G Protein-Coupled Receptor) ligands, the most important source of current medicines, only methods that analyze discrete fragments have been described. For instance, a property-based scoring scheme was constructed for the classification of GPCR ligands, intended for the creation of focused libraries.[31] Similarly, Schnur *et al.* identified frameworks, or substructure classes that are common for families of ligands. In the context of GPCR ligands, these were defined as privileged structures albeit that these frameworks were not selective for GPCRs.[32]

While previous studies were limited to analysis of pre-defined fragments, in this chapter, we will use a complete method (i.e. frequent substructure mining) to analyze the structural features of GPCR ligands. This method represents an unbiased as well as exhaustive way to mine information contained in chemical datasets of GPCR ligands. We build upon the approach described by Kazius *et al*.[28] to find frequently occurring substructures that are discriminative for GPCR ligands. This is accomplished by comparing the ligands against a control group and analyzing the frequencies of all possible substructures that occur in the sets. To include additional chemical details, both normal and elaborate chemical representations (atom and bond type

abstractions with atom labels) were used. However, abstractions for molecular parts, such as special types for rings or chains, were omitted since these depend on the choice of the chemist, thereby introducing a bias. In addition, with reduced graph representations, information such as bond distance or substituent positions is lost, which led us to believe that the choice of the current algorithm is appropriate for the work performed here. To derive the significant features common to specific groups of ligands, we conducted two additional experiments on subsets of the original sets. For the first experiment, subsets were based on the presence of the previously found most-significant substructure. For the second experiment, ligands were grouped into subsets according to the hierarchical classification of their target GPCRs. In addition to the work of Kazius *et al*.,[28] we also analyzed which substructures are rarely found in the GPCR ligands compared to the control group. This type of analysis would be less useful for prediction of mutagenicity, but has added value for prediction of receptor binding. In the latter case, there will not only be substructures that contribute to binding, but also ones that lower this possibility (e.g. steric hindrance, unfavorable pharmacophoric features). From this two-sided analysis, we established a comprehensive analysis of favorable and unfavorable features of this important ligand class.

## 3.3 Materials and Methods

### 3.3.1 Datasets.

GPCR ligands were collected from two publicly available online resources: the GPCR-ligand database (GPCR-Ligand Database, or GLIDA) from the University of Kyoto,[33] and a database for human GPCRs and their ligands (hGPCR-lig).[34] The set from GLIDA consisted of 22,122 ligands for human, mouse and rat receptors, collected from various public data sources, such as PubChem,[35] Ki Database,[36] and scientific literature. The 17,908 GPCR ligands from the hGPCR-lig database are taken from scientific literature and the MDL Drug Data Reports (MDDR). Although the sets do overlap, the

first one is more illustrative for published research from academia while the second is more representative for any patented drugs recently launched or under development, i.e. commercial drugs, preclinical candidates etc. These two sets were compared against a control set, denoted as the background set. For this, 15,993 compounds from ChemBridge's DIVERSet screening collection were used.[37] This set was meant to provide a suitable contrast to the GPCR ligands since we were interested in how GPCR ligands differ from a varied collection of small molecules. In GLIDA, ligands are grouped according to the classification of their targets. Targets follow the pharmacological classification of GPCRs as used by the International Union of Pharmacology (IUPHAR).[38, 39] Targets are arranged into a hierarchy of subfamilies, families, and classes, as used by the GPCRDB information system.[40] In GLIDA, some receptor classes are missing due to the low number of known ligands, e.g. trace amines in amine-binding GPCRs. Each ligand-target pair is annotated with an activity type, namely: full agonist, partial agonist, agonist, antagonist or inverse agonist. Since the annotation of GLIDA is not entirely completed yet, we only used 'agonist' (for full, partial, and agonist) and 'antagonist' (for inverse agonist and antagonist). Classification of compounds as active depends on the origin of the compounds. A reported affinity in one of the source databases classified a compound as active, independent on the reported binding affinity. The sets were cleaned and standardized using Scitegic's Pipeline Pilot 6.1.5.0 Student Edition.[20] Salts, counter-ions, and other small fragments associated with the molecules were removed and zwitterions neutralized. Charge and stereochemical information was discarded and bonded hydrogen atoms were omitted from the representation. After that, ChemAxon's standardizer[41] was used (for consistency with existing databases) to convert the structures into a uniform representation and to filter out duplicates. Only structures with molecular weight below 1000 Da were used. The final GPCR ligands sets consisted of 21,619 compounds for GLIDA, 16,509 compounds for hGPCR-lig, and 15,983 compounds for the background set. In some cases, analysis of large datasets using elaborate representation (see below) proved to be difficult since physical limits of system resources (maximum file size) were reached. In these cases, the experiment was

continued using a sampled set of 5k ligands. These 'sampled sets' were constructed using Pipeline Pilot's Random Percent Filter.[20]

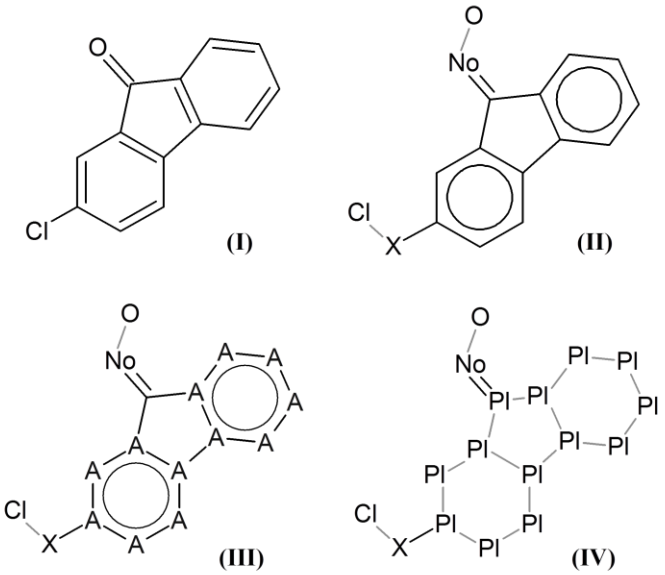



**Figure 1.** Example molecule in normal **(I)** and two elaborate chemical representations: one for aromatic bonds **(II)**, one for aromatic atoms and bonds **(III)**, and one for planar ring systems **(IV)**. In the normal representation **(I)**, aromatic bonds are represented as alternating single and double bonds, whereas in the first elaborate representation **(II)**, a special type for aromatic bonds is used. In addition to this representation, the representation in **(III)** adds a special type for aromatic atoms (A). Since this example molecule has a planar ring system, all atoms that constitute the system are denoted as 'Pl' in the planar representation **(IV)**. In both elaborate chemical representations, wildcards are used for heteroatoms ('No') and for halogens ('X') with a label attached specifying the actual atom-type.

### 3.3.2 Chemical representation.

Molecular structures are represented as labeled graphs. Hydrogen atoms were excluded from representation. Four types of chemical representation were used: the initial chemical structure representation with the atom and bond types unchanged, and three 'Elaborate' Chemical Representations (ECRs).[28] Figure 1 offers an example

that accompanies the following description of the representations. Elaborate representation is a method to include extra information about the molecule by using abstractions, translations, and/or extra labels. The first elaborate representation includes a special bond type for aromatic bonds. In addition, the second one has a special type for aromatic atoms. The third representation offers a special type for planar ring systems, which has been successfully applied previously to predict the mutagenicity of compounds.[28] In elaborate chemical representation, aliphatic Nitrogen, Oxygen, and Sulfur atoms were represented as aliphatic heteroatom by replacement with the symbol No. An extra label was attached to N and O to indicate the type and number of bound hydrogens, 'Ze' (zero) for no bonded hydrogens, 'On' for one bonded hydrogen, and 'Tw' for two bonded hydrogens. The halogen atoms, Cl, Br, I, and F, were replaced by X and an extra label was attached to indicate their type. Note that the above-mentioned representation differs from (Kazius *et al*.) in that only one hetero-atom type was used and not two types for 'small heteroatom' (No) and 'large heteroatom' (Ps). In addition, fluor (F) was included as part of the halogen abstraction. Aromatic atoms and bonds were detected with basic aromaticity. Figure 1 has an example of a molecular structure in normal and chemical representation. The use of alternate representations may cause the same graph to appear multiple times. The aim of abstractions for atom and bond types is to raise the occurrence of similar substructures above the support threshold. Individually, these substructures might go undetected; however, the occurrence of their common representation sums the individual frequencies.

