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Adamantyl thioureas as soluble epoxide hydrolase inhibitors

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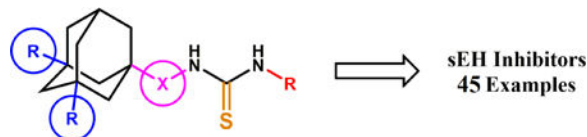
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Abstract

A series of inhibitors of the soluble epoxide hydrolase (sEH) containing one or two thiourea groups has been developed. Inhibition potency of the described compounds ranges from 50 μ M to 7.2 nM. 1,7-(Heptamethylene)bis[(adamant-1-yl)thiourea] (**6f**) was found to be the most potent sEH inhibitor, among the thioureas tested. The inhibitory activity of the thioureas against the human sEH is closer to the value of activity against rat sEH rather than murine sEH. While being less active, thioureas are up to 7-fold more soluble than ureas, which makes them more bioavailable and thus promising as sEH inhibitors.

Graphical Abstract



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Keywords

soluble epoxide hydrolase; inhibitor; adamantine; isothiocyanate; thiourea

Mammalian soluble epoxide hydrolase (sEH, E.C. 3.3.2.10) is involved in the metabolism of epoxy-fatty acids to the corresponding vicinal diols through the reaction with a water molecule.^{1,2} Endogenous substrates for the sEH include cytochrome P450 metabolites of arachidonic (epoxyeicosatrienoic acids, EETs) and docosahexaenoic (epoxydocosatrienoic acids, EDPEs) acids.^{3,4} EETs possess vasodilatory effects through the activation of the calcium dependent broad K⁺-channels in endothelial cells, which are beneficial in many renal and cardiovascular diseases.^{5,6} Furthermore, the EETs have some anti-inflammatory and analgesic properties.⁷ Their conversion to dihydroxyeicosatrienoic acids (DHETs) by sEH reduces those beneficial activities. The inhibition of sEH *in vivo* by highly selective inhibitors results in an increase of the concentration of the EETs and other epoxy fatty acids and is accompanied by a reduction in angiotensin driven blood pressure in rodents, but also reduction of inflammatory and painful states, thereby suggesting that sEH is a target for the treatment of hypertension, inflammatory diseases and pain.^{8–10}

Most of compounds reported as sEH inhibitors are 1,3-disubstituted ureas.^{11–15} To our knowledge, only 10 thioureas have been reported as sEH inhibitors^{16,17} compared to thousands of ureas. Thus, a systematic investigation of thioureas as sEH inhibitors is needed. Separately, ureas are difficult to formulate because of their high melting points and low water solubility. Herein, we investigate the influence of a thiourea function on the physical properties in comparison to ureas.

The common structure of known sEH thiourea based inhibitor is Ad-NHC(S)NH-R, where Ad is adamantan-1-yl, R is alkyl, aryl or heterocyclic group.^{18–20} While the R-group was altered, the left (adamantane) part of the thiourea molecules was the same in almost all known thioureas based sEH inhibitors. Thus, the impact of alterations in adamantyl part of the thioureas on their potency and properties has never been investigated.

In this work we prepared and systematically studied new structural types of adamantyl thioureas with the following features: (*i*) spacers between adamantyl substituent and thiourea group to enhance conformational mobility; (*ii*) alkyl substituents in the bridgehead positions of adamantane to alter its lipophilicity; (*iii*) two adamantyl parts in a single molecule; (*iv*) adamantyl fragment linked with thioureas group by the bridge carbon; (*v*) aromatic fragments to regulate lipophilicity.

Reaction of amines with isothiocyanates is among the most widely used procedures for the preparation of thioureas. In contrast to isocyanates, isothiocyanates are less reactive and do not react with water in common conditions²¹. Due to the bimolecular nature of this reaction, the adamantane moiety can be introduced in to the molecule of thiourea either with adamantyl amine or with adamantyl isothiocyanate. In this case, it is reasonable to use adamantyl amines, which are quite available in contrast to the adamantyl isothiocyanates.²² Since the mechanism of thiourea formation is nucleophilic addition to thiocarbonyl group, the most significant factors affecting the reaction speed are the amine nucleophilicity,

positive charge on a thiocarbonyl carbon atom and steric factors in a transition state. On the one hand, the basicity of amino group in adamantyl amines is relatively high²³ due to the donor effect of adamantane fragment, which should have positive effect on reactivity. On the other hand, steric hindrance in transition state caused by large adamantane fragment could lower reactivity of such amines despite of high basicity. Introduction of spacers between the amino group and adamantane will decrease basicity as well as steric hindrance in transition state.

Previous research²⁴ showed that selectivity of the reaction under consideration is highly affected by the reagents ratio and the solvent used. So when carrying reaction with equimolar amount of adamantyl amine or in excess of aromatic isothiocyanate, an adverse reaction leading to the formation of corresponding adamantyl isothiocyanate takes place. This metathesis effect is possible due to the consecutive route of the reaction when thioureas are involved in the reaction with excessive isothiocyanate. Hence, it is expedient to use solvents, which cannot dissolve thioureas and thereby exclude it from the reaction zone (for instance, hexanes). It is necessary to mention that metathesis of amines into isothiocyanates was discovered in reactions carried out without triethylamine.²⁴ Introduction of strong base such as Et₃N or DIPEA in most cases suppresses the adverse reaction between thioureas and isothiocyanate.

Thioureas bearing aromatic moiety are the most intensively investigated as biologically active compounds among the thioureas.^{25–27} Combination of a benzene ring and an adamantyl fragment in one molecule is of great interest. Among ureas and thioureas containing aromatic moiety those with EWG (Cl, F, F₃C and F₃CO) were the most potent as sEH inhibitors.²⁸ Taking into account that interaction of urea-type inhibitor with the sEH domain is NH-acidity dependent, we developed thioureas containing aromatic ring substituted with EWG (**1j–q**, Scheme 1).

Thioureas **1a**, **1d–1h** are poorly soluble in water due to hydrophobic nature of both the adamantyl and aromatic sides of the molecules. Insertion of one methylene bridge between thiourea group and benzene ring leads to the 4-fold decrease in water solubility and to an increase of activity (**1a** vs. **1d**) while addition of second one does not affect solubility and leads to a slight decrease of activity (**1d** vs. **1f**). A 3-fluoro substituted aromatic part does not change water solubility (**1j–m**) while fluorine in 4-position makes the thioureas 4-fold more soluble in water (for example, **1a** vs. **1n**). Introduction of fluorine into the 4-position of the aromatic nucleus also leads to the most notable increase of activity. Thioureas with methyl substituents in bridgehead positions in adamantane as well as those with methylene spacers at the thiourea groups possessed lower melting points. For example, in a row of thioureas **1b**, **1e** and **1h** introduction of methylene spacer leads to 39 °C decrease in melting point and for another 9 °C with a 1,2-ethylene spacer. The most significant melting point decrease (108 °C) is observed when two 1,2-ethylene spacers were introduced at both sides of the thiourea groups when compound **1i** compared to 1-(adamantan-1-yl)-3-phenyl thiourea.³⁰ More substituents in the adamantane part of the thiourea molecule as well as complication of the spacer at thioureas group leads to lower inhibitory activity. In this case complicated spacers have the worst impact on activity decrease. Compounds **1a** and **1b** have the same molecular mass but **1a** (no spacer between adamantane and thiourea, two methyl substituents in

adamantane) is 2-fold more potent than **1b** (1,1-ethylene spacer between adamantane and thiourea, no substituents in adamantane). Compound **1c** (-CH₂-CH(C₂H₅)- spacer between adamantane and thiourea, no substituents in adamantane) is 25-fold less potent than **1a**. Similar trend was observed for compounds **1d** and **1e**; **1g**, **1f** and **1h**; **1o**, **1n** and **1p**. The only exception from this empirical rule are the compounds with a 1,2-ethylene spacer between the adamantane and thiourea groups (**1m** and **1q**) being more potent than thioureas with single methylene spacer (**1k**) or with two methyl substituents in adamantane (**1n**).