### 3.3.3 Frequent substructure mining.

The frequent subgraph-miner Gaston was used to find all frequently occurring substructures in the datasets.[26, 27] Frequent subgraph miners such as Gaston iterate over all molecules, extracting all possible substructures per molecule. Current subgraph miners utilize several approaches to keep the number of found substructures to a minimum. One reasoning is that a larger substructure can never occur more frequently than the smaller substructures it consists of. This allows numerous

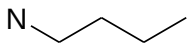
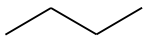
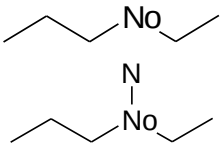
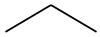
substructures to be pruned before being considered. Compared to other algorithms, Gaston is more efficient since computationally expensive operations take place in the last steps, when a large number of possible substructures has already been discarded. For a quantitative comparison of Gaston with other frequent subgraph miners, see Wörlein *et al*.[22] The importance of a substructure was determined by comparing its frequency against the frequency of occurrence in the control set. The most revealing substructures are those that occur frequently in one set and not in the other. As a measure of the importance of a substructure, the significance of association with one of the sets was determined by calculating the p-value of the finding. The p-value as used in this study is defined in page 3 of the Supporting Information of Kazius *et al*. 2005.[42] It is the probability to find a statistical association with one of the two groups based on chance alone. On the assumption of a binomial distribution, it was calculated based on the number of ligands versus control group that were detected using that substructure. While this measure makes assumptions such as to the underlying distribution of features in each database, we still found it to be useful also in the ranking scheme described here. Using the p-value, the lists of frequently found substructures were ordered according to significance with the most-discriminating substructures at the top. The substructure with the lowest p-value was considered the most significant finding. P-values of substructures are the same if they have the same occurrence. When two substructures had the same p-value and one substructure was a substructure of the other substructure, only the larger substructure was kept. On average, this was the case in 25% of all substructures. In case of substructures with equal p-values that were not substructures of each other, the larger substructures had preference over smaller ones in the list ordering. For example, if alanine and valine would be substructures with equal occurrences, and hence equal p-values, only the valine substructure would be kept in the list since alanine is a substructure of valine. In the case of a leucine instead of alanine, both substructures would be kept since neither of the two is a substructure of the other. Another important parameter in frequent subgraph mining is the minimum support value, which is the relative number of molecules a substructure should occur in to be detected by the algorithm. Lowering

the minimum support will result in finding an equal or higher number of substructures. A higher number of substructures increase the chance of finding a substructure that is more significant. However, there is a balance between minimum support and p-value of the most significant substructure. This will be illustrated with the following example of two sets of 100 compounds each. Presume that the most significant substructure found at a support threshold of 30 compounds occurred in 60 active and 20 control compounds. The p-value for this substructure is 5.32e-09, which is the chance of finding this substructure based on chance alone. Lowering the minimum support from 30 to 20 means that a new set of substructures is added to the already generated set. In theory, the most significant substructure that could be added with the new set has an occurrence of 29 active and 0 control compounds. The p-value of this theoretical substructure is 1.73e-10, which is more significant than the actual found substructure (with a p-value of 5.32e-09). Therefore, the experiment should be repeated at a lower minimum support of e.g., 20 to examine whether this theoretical substructure does actually exist. The experiment is completed if it results in the same substructure as found in the first run. This is because a more significant substructure cannot be found by lowering the support, i.e. the best theoretical substructure (19 active, 0 control) has a p-value of 7.42e-07. When another, more significant substructure is found at a lower support, the process is repeated until no theoretical substructure can be found that is more significant. Concluding, the minimum support value was chosen by iteratively lowering it per run until no better (more significant) substructures could be found, resulting in practice in support values between 10% and 30% for the datasets used here.

### 3.3.4 Software

For translating the molecules into elaborate representation, for partitioning the graph sets, and for the p-value calculations simple awk and bash scripts were used. These scripts and the substructure mining were run on Scientific Linux. (Sub)structures were edited and visualized using (MS Windows based applications) Pipeline Pilot 6.1.5.0 Student Edition and MDL ISIS/Draw 2.5.[20, 43]

**Table 1.** Best discriminating substructure for each of the four elaborate representations of the GPCR ligands in hGPCR-lig ('Active' column; upper line) and in the GLIDA collection ('Active' column; lower line). For each representation, the same substructure is found in both databases, except for the 'aromatic atoms and bonds' representation. Here, the substructures differ in the presence of an extra atom label. The column labeled 'Control' refers to the background DIVERSet compound library. See Materials & Methods for explanation of p-value calculation.

| Representation | Substructure | Active | Control | P-Value |
|---|---|---|---|---|
| **Normal** |  | **12,162 (74%)**<br>**16,434 (76%)** | **4,081 (26%)** | **7.35e-1707**<br>**3.46e-2138** |
| **Ar-Bonds** |  | **11,926 (72%)**<br>**16,142 (75%)** | **4,065 (25%)** | **7.25e-1611**<br>**3.04e-2026** |
| **Ar-Atoms / Bonds** |  | **8,627 (52%)**<br>**11,166 (52%)** | **1,977 (12%)**<br>**1,517 (9%)** | **1.06e-1354**<br>**1.50e-1760** |
| **Planar** |  | **13,050 (79%)**<br>**17,739 (82%)** | **5,534 (35%)** | **7.45e-1479**<br>**2.28e-1959** |

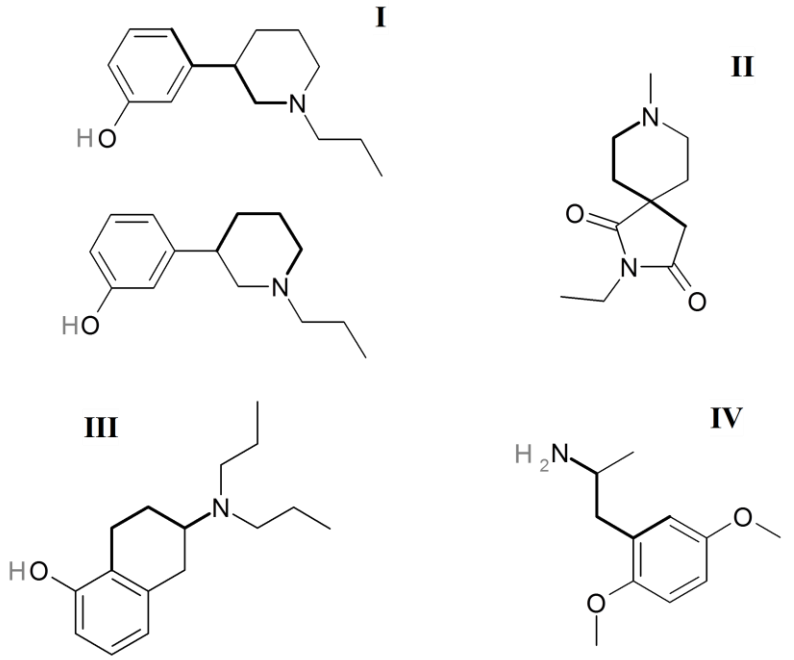



**Figure 2.** Example overlays on GPCR ligands of the most discriminative substructure, 'normal' representation. For the first ligand (I), two possible overlays are shown; multiple overlays are also possible for the ligands II and III. Note that the 'normal' representation uses Kekulé structures for aromatic systems and not separate types for delocalized bonds and aromatic atoms. This results in some interesting examples where the single bond of an aromatic ring is part of the aliphatic chain of the overlaid substructure, i.e. in structures I and IV.

## 3.4 Results and Discussion

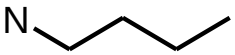
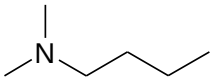
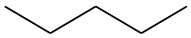
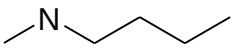
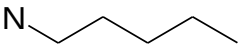
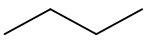
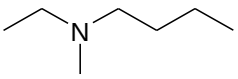
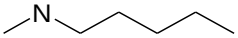
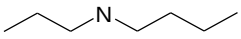
### 3.4.1 Mining of characteristic substructures of GPCR ligands.