As previously reported, 2-adamantyl ureas usually were more potent than corresponding 1-adamantyl analogs.¹⁸ Thus, we carried out reactions of 2-adamantyl amines with aromatic isothiocyanates (Scheme 2).

Water solubility of thioureas predictably lowers with each new methylene group between thiourea group and the benzene ring. Besides that, addition of branched spacer between the thiourea and adamantane part led to 4–9-fold decrease of solubility. For compounds **2a-c** regulations concerning the effect of spacers on inhibition potency against sEH are the same as for compounds **1a-s**. For compounds **2a-c** inhibition potency increased 6.7-fold when a methylene spacer was added between the thiourea groups and the benzene rings (from 229 nM for compound **2a** to 34 nM for **2b**) and 4-fold more with 1,2-ethylene spacer (up to 8.2 nM for compound **2c**). IC₅₀ of 8.2 nM for compound **2c** is extremely high activity for thioureas and even good enough for ureas.

A molecular docking in the sEH active site was performed for compounds **2a**, **2b** and **2c**. For this, the published X-ray crystal structure of the human sEH complexed with a thioureas-based ligand (PDB accession number 4JNC) was used.³¹ For compounds **2b** (Fig. 1) and **2c** (Fig. 2) the known¹⁹ complexation model was confirmed and the potency difference between these thioureas probably is connected with their lipophilicity. The 3D depictions of the binding site (Fig. 1–3) were created with VIDA 4.3.0 (OpenEye Scientific Software, Santa Fe, NM <http://www.eyesopen.com>).

Compared to its urea analog³², **2a** is 13-fold less potent. The molecular docking of **2a** (Fig. 3) suggests that it is twisted upside down, unlike **2b** and **2c** (fig. 1 & 2). In such position, there is no bonding possible between the thiourea and the protein, which probably explain **2a** low inhibitory activity (Table 2). Besides **2a**, such positioning in the active site could explain why thioureas were found previously to be poor sEH inhibitor^{16,19}, as the hydrogen bonds between the chalcogen and Tyr383 and Tyr466 are essential to yield highly potent inhibitor of sEH³³.

The X-ray crystal structure of compound **2a** (Fig. 4) reveals that the molecule is bent against the conformation proposed above. Prevalence of this conformation could also explain the low activity of thioureas due to the difficulty of hydrogen bond formation between NH and Asp335.

Thiourea **2a** crystallizes in the monoclinic space group $P2_1/c$ with one molecule in the asymmetric cell. The molecular structure of **2a** in the crystalline phase is characterized by the antiperiplanar torsion angle of the C21–N2–C1–S1 moiety { $\tau = 173.8(3)^\circ$ } and the

synperiplanar angle of the C101–N1–C1–S1 moiety $\{\tau = 7.2(5)^\circ\}$. The geometry of the symmetrically independent molecule **2a** is shown in Fig. 4. The main supramolecular motif in the crystals is a centrosymmetric dimer, which is formed by the intermolecular hydrogen bonds N2–H2...S1 $\{N2-H20.868(18) \text{ \AA}, H2...S1 2.59(2) \text{ \AA}, N2...S1 3.425(3) \text{ \AA}, N2-H2...S1 162(3)^\circ\}$; symmetry code: $-x+1, -y+2, -z+1$. A potential H-bond donor group N2–H2 has no acceptor due to steric obstacles caused by the phenyl and adamantan-2-yl substituents. The fragment of molecular packing of **2a** in the crystals is presented in Fig. 5. The figure shows the significant contribution of N–H...S and C–H...S interactions to the stabilization of the molecular conformation. The mentioned distance H2...S1 is shorter than the sum of the contact radii of hydrogen (1.20 Å) and sulfur (1.80 Å).³⁴ The three-dimensional crystal structure is arranged by the association of the zero-dimensional dimers by weak dispersion interactions of the peripheral fragments. The packing index is equal to 67.9%. The crystallographic data for the investigated compound have been deposited in the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 1824337.

It was previously shown that 1,3-disubstituted ureas with two adamantyl fragments in their structure are very potent soluble epoxide inhibitors.¹⁷ We used adamantyl isothiocyanates along with adamantyl amines to synthesize diadamantyl thioureas **3a-j**, **4a-f** and **5** (Scheme 3–5).

Diadamantyl thioureas could be divided into three groups based on connection of adamantane with thioureas group. In thioureas **3a-j** both adamantanes linked with thiourea group by its bridgehead carbons while in thioureas **4a-f** one adamantane linked with its bridgehead carbon and second with bridge carbon. In compound **5** both adamantanes linked with thioureas by its bridge carbons.

Thioureas with two adamantyl fragments with some exceptions possess water solubility of 30–120 μM and inhibitory potency up to 11.2 nM. Intramolecular interactions in symmetrical molecules are relatively high, and therefore introduction of methylene spacer between adamantyl and thiourea group increases solubility (**3a-c**) but has almost no effect on activity. A 1,4-phenylene spacer in the same position does not affect water solubility (**3a** and **3e** or **3c** and **3g**) but dramatically decreases inhibitory activity against the sEH. Introduction of two 1,4-phenylene spacers from both sides of thiourea group leads to 3-fold increase in water solubility accompanied with 1367-fold decrease in inhibition potency (**3a** vs. **3j**). It should be noted that synthesized thioureas are 4–5-fold more water soluble than ureas of corresponding structure. For example diadamantyl thiourea **3a** is 4.7-fold more soluble than corresponding diadamantyl urea.¹⁷

Interestingly, quantum chemical calculations for **DAU** and **3a** indicate that the presence of the sulfur atom in place of the oxygen in **DAU** yields a change in conformation of the chemicals. As shown on Fig 6, while conformer A is the most stable for **DAU**, conformer C is the preferred form of **3a** (MP2, def2-SVP, Orca 4.0.1³⁷). This could in part explain the difference in potency observed between **DAU** and **3a**.

Thus, in some cases loss of potency for thioureas in comparison with the ureas can be explained by the change in the conformational population.

The impact of asymmetry combined with the ability of the molecule to create intramolecular hydrogen bonds could be traced on thioureas **3a**, **4a** and **5**. These three thioureas have two adamantyl fragments directly linked with thiourea group without any spacers or substituents. Only difference is the connection between adamantyl and thiourea group in compound **3a**, where both adamantyls are linked by bridgehead carbons, whereas in compound **5**, both the adamantyls are linked by bridge carbons. Similarly in compound **4a**, one is linked by bridge carbon whereas the other one by bridgehead. Thiourea **5** is 1.5-fold more soluble than thiourea **3a** while asymmetric thiourea **4a** is 1.8-fold more soluble than **3a**.

Previously, we reported that diureas containing two urea groups linked with aliphatic spacer are very potent soluble epoxide hydrolase inhibitors.¹⁸ High activity of these compounds supposed to be due to the ability of second urea group to bind with Ser374 of the sEH while the second adamantyl contacts the inner hydrophobic areas of the protein.

Synthesis of symmetric dithioureas was carried out by the reaction of 1-adamantylisothiocyanate with aliphatic diamines (Scheme 6).

Water solubility of dithioureas decreases with each new methylene group introduced between thiourea groups and there is 3-fold difference between compound **6a** with 1,2-ethylene spacer and compound **6h** with 1,10-decylene. Thiourea **6a** is 10–137-fold less potent than the rest of thioureas in this series. Such a big difference support our hypothesis about formation of hydrogen bonds between second thiourea group and Ser374 and propylene linker is the required minimum while 6–8 carbon linkers preferred. When extending spacer between thiourea groups inhibition potency grows and comes to a maximum of 7.2 nM with 1,7-heptylene spacer (**6f**). Further elongation leads to a slight decrease of activity.