Frequent subgraph mining was first applied to the complete sets, both hGPCR-lig and GLIDA, for a broad analysis of structural features in GPCR ligands. Analysis of the substructure distributions revealed the best discriminating substructure for each of the four elaborate representations (see Materials & Methods). The substructure

originating from the 'normal' representation performed best in discriminating GPCR ligands (both from hGPCR-lig and from GLIDA) from background compounds, i.e. the ChemBridge DIVERSet library. The statistics for all representations are summarized in Table 1, demonstrating that within each of the four representations highly significant substructures are occurring. The largest substructure was found in the 'aromatic atoms and bonds' representation, and suggests a symmetrical organization of lipophilicity (through aliphatic carbon atoms) around a heteroatom, which was specified as nitrogen for GLIDA. This chemistry implies that at physiological pH the nitrogen heteroatom is likely to be protonated and charged, in line with the notion that many GPCR ligands interact with biogenic amine receptors. Strader *et al.*,[44] in one of the first mutagenesis studies on GPCRs, identified a negatively charged aspartic acid residue in transmembrane domain 3 of the β-adrenoceptor to form a salt bridge with the ligands' protonated amino group. A similar, though one atom smaller, substructure is found in the 'normal' representation. Consequently, this substructure is also at the top of the list of most significant substructures of the normal representation (hGPCR-lig: Table 2), and is found in 74% of GPCR ligands in hGPCR-lig compared to only 26% of background compounds. Figure 2 shows some examples of GPCR ligands with this substructure overlaid, in sometimes unanticipated ways. These types of overlay also illustrate the completeness of coverage compared to the chemical fragment approach discussed in the Introduction. The other significant substructures in Table 2 are essentially variations of the first; the only differences are in number and length of carbon chains/atoms attached to the nitrogen atom. By scanning through the lists (e.g. Table 1 & 2, and Table 1 & 2 in Supporting Information), a recurring theme becomes apparent. The top most significant substructures are alkyl chains, some in combination with nitrogen, aromatic bonds or combinations of these. Note that these lists represent substructures that occur often in GPCR ligands and not in the background molecules thus being the distinguishing features that set these ligands apart from other organic compounds. In the 'normal' representation, a recurring theme is the alternating single/double bond feature, most likely being the substitute for aromatic bonds. Furthermore, the top significant substructures in this representation are alkylamines,

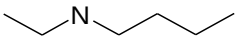
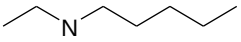
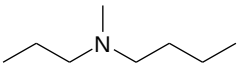
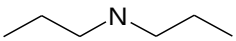
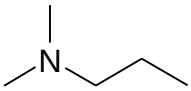
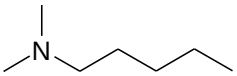
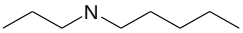
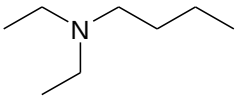
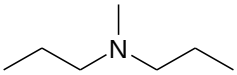
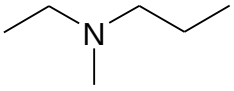
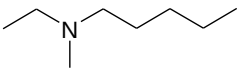
chains of single-bonded carbon atoms, or combinations of both. The amine-containing substructures differ in number and length of bonded alkyl substituents; similarly, the length of the carbon tail differs, as well as the position of the nitrogen within the tail. The substructure profiles for hGPCR-lig and GLIDA ligands were nearly identical, which implies that the method is stable for either dataset. For instance, there was only one difference in the top 20 most significant substructures, and the ordering was virtually the same (compare Table 2 with Table 1 in Supporting Information). This means that the most significant substructures cover the same area of chemical space in both the clinically promising candidates (hGPCR-lig) and compounds originating from scientific and patent literature (GLIDA). The top most significant substructures are dominated by a few substructural themes that are common to the group as a whole. The hypothetical 'parent' fragment from which all frequent substructures derive would be an amine connected to an aromatic system through a carbon chain. This complies with the most common substructure, as defined by Sheridan *et al*.,[18] which was found in 21% of the ligands in the GPCR set. Again, the abundance of this substructure is probably due to the high number of aminergic receptor ligands present in the database (see also below). Even though the top significant substructures provide chemical insights found in the largest number of compounds, they also might reflect an obvious bias. A very simple one might be the commercial availability of reagents. Also, the hGPCR-lig database is largely filled with drug candidates that have reached the market or later-stage clinical trials. Due to the high attrition rates in drug discovery and development[45] these advanced compounds must have additional features for drug-likeness that made them not fail beforehand, reducing the 'randomness' of substructure occurrence.

**Table 2.** List of the 20 most frequent substructures found in GPCR ligands (hGPCR-lig) compared against the DIVERSet background compounds ('normal' representation). Substructures were sorted according to significance, with the most significant substructure at the top. Thus substructure 1 (in bold) is found in 12,162 GPCR ligands (74%) compared to 4,081 background compounds (26%). This finding is highly significant as judged from the corresponding p-value (7.35e-1707).

| Nr | Substructure | Occurrence | | P-Value |
|---|---|---|---|---|
| | | GPCR ligands | Background compounds | |
| **1** |  | **12,162 (74%)** | **4,081 (26%)** | **7.35e-1707** |
| **2** |  | **8,397 (51%)** | **1,273 (8%)** | **4.70e-1696** |
| **3** |  | **12,028 (73%)** | **4,128 (26%)** | **2.72e-1626** |
| **4** |  | **11,432 (69%)** | **3,741 (23%)** | **2.61e-1551** |
| **5** |  | **9,298 (56%)** | **2,096 (13%)** | **2.62e-1535** |
| **6** |  | **14,900 (90%)** | **7,844 (49%)** | **6.02e-1522** |
| **7** |  | **7,847 (48%)** | **1,225 (8%)** | **1.39e-1520** |
| **8** |  | **8,669 (53%)** | **1,900 (12%)** | **1.30e-1411** |
| **9** |  | **7,443 (45%)** | **1,178 (7%)** | **3.46e-1405** |

*(continued, see next page→)*

***Table 2 continued.***

| Nr | Substructure | Occurrence | | P-Value |
|---|---|---|---|---|
| | | GPCR ligands | Background compounds | |
| 10 |  | 10,355 (63%) | 3,152 (20%) | 1.00e-1400 |
| 11 |  | 7,724 (47%) | 1,539 (10%) | 2.31e-1282 |
| 12 |  | 5,707 (35%) | 594 (4%) | 4.60e-1218 |
| 13 |  | 8,210 (50%) | 2,039 (13%) | 1.12e-1179 |
| 14 |  | 10,433 (63%) | 3,761 (24%) | 1.75e-1166 |
| 15 |  | 5,403 (33%) | 534 (3%) | 5.96e-1163 |
| 16 |  | 5,579 (34%) | 666 (4%) | 7.51e-1120 |
| 17 |  | 5,532 (34%) | 712 (4%) | 3.68e-1071 |
| 18 |  | 6,386 (39%) | 1,147 (7%) | 4.91e-1067 |
| 19 |  | 9,829 (60%) | 3,559 (22%) | 6.48e-1045 |
| 20 |  | 4,901 (30%) | 516 (3%) | 4.27e-1011 |

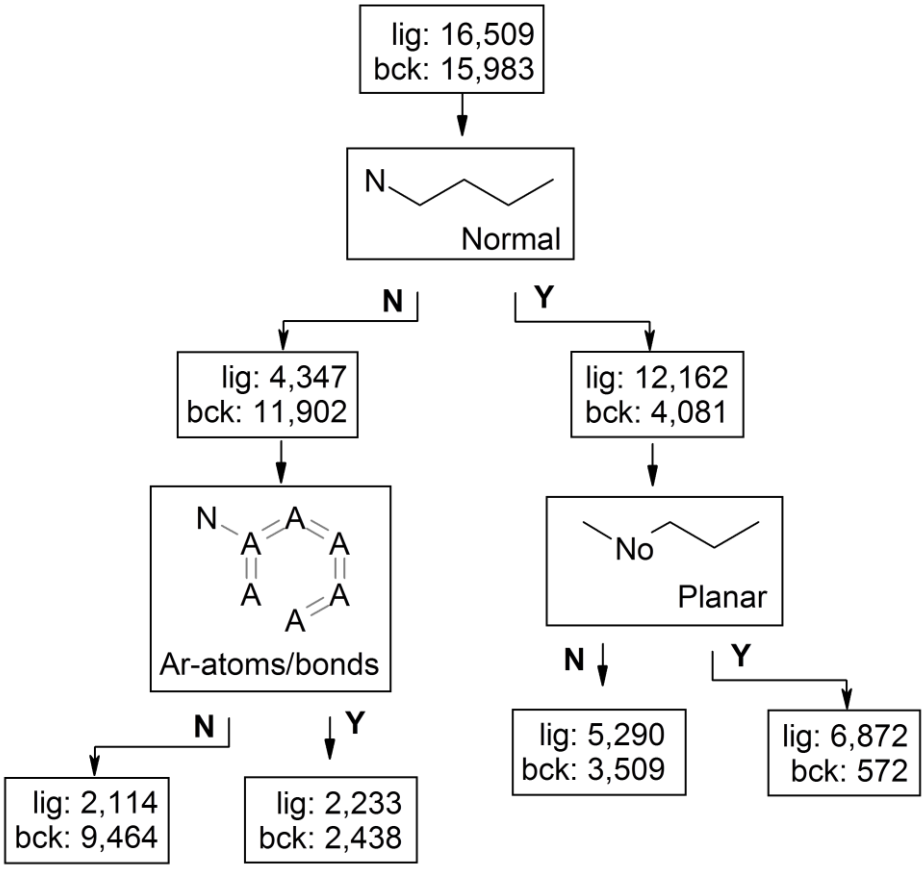



**Figure 3.** Hierarchical partitioning of GPCR ligands (hGPCR-lig – lig) versus background compounds (DIVERSet - bck). Ligands having the first substructure are taken out of the two sets (left path, N for 'No') or rather kept (right path, Y for 'Yes'), and the process of substructure mining is repeated. Numbers given are the absolute numbers of molecules used. Most discriminative substructures in any elaborate representation are shown. The name of the representation that yielded this substructure is given at the bottom of the box surrounding the substructures.

### 3.4.2 Hierarchical partitioning to distinguish GPCR ligands from other ligands.

For the first hierarchical analysis (the hierarchical partinioning), we used the hGPCR-lig and DIVERSet compounds. To find a new set of significant features, the experiment was repeated on subsets of the original sets: a subset in which all structures contained the substructure and another set in which they did not. The most significant substructure from the best performing representation was used to split the sets and substructure mining was then repeated for the two subsets. The results are represented hierarchically as a tree (Figure 3). At the top of this hierarchy, we find the butyl amine substructure ('normal' representation) which occurs in 76% of GPCR ligands compared to 24% of background compounds. More than half of the molecules containing this aminergic tail also contain a substructure consisting of a heteroatom substituted with a methyl group and a propyl group. This substructure may overlay the butyl amine group or may be located elsewhere in the molecule. Molecules without the butyl amine substructure have a six atom aromatic system, of which one is nitrogen, as most important structural feature. This aromatic chain is not closed to form a six-membered ring, meaning that this substructure is found not only in six-membered rings but also in aromatic systems containing fused five-membered rings etc. Since the 'normal' representation does not discriminate between aliphatic and aromatic bonds, it may lead to substructure contributions that are part of an aromatic ring system (Figure 2, e.g., compound IV). We therefore tested whether the use of a special aromatic bond type was the more suitable representation. Substructure occurrences for the aromatic bonds representation are listed in Table 3. When comparing the substructures of this representation with those of the 'normal' representation, a large overlap of the common motif was observed. For both representations, the most important moiety is the nitrogen substituted with one or more alkane chains. From this data, a second hierarchy (Figure 4) was constructed that did not consider the 'normal' representation. This approach resulted in a different substructure set than with the 'normal' representation. Compared to the amine from

the 'normal' representation, the best-discriminating substructure now had a shorter tail attached to the nitrogen. This carbon tail is probably shorter due to the absence of contributing aromatic bonds that were represented as single bonds in the 'normal' representation. The first substructure with an aromatic bond was found at position 24 in the substructure list in Table 3, whereas in the 'aromatic atoms and bonds' representation the first aromatic substructure was found much higher in the substructure list, i.e. at the third position. Iterating over the substructures with the 'aromatic atoms and bonds' representation leaves the impression that an aromatic ring is forming (Supporting Information: substructures 3 to 5 and 10 to 15 in Table 2). However, the fully closed ring is found much further down the list. The reason for this is that aromatic rings that differ in size contribute the same open substructures to the appearance lists. Since closed five-membered rings and closed six-membered rings cannot be overlaid, the support for these substructures is much lower. Note that even though the 'normal' representation may seem naïve, it still yielded the most significant result for GPCR ligands. Using this representation, a significant enrichment of the source sets can be accomplished, provided that bonds may be part of an aromatic system (Figure 1). Complementary to the analysis of typical substructures for the GPCR ligands was our analysis of substructures occurring more frequently in the DIVERSet background library. Thus, repeating the experiment for the background compounds yielded structural features that have low abundance in GPCR ligands. Table 4 gives an overview of the most-significant substructures for each representation. Similar as for the GPCR ligand analysis, a hierarchy was constructed for the background compounds (Figure 5). The tree is the same for analysis with and without the results of the 'normal' representation, since this representation did not produce any most significant substructures. The substructures that occurred often in the background set and not in the ligands are those that should be avoided when searching for GPCR ligands (since they are not commonly found in the ligands). A carboxamide substructure was the most significant substructure found for the background (DIVERSet[37]) set. For this set, the aromatic bonds representation yielded the most significant finding (Table 4). Almost two-third of the compounds (depending on definition) in this set have a

carboxamide or ester at the core of their scaffold, either linking two ring systems or linking a ring system with an aliphatic group. The high number of carboxamide and ester groups at the core of the molecules may reflect the simple organic reactions between alcohols and acids that have been used to construct the library. GPCR ligands differ not only in lower number of occurrence but also in position of these motifs. Where carboxamide and ester groups mainly form the linking groups between fragments in DIVERSet compounds, these motifs are also found as side-groups in GPCR ligands. This possibly reflects efforts to make drugs that are more soluble (for increased bioavailability) or to create prodrugs. The substructures in the GPCR hierarchy contained zero (Figure 4) or one heteroatom (Figure 3 and Figure 4), whereas those for the background set contained one, two, or three heteroatoms (Figure 5). This might reflect the lower number of hydrogen bond donors/acceptors in GPCR actives compared to inactives which was already noticed by Balakin *et al*.[31] Normally, as Feher and Schmidt pointed out,[46] the number of (small) heteroatoms is roughly equal in drugs as well as compounds in combinatorial libraries.

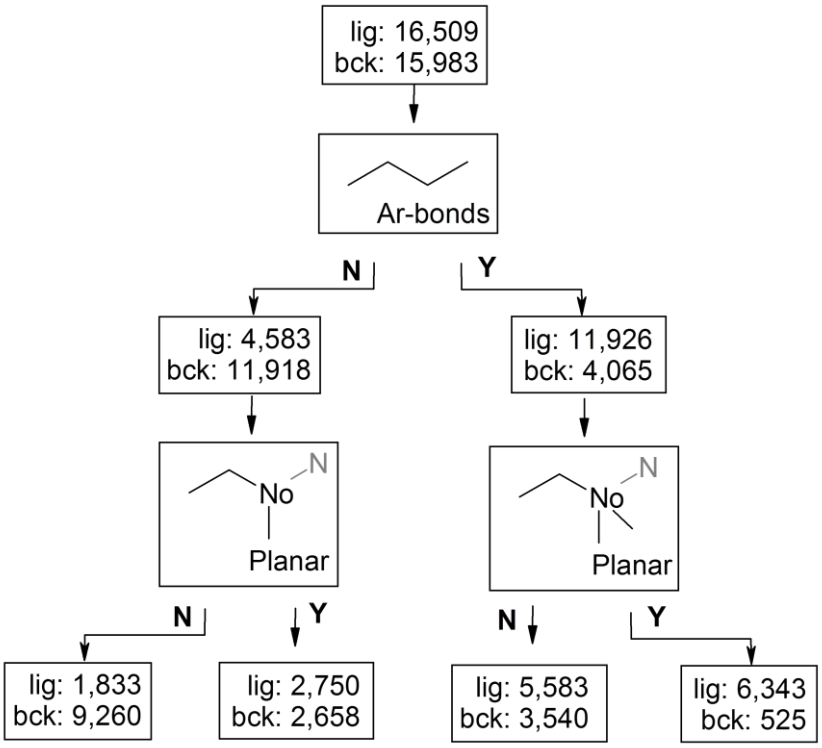



**Figure 4.** Hierarchical representation of the splits for GPCR ligands (hGPCR-lig) compared to the DIVERSet background collection, constructed without using the normal representation as in Figure 3. See legend to Figure 3 for further explanation. As opposed to Figure 3, we now see a butyl chain without the amine group at the top of the hierarchy. Substructures containing the amine group are found one level down in the hierarchy.