Trying to compare water solubility of our adamantyl thioureas with known ureas of corresponding structure, we discovered the lack of such data in the literature. Thus we synthesized four dithioureas **6i-l** similar to the diureas with known water solubility (Table 6).

Data in Table 6 show that thioureas are 2.5–7-fold more soluble in water than ureas of similar structure. Despite of lesser inhibition potency against human sEH than ureas, thioureas more soluble in water. Thus, the total effectiveness of thioureas when compared to ureas seems to be on the same level with the correct approach to the design of the molecule.

During the transition from *in vitro* testing to *in vivo*, first series usually carried out on laboratory animals, specifically mice and rats. To confirm extrapolation possibility of rodent *in vivo* testing to humans, we investigated inhibition potency of thioureas against mouse and rat sEH (Table 7).

Data (Table 7) show that thioureas in general are less active against rodent sEH. Therefore, one must be careful while transferring the effects found *in vivo* on rodents to humans. In addition, in most cases, the inhibitory activity of thioureas against the human sEH is closer

to the value of activity against rat sEH rather than murine sEH. Therefore, it is advisable to conduct *in vivo* sEH inhibition tests on laboratory rats.

Thus, a series of thioureas and dithioureas has been developed and systematic study of adamantyl thioureas as inhibitors of the soluble epoxide hydrolase (sEH) was performed. Inhibition potency of the described compounds ranges from 50 μM to 7.2 nM. Among the thioureas, compound **2c** (IC_{50} = 8.2 nM) and compound **6f** (IC_{50} = 7.2 nM) were found to be the most potent sEH inhibitors. While being less potent, the thioureas are up to 7-fold more soluble than the corresponding ureas, suggesting the sulfur containing compounds could be easier to formulate and probably more bioavailable than the corresponding ureas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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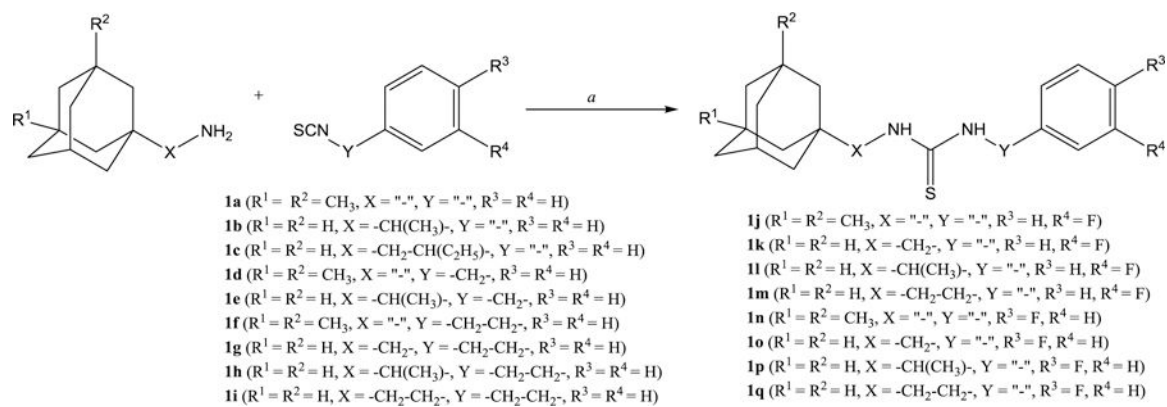
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Thioureas with aadamantane fragment were systematically studied as sEH inhibitors.