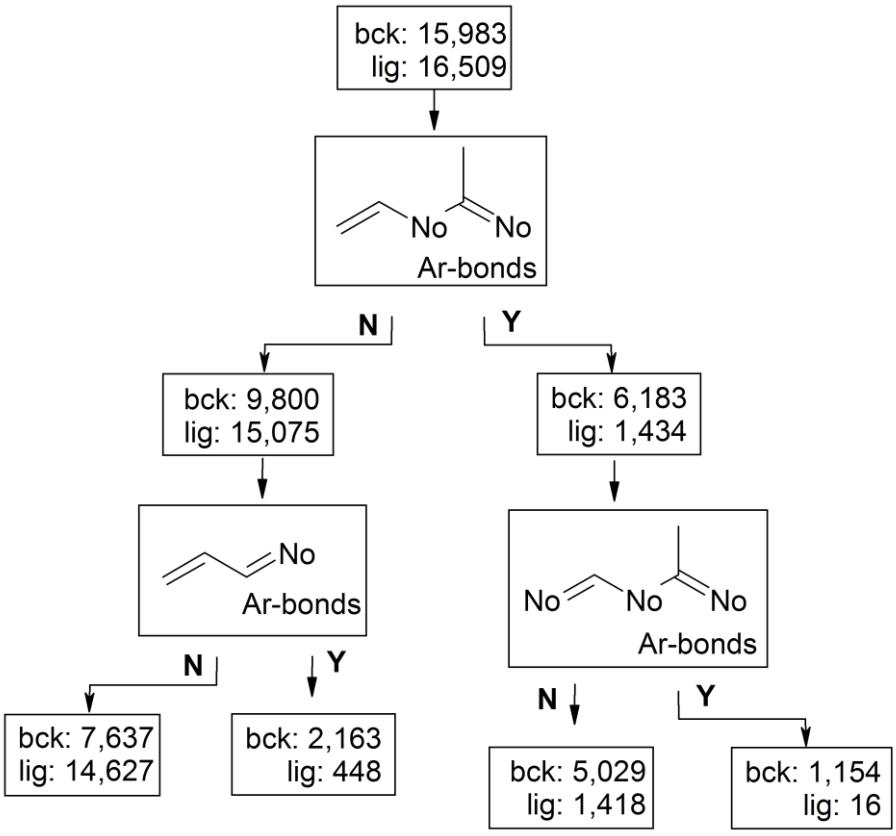
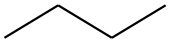
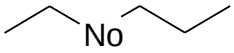
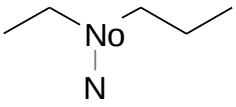
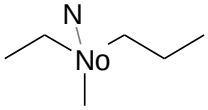
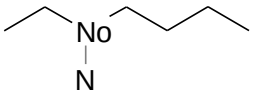
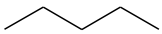
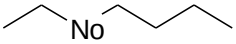
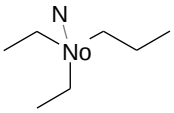
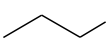



**Figure 5.** Hierarchical splits for the DIVERSet background compounds compared to GPCR ligands (hGPCR-lig). See legend to Figure 3 for further explanation. Here, it is particularly remarkable that the substructures all contain a double bonded heteroatom. The substructures at the top and right path match the carboxyl and ester groups, which are abundant in the DIVERSet.

**Table 3.** Frequent substructures for the GPCR ligands (hGPCR-lig) found with the 'aromatic bonds' representation. The first occurrence of an aromatic bond is found in the 24th substructure. See for further explanation the legends of Table 1 and Table 2.

| Nr | Substructure | Occurrence | | P-Value |
|---|---|---|---|---|
| | | GPCR ligands | Background compounds | |
| 1 |  | 11,926 (75%) | 4,065 (25%) | 7.25e-1611 |
| 2 |   | 10,344 (65%) | 2,828 (17%) | 2.11e-1552 |
| 3 |   | 9,474 (59%) | 2,257 (14%) | 4.59e-1514 |
| 4 | | 14,928 (93%) | 8,258 (50%) | 6.72e-1383 |
| 5 |   | 7,290 (46%) | 1,241 (8%) | 5.53e-1311 |
| 6 |   | 7,040 (44%) | 1,168 (7%) | 8.48e-1271 |
| 7 |  | 9,056 (57%) | 2,444 (15%) | 8.58e-1268 |
| 8 |   | 7,587 (47%) | 1,574 (10%) | 4.22e-1216 |
| 9 |   | 5,498 (34%) | 525 (3%) | 1.45e-1202 |
| 10 |   | 12,898 (81%) | 6,091 (37%) | 3.78e-1202 |

*(continued, see next page→)*

***Table 3 continued.***

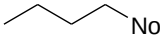
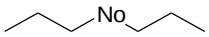
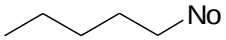
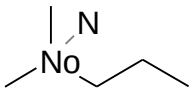
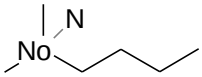
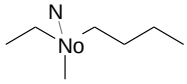
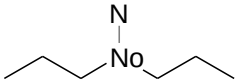
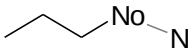
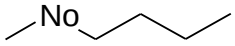
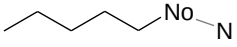
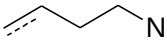
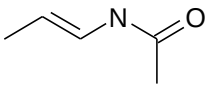
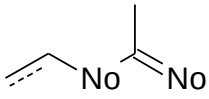
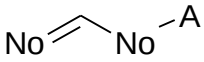
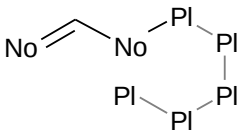
| Nr | Substructure | Occurrence | | P-Value |
|---|---|---|---|---|
| | | GPCR ligands | Background compounds | |
| 11 |  | 9,962 (62%) | 3,286 (20%) | 3.25e-1200 |
| 12 |  | 5,626 (35%) | 606 (4%) | 4.33e-1181 |
| 13 |  | 7,222 (45%) | 1,467 (9%) | 1.76e-1154 |
| 14 |  | 7,863 (49%) | 1,868 (11%) | 2.25e-1151 |
| 15 |  | 5,774 (36%) | 736 (4%) | 1.11e-1133 |
| 16 |  | 5,441 (34%) | 590 (4%) | 2.31e-1131 |
| 17 |  | 5,159 (32%) | 514 (3%) | 2.26e-1097 |
| 18 |  | 11,202 (70%) | 4,916 (30%) | 4.81e-997 |
| 19 |  | 8,939 (56%) | 2,999 (18%) | 2.10e-985 |
| 20 |  | 5,998 (38%) | 1,110 (7%) | 1.64e-966 |
| … | … | … | … | … |
| 24 |  | 10,819 (66%) | 4,728 (30%) | 2.04e-937 |

**Table 4.** Best discriminating substructure per elaborate representation for the DIVERSet background compounds compared to the hGPCR-lig collection. Note that the substructures do not have a geometric arrangement; the layout of double bonds and aromatic bonds is arbitrary.

| Representation | Substructure | Background compounds | GPCR ligands | P-Value |
|---|---|---|---|---|
| **Normal** |   | **6,247 (39%)** | **1,511 (9%)** | **9.03e-920** |
| **Ar-bonds** |   | **6,183 (39%)** | **1,434 (9%)** | **2.65e-938** |
| **Ar-Atoms & Bonds** |   | **6,998 (44%)** | **2,140 (13%)** | **1.92e-863** |
| **Planar** |   | **6,218 (39%)** | **2,101 (13%)** | **4.39e-658** |

### 3.4.3 GPCR Subfamilies.

We continued our analysis by focusing on individual classes of GPCR ligands. For the subfamily analysis, we continued with GLIDA and DIVERSet. For this second analysis, we derived smaller, sampled sets next to the two original full sets. The sampled sets, which were more convenient to work with, had the same substructure profiles as the full sets. For instance, the substructure lists of the full (Table 1 in Supporting Information) and sampled set (Table 3 in Supporting Information) of GLIDA are nearly identical. Substructures have the same order, e.g. only two shifts in positions occur in the 20 topmost structures for the 'normal' representation. Ligands were grouped

hierarchically, based on the classification of the target GPCRs. The hierarchical grouping and levels where substructure analysis was performed are schematically presented in Figure 6. Similar to the analysis of GPCR ligands against a background set we now compared the ligands of a subgroup against all other ligands in the entire group. A group within a group is called a *subgroup*; the group that contains the subgroup is denoted as the *supergroup*. Substructures that have a significant preference for either the subgroup or the supergroup are denoted as either *specific* or *avoiding*, respectively. Substructures that are specific for the supergroup are denoted as *generic*. Specific substructures are those that set ligands from one subgroup apart from ligands of the neighboring subgroups. Avoiding substructures are those that seem to avoid ligands of the subgroup but do occur in neighboring subgroups. Generic substructures are those that are common to a subgroup and the neighboring subgroups. Substructure lists not provided in this article are available as Supporting Information.

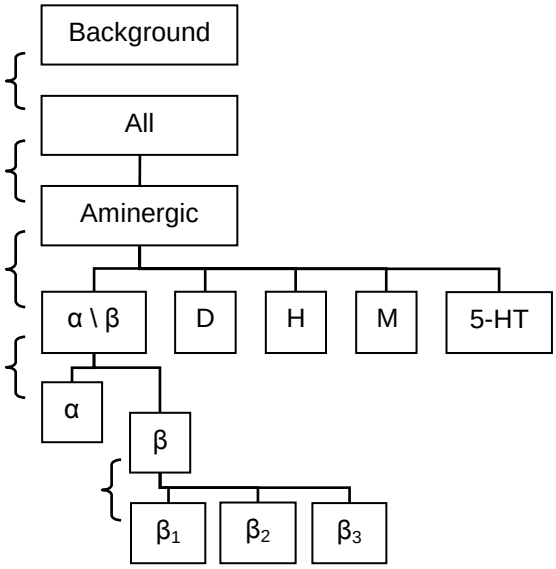



**Figure 6.** Schematic drawing of the subfamily hierarchy indicating the levels at which substructure analysis was performed (denoted by braces on the left side of the hierarchy). Boxes represent the sets, and use the following labels: 'Background' – The ChemBridge DIVERSet, 'All' - total set of GPCR ligands found in GLIDA, 'Aminergic' – all aminergic receptor ligands, 'α \ β' – Adrenoceptors, 'D' – Dopamine receptors, 'H' – Histamine, 'M' – Muscarinic Acetylcholine receptors, '5-HT' – Serotonin receptors, 'α' – α-adrenoceptors, 'β' – β-adrenoceptors, '$\beta_{1\text{-}3}$' – β-adrenoceptor subtypes 1 to 3.

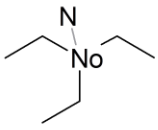
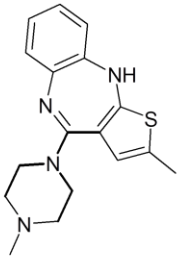
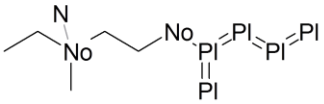
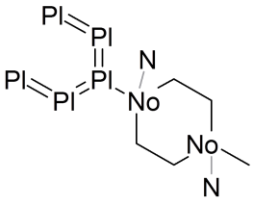
| Motif - Description | Example Substructures | Example Molecule |
|---|---|---|
| **I** - Nitrogen substituted with one or more alkanes, that is, methyl, ethyl, and/or propyl groups. |  17. 2,349 (46%) / 1,575 (31%) |  |
| **II** - An aromatic chain, 5 or 6 atoms long, connected to a heteroatom that is linked to another heteroatom trough one or two ethyl groups (e.g. piperazine). The second heteroatom may be substituted with methyl or ethyl groups. |  37. 1,107 (22%) / 574 (11%) | |
| |  46. 862 (17%) / 397 (7%) | |

**Figure 7.** Common motif and example substructures for most significant substructures of aminergic ligands compared against all other GPCR ligands (GLIDA, 5k sampled), in planar ring systems representation. The 'Motif' number (Roman number, in bold) indicates number of the found motif or structural theme. The motif number is followed by a short description and one or more example substructures are provided. Below each example substructure, the position, occurrence in the active set (absolute and percentage) and occurrence in the control set (absolute and percentage) are listed. See the Materials & Methods section for further explanation about the representation of the substructures. For some motifs, an example molecule from the same class is provided, with the example substructure overlaid in bold. Here, an example drug containing motif I is olanzapine, which is used to treat schizophrenia, acting on dopamine $D_1$, $D_2$, and serotonin 5-$HT_2$ receptors (taken from Klabunde *et al.*[51]).

First, the differences between aminergic receptor ligands and all other GPCR ligands in GLIDA were analyzed (Figure 7). The 'planar ring' representation yielded the most

significant substructures, even though substructures with parts of a planar ring were found at lower positions in the list. The best-discriminating substructures were methyl- and ethyl-substituted amines; the amine group of the endogenous ligands (e.g., dopamine, (nor)epinephrine, acetylcholine, etc.) is the common motif that accounts for naming of this group as biogenic amine receptors. The high occurrence of these substructures reflects efforts to mimic endogenous ligands by making analogs of these ligands (e.g., isoproterenol based on epinephrine). The opposite analysis was also conducted, yielding the structural features common to GPCR ligands excluding the aminergic ligands. The first, most significant, structural feature was a carbon atom connected to both a single-bonded heteroatom and to a double-bonded heteroatom. In the following positions, this heteroatom was specified as being a nitrogen atom, the second one as an oxygen atom. This reflects the carboxamide motif, found in peptide ligands (MW < 1000), which are part of other, non-aminergic, classes in GLIDA. The second important substructure consisted of two aromatic systems connected by a methylene group or by a single bond.

We continued by analyzing the five major aminergic targets individually against the other four. These five are the adrenoceptors (both alpha- and beta-), the dopamine receptors, the histamine receptors, the muscarinic acetylcholine receptors, and the serotonin receptors. Octopamine and trace amine receptors were not included due to scarce ligand information. For each analysis, the size of the aminergic control group was different due to the removal of duplicate entries, i.e. compounds that bind to more than one class. Although substructures found for the control group may be common to multiple GPCR targets, these are different from privileged substructures. Privileged substructures are discrete fragments, often scaffolds, found in one or more ligands for more than one target in the family.[47] Our analysis considers all possible substructures, and yields only the most frequent substructures among the targets.

### 3.4.4 Adrenoceptor ligands.

An important feature of the adrenergic receptor ligands vs. all other aminergic ligands is a substructure consisting of two heteroatoms connected by an ethyl group (Figure 8). The first heteroatom of this substructure is an oxygen atom specified as hydroxy-group, the second is a nitrogen atom with a single hydrogen atom attached, meaning that this nitrogen is secondary. This chemical signature is representative for the motifs found in both β-adrenoceptor agonists and antagonists. An example containing this substructure is metoprolol, a $\beta_1$ antagonist (beta-blocker) used to treat hypertension. The second example substructure for motif I in Figure 8 has no atom specifiers for the heteroatoms, which means that this substructure also overlaps with the 1,2 diaminoethane substructure. A search for adrenoceptor (ant)agonists that have this substructure and not the hydroxyethylamine returned 58 hits, most of them specified as α-adrenoceptor ligands in the database (second example structure of motif I). Note that both aforementioned substructures in the query had heteroatoms with one explicit hydrogen atom. At lower positions, the hydroxyethylamine motif reappears bonded to an aromatic system at the carbon atom that has the hydroxyl-group attached. This is an exclusive element in β-adrenoceptor agonists. The substructures are essentially all part of the example substructure given for motif II which is found in 27% of the aminergic ligands. An example drug that has this motif is terbutaline, a $\beta_2$-adrenoceptor agonist used in the treatment of asthma. Substructures found less frequently in adrenergic ligands compared to aminergic ligands consisted of a nitrogen atom substituted at two or three positions, some as part of a largely saturated five- or six-membered ring, as found in e.g., apomorphine, a dopamine receptor ligand.