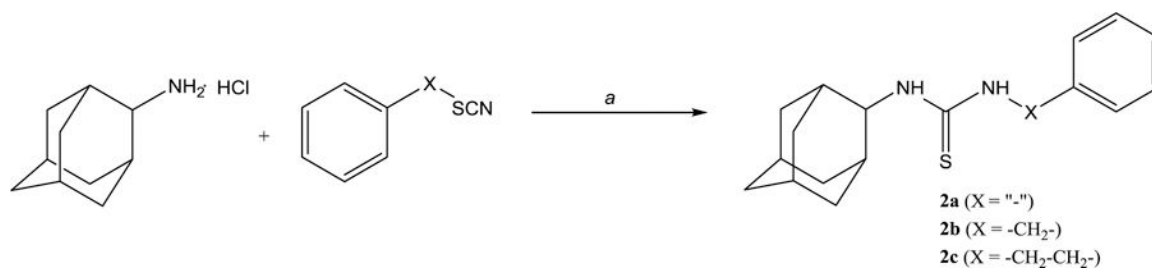
45 adamantyl thioureas were synthesized.

Difference of activity against human, rat and murine sEH were investigated.

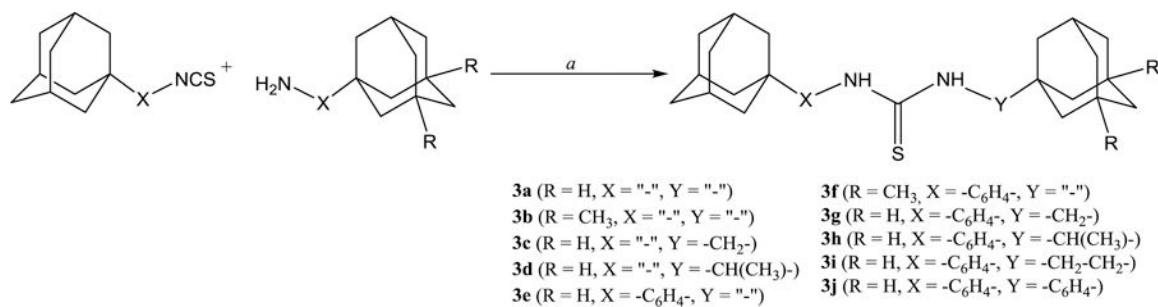
Discovered that thioureas are up to 7-fold more soluble than corresponding ureas.

**Scheme 1.**

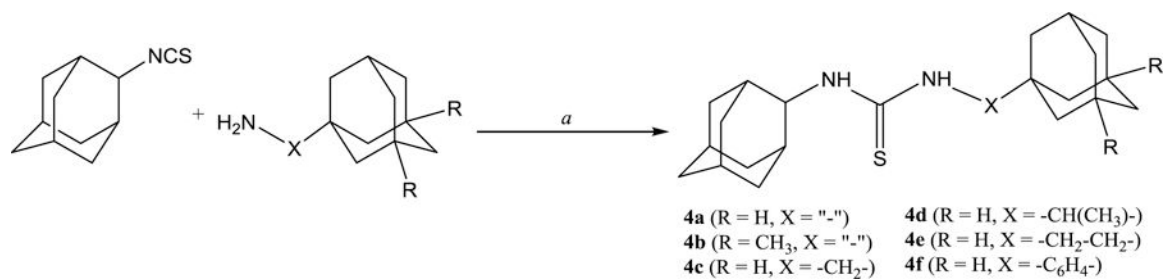
Reagents and conditions: *a*. DMF, Et₃N, 80 °C, 12 h.

**Scheme 2.**

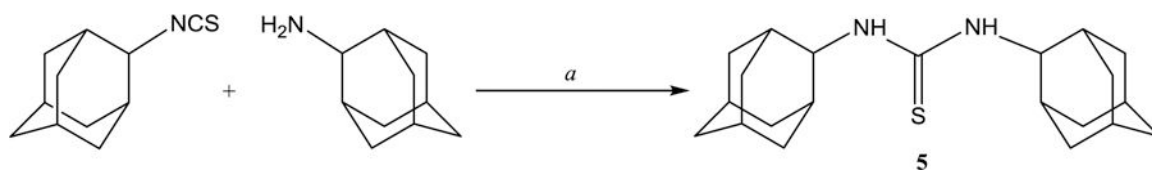
Reagents and conditions: *a*. DMF, Et₃N, 80 °C, 12 h.