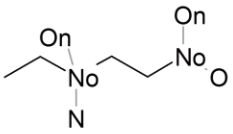
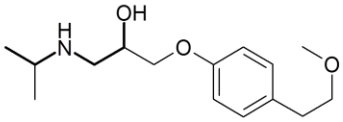
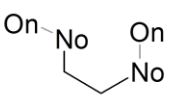
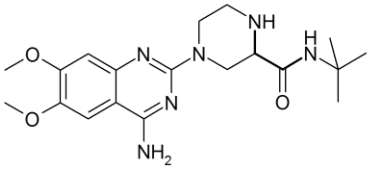
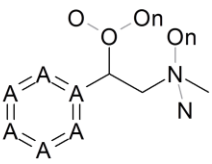
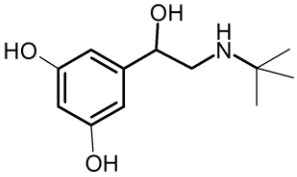
| Motif - Description | Example Substructures | Example Molecules |
|---|---|---|
| **I** - Two heteroatoms connected by an ethyl linker. Both heteroatoms have one hydrogen attached. |  <br>1. 1,003 (44%) / 57 (0%) |  |
| |  <br>5. 1081 (48%) / 148 (1%) |  |
| **II** - Chain of six aromatic atoms attached to the hydroxy ethyl amine substructure (motif I) |  <br>29. 611 (27%) / 16 (0%) |  |

**Figure 8.** Common motif and example substructures for most significant substructures of the adrenoceptors ligands, in aromatic atoms and bonds representation. See legend of Figure 7 for further explanation. First example structure (for motif I) is metoprolol, a $\beta_1$-adrenoceptor antagonist (beta-blocker) used to treat hypertension (taken from Klabunde *et al.*[51]). The second example structure for motif I is 4-(4-amino-6,7-dimethoxyquinazolin-2-yl)-N-tert-butylpiperazine-2-carboxamide, an α-adrenoceptor ligand and prazosin derivative found in GLIDA.[33] The example structure for motif II is terbutaline, a $\beta_2$-adrenoceptor agonist used in the treatment of asthma.

### 3.4.5 Alpha- and beta-adrenoceptor ligands.

We further examined the adrenergic receptor ligands, where we distinguished between α- and β-adrenoceptors. The most significant features specific for the α-adrenoceptor ligands (Figure 9) consist of a nitrogen atom substituted at three positions with methyl and ethyl groups (73% of ligands). One ethyl group can be connected to an aromatic system (33%), or to a heteroatom that is connected to an aromatic system (29%). An example drug containing this substructure is phenoxybenzamine, an $\alpha_1$-adrenoceptor antagonist used in the treatment of hypertension. The most significant substructures specific for β-adrenoceptor ligands (Figure 10) were all based on the 1-(ethylamino)propan-2-ol moiety (86% of ligands). An example drug containing this substructure is propranolol, a non-selective beta-blocker, used in the treatment of hypertension. The most significant substructures specific for the $\beta_1$-adrenoceptor were all parts of a methylaminopropane substructure (81% of ligands). Here it should be noted that commercially available $\beta_2$-adrenoceptor ligands are *agonists* having a structure such as terbutaline (Figure 8, first example motif II), whereas $\beta_1$-adrenoceptor ligands are mostly antagonists such as metoprolol (Figure 8, first example motif I). The most significant *avoiding* substructure for $\beta_1$-adrenoceptor ligands (50% of ligands), which at the same time occurs in $\beta_2$- and $\beta_3$-adrenoceptor ligands, consisted of an aromatic chain linked by an ethyl group to nitrogen that was linked by an ethyl group to an oxygen.

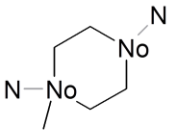
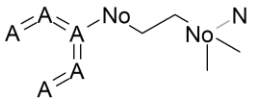
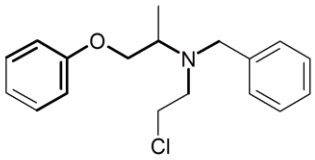
| Motif - Description | Example Substructures | Example Molecule |
|---|---|---|
| **I** - Nitrogen substituted with methyl ethyl groups. Ethyl can be connected to an aromatic chain of five atoms. This connection is either directly or indirectly through a heteroatom. |  11. 317 (27%) / 8 (1%)<br>  13. 331 (32%) / 15 (1%) |  |

**Figure 9**. Common motif and example substructures for most significant substructures of the α-adrenoceptors ligands versus β-adrenoceptor ligands, in aromatic atoms and bonds representation. An example is phenoxybenzamine, a $\alpha_1$-receptor antagonist used to treat hypertension. See legend of Figure 7 for further explanation.

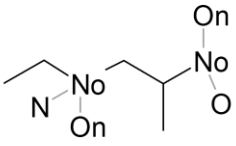
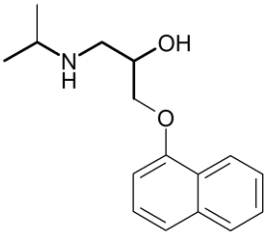
| Motif - Description | Example Substructure | Example Molecule |
|---|---|---|
| **I** - Hydroxygroup linked with substituted amine group. Mainly 1-(ethylamino)propan-2-ol substructure. |  1. 996 (86%) / 27 (2%) |  |

**Figure 10.** Common motif and example substructures for most significant substructures of the β-adrenoceptor ligands versus α-adrenoceptors ligands, in aromatic bonds representation. An example drug containing this substructure is propranolol, a non-selective β-adrenoceptor antagonist (beta-blocker). See legend of Figure 7 for further explanation.

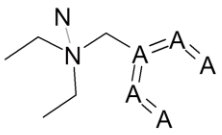
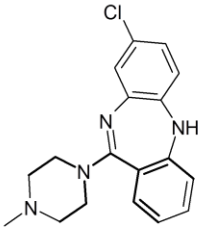
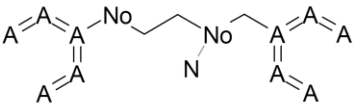
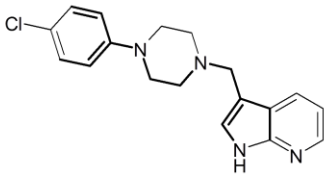
| Motif - Description | Example Substructures | Example Molecules |
|---|---|---|
| **I** - Chain of 5 aromatic atoms linked through one carbon to a nitrogen that is substituted with two methyl/ethyl groups. |  1. 572 (30%) / 718 (8%) |  |
| **II** - Two chains of five/six aromatic atoms, linked by a linker of a heteroatom connected to N-methylethyleneamine. |  1. 226 (12%) / 68 (0%) |  |

**Figure 11**. Common motif and example substructures for most significant substructures of the dopamine receptor ligands, in aromatic atoms and bonds representation. An example drug that has motif I is clozapine, an antipsychotic agent used in the treatment of schizophrenia. [52] Another example for motif I and also for motif II is compound L-745,870, a selective dopamine $D_4$ receptor antagonist.[53] See legend of Figure 7 for further explanation.

### 3.4.6 Dopamine receptor ligands.

For the dopamine receptor ligands, two types of specific substructures were identified (Figure 11). The first substructure (in 30% of the ligands) consists of a chain of 4 to 5 aromatic atoms, connected to a nitrogen atom through a single carbon atom. This nitrogen is tertiary, as it is substituted with either two ethyl groups, or one methyl and one ethyl group. The second substructure (12% of the ligands) consists of two aromatic chains of five or six atoms long that are linked through a heteroatom connected to N-methylethyleneamine, e.g. an N-methylethylenediamine linker. The terminal nitrogen of this linker may be substituted with an ethyl group. In both example molecules in Figure 11, the substructures overlap with the piperazine ring. The fact that the most

significant substructures do not 'use' the entire piperazine moiety suggests that variations on the piperazine theme are possible when designing dopaminergic drugs. Similarly, the aromatic chains in motifs I and II overlap with several types of aromatic systems, e.g. five-membered or six-membered rings, containing either carbon or hetero-atoms. This implies that aromaticity is the important feature and not so much the type of ring system that is used. Again, this finding offers further options for drug design.

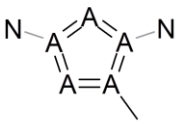
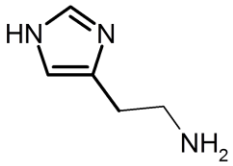
| Motif - Description | Example Substructure | Example Molecule |
|---|---|---|
| **I** - Chain of five aromatic atoms, one or two being nitrogen separated by one atom, connected to an alkyl group that is one to four carbons long. In case of a propyl, it is connected to a heteroatom. |  1. 257 (42%) / 562 (6%) |  |

**Figure 12.** Common motif and example substructures for most significant substructures of the histamine receptor ligands, in aromatic atoms and bonds representation. Histamine is provided as example molecule containing this motif. See legend of Figure 7 for further explanation.

### 3.4.7 Histamine receptor ligands.

The most common motif (almost 50%) specific for histamine receptors is a chain of five aromatic atoms (Figure 12), where one or two aromatic atoms are specified as nitrogen atoms. These nitrogen atoms are separated by one aromatic atom; in some cases, one of the other neighboring aromatic atoms has an ethyl group attached. The majority of the significant substructures are chains, and actual ring closures, forming e.g. the imidazole ring as in histamine, are scarce. This seems counterintuitive at first sight, since the five-membered aromatic heterocycles are among the most obvious features when visually inspecting the set. Although a common theme, the heterocycles

in histamine receptor ligands all differ in size, ring-fusions, and heteroatoms. By considering substructures instead of complete ring fragments, it was thus possible to find structural similarities that have a much higher support among the ligands. This causes the high occurrence of the 'aromatic chains', since it is the most common feature among the diverse heterocycles.