**Scheme 3.**

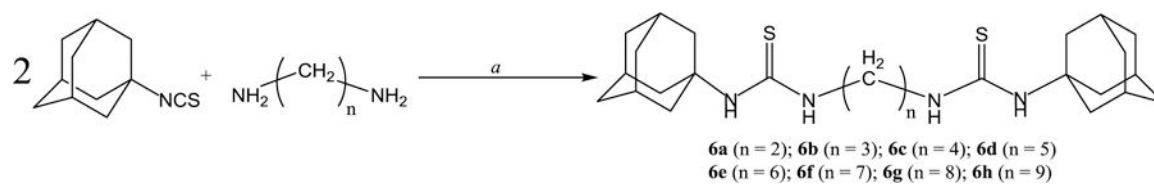
Reagents and conditions: *a*. DMF, Et₃N, 80 °C, 12 h.

**Scheme 4.**

Reagents and conditions: a. DMF, Et₃N, 80 °C, 12 h.

**Scheme 5.**

Reagents and conditions: *a.* DMF, Et₃N, 80 °C, 12 h.

**Scheme 6.**Reagents and conditions: *a*. DMF, Et₃N, 80 °C, 12 h.

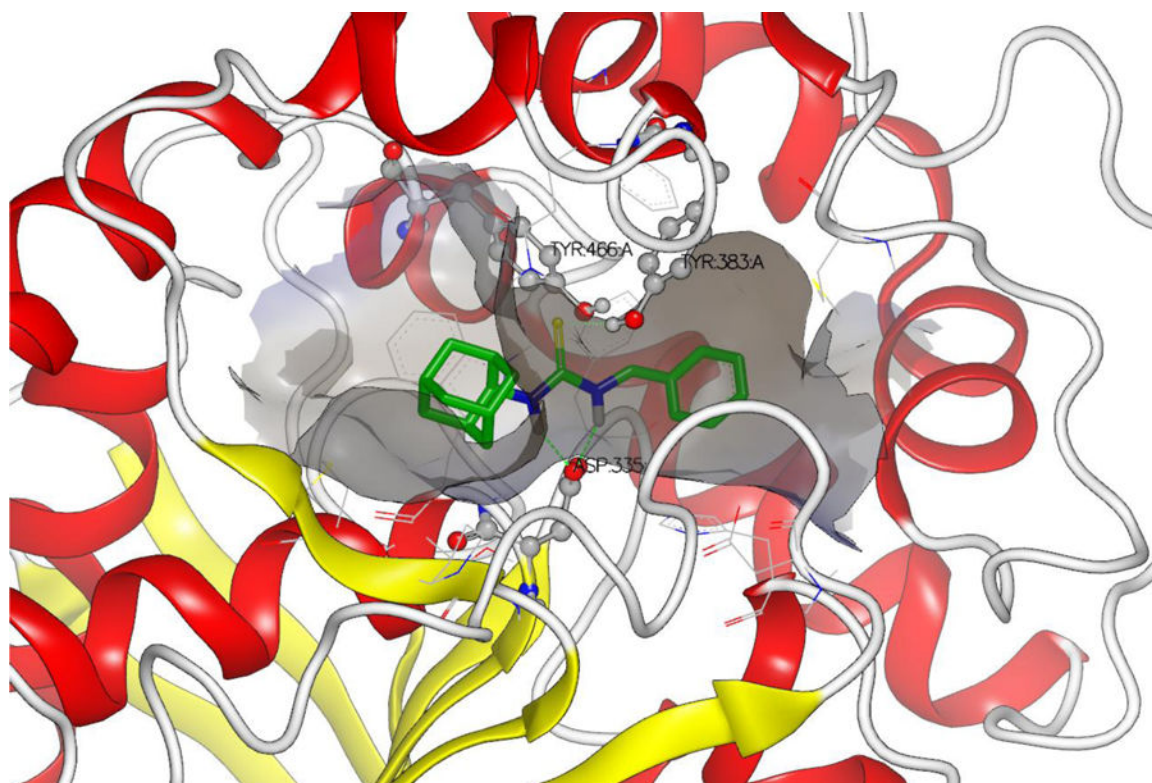


Figure 1.
Compound **2b** (green) docked into the active site of human sEH.