### 3.4.8 Muscarinic Acetylcholine receptor ligands.

Substructures of derivatives of the quinuclidine ring are the most common substructures for the muscarinic acetylcholine receptor ligands (Figure 13). It occurs in 19% of ligands for this class compared to 0% in other aminergic ligands. A second heteroatom may be attached, separated two carbon atoms from the nitrogen. A typical example is civemeline, a muscarinic $M_3$ receptor agonist (Figure 13, first example). In this case, the second heteroatom would be the oxygen atom.

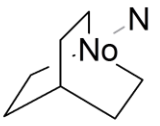
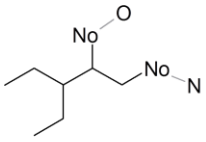
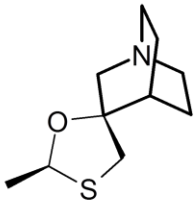
| Motif - Description | Example Substructures | Example Molecule |
|---|---|---|
| **I** - Derivatives of quinuclidine ring, either complete or substructural pieces. Some substructures have two heteroatoms separated by two carbon atoms. |  1. 260 (19%) / 17 (0%)<br>  14. 166 (12%) / 5 (0%) |  |

**Figure 13**. Common motif and example substructures for most significant substructures of the muscarinic acetylcholine receptor ligands, in aromatic bonds representation. An example molecule containing the quinuclidine ring is cevimeline, a muscarinic $M_3$ receptor agonist. See legend of Figure 7 for further explanation.

### 3.4.9 Serotonin receptor ligands.

For the serotonin receptor ligands (Figure 14), the best specific substructure resembles the shape of the 2-ethyl-indole moiety (31% of ligands) that forms the core of serotonin, although a label specifying the nitrogen atom is missing. This is because this substructure covers the largest set of serotonin ligands (without becoming too general). In some cases, the ethyl group is attached to C3 rather than N1 of the core, which means that either the ethyl group or the atom specifier is not part of the substructure. The nitrogen atom can also be replaced by other heteroatoms, or be absent in scaffolds that consist only of carbons, forming a planar ring system. This ring system consists of one aromatic ring instead of two. In fact, examples of all three cases were found among the ligands. At lower positions in the lists (position 22 in Table 16, Supporting Information) the same substructure (25% of ligands) is found with the nitrogen atom specifier. Examples containing this substructure are the endogenous ligand serotonin and the triptan anti-migraine drugs such as Sumatriptan (Figure 14).

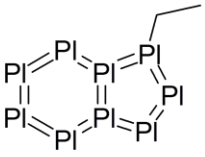
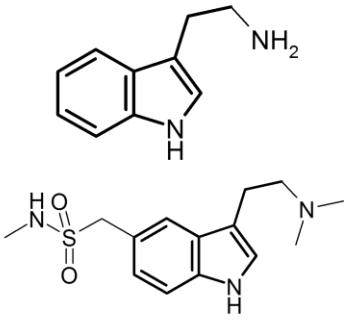
| Motif - Description | Example Substructure | Example Molecules |
|---|---|---|
| **I** - Substructures of a planar six ring fused with a five ring that has an ethyl group attached. |  1. 1,281 (31%) / 380 (6%) |  |

**Figure 14.** Common motif and example substructures for most significant substructures of the serotonin ligands, in planar ring systems representation. An example substructure is the endogenous ligand serotonin (above), or the anti-migraine drug sumatriptan (below). See legend of Figure 7 for further explanation.

### 3.4.10 Aminergic receptor ligands.

When comparing the aminergic subgroups against all other aminergic ligands, the most significant features of the aminergic supergroup always have a low occurrence. This low occurrence is probably because these features are actually the features of one of the other subgroups of this class. For instance, the *avoiding* substructures for the serotonin receptor ligands are, among others, motif I from the adrenoceptor ligands (Figure 8). Therefore, these features can be considered substructures to avoid since they indicate possible side effects on other aminergic receptors. This is a more generalized way of a so-called anti-target analysis, to avoid GPCR-mediated side effects (Klabunde *et al*. 2005)[48]. For dopamine, histamine, serotonin receptor ligands, the most significant substructures of the other aminergic ligands (the *avoiding* substructures) are dominated by a motif of two heteroatoms connected by an ethyl linker. Since this resembles the substructures found for the (β) adrenoceptors (Figure 8), in both frequency and shape, this class probably dominates the avoiding substructure lists of the other four classes.

### 3.4.11 General observations.

In the following, we will discuss the representations and substructure selection criteria employed and their likely influence on the results obtained. Firstly, the extraction of substructures discards any geometric information such as bond orientation. This loss of information may be appreciated, however, as it is beneficial for extracting more 'abstract' features in molecules. For instance, opposite *cis-trans* isomers may contribute to the same double bond in a substructure. Similarly, a chain of aromatic bonds may be part of one or multiple fused ring systems. Chirality is also lost in our approach, an issue that holds for all substructure search methods. Inclusion of 3D-conformational aspects in substructure searching is an open area for further research. Secondly, the p-value was used to sort the substructures according to significance. However, this value is very small for the top findings, and the differences between substructures are small. Therefore, choosing the most significant substructure to split

the set is arbitrary; all substructures at the top of the occurrence lists would be a very good choice. Not only the significance of the finding is important but also what the finding predicts. It might therefore be better to focus less on the p-value and more on the ratio of retrieval of GPCR ligands and background compounds. In the end, a scientist might be more interested in the percentage of GPCR ligands that can be identified from a set of molecules. Moreover, our approach focuses on selective substructures only; substructures that occur frequently in both sets are discarded. Although not selective, these substructures are still common structural features for GPCR ligands. Therefore, for the design of GPCR screening libraries, we also analyzed the features common in GPCR ligands in general, which were selected based on a minimum number of atoms/bonds (to remove trivial fragments) and frequency. A list of frequent, non-selective substructures is provided in Table 22, Supporting Information. This table lists all frequent (≥60% of molecules) substructures of more than six atoms that are found in GLIDA (5k sampled, normal representation). Some of the substructures (such as no. 23) can be associated easily with ligands of the aminergic receptor class, while other substructures (such as no. 1) seem to describe a more generic pattern of GPCR ligands. While this table will not be analyzed further by us at the current stage, fragments of the above type should be beneficial to be included in the design of GPCR libraries of any of the subtypes discussed in this chapter. As a final point, almost all significant findings were found when we used elaborate chemical representations, mainly with 'aromatic atom and bond types'. This suggests that this representation might be best for the application domain (i.e. GPCR activity prediction). Apparently, elaborate chemical representations add substantial value when searching for structural features typical for active compounds. Suggestions for further research would therefore be to extend the types of representations used, for instance by encoding the electronic properties of a molecule (for example, see: Martin *et al*., 2008)[49].

## 3.5 Conclusions

In this study, we analyzed frequently occurring substructures in GPCR ligands by comparing these with various control groups of compounds. Our analysis is complementary to employing privileged structures in ligand design,[50] since it is not restricted to existing scaffold structures. It therefore offers further opportunities for introducing novelty in new chemical entities. We used different chemical representations for the molecules under consideration. As a result, we derived generalized substructural features for both ligands and control groups. The substructures found in the background set reflected the use of simple reactions that may have been employed to construct the library, for instance, the ester and carboxamide groups. In the GPCR ligand group, we found common as well as 'novel' substructures. First of all, our analysis identified well-known motifs (e.g. the side chain in β-adrenoceptor antagonists), which we considered a validation of our approach. In fact, the butylamine substructure (often linked to an aromatic moiety) occurred in 74% of the GPCR ligands compared to 26% of the background control group. Secondly, new structural patterns were also found, which may help medicinal chemists in their design efforts. As a typical example, we found fused 5:6 bicyclic ring systems in serotonergic ligands. These were identified in the so-called planar representation, indicating that aromaticity is not essential for both rings and that the precise location and nature of a heteroatom in the bicyclic core is not fixed. Indeed, the use of elaborate chemical representation gave the best, i.e. the most significant, description of the structural features that are important for a (sub)class of GPCR ligands.

## 3.6 Supporting Information Available

Lists of most significant substructures per compound set. This material is available free of charge via the Internet at http://pubs.acs.org.

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