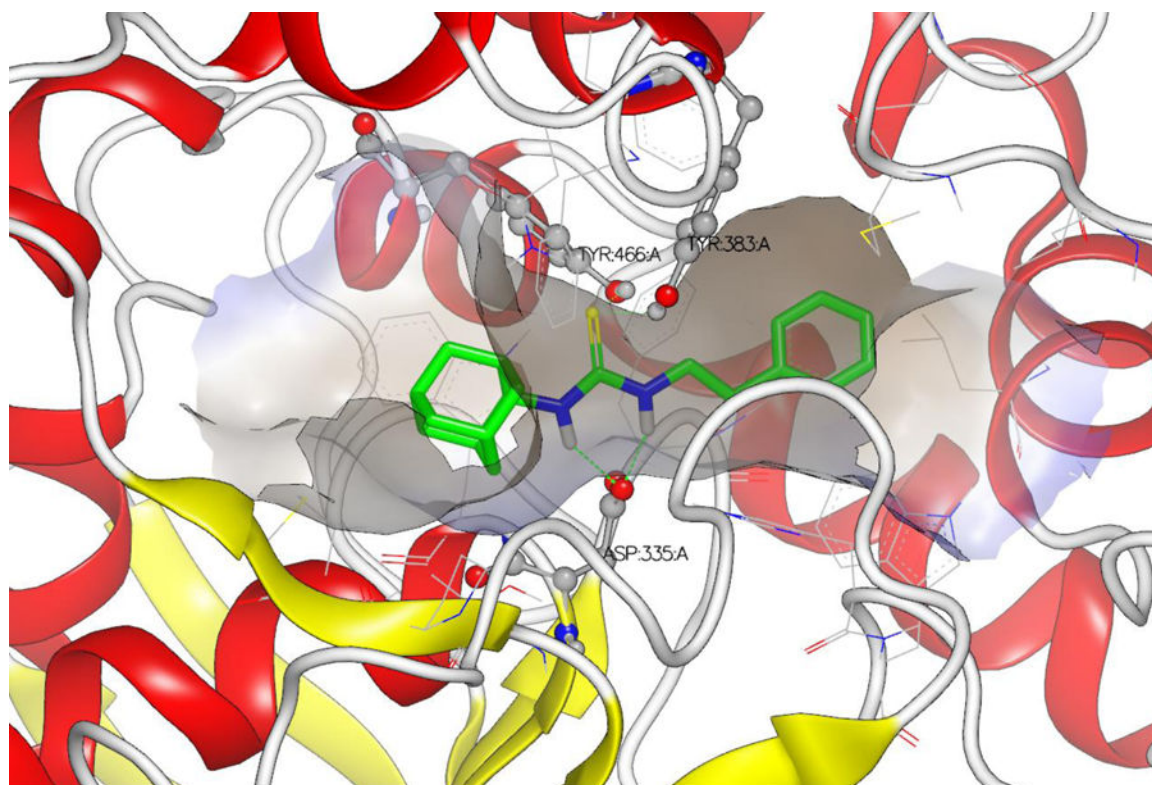


Figure 2.
Compound 2c (green) docked into the active site of human sEH.



Figure 3. Compound **2a** (green) docked into the active site of human sEH. Upside down orientation of the molecule and the absence of hydrogen bonds (dashed green lines) between the sulfur and Tyr383 and Tyr466 observed.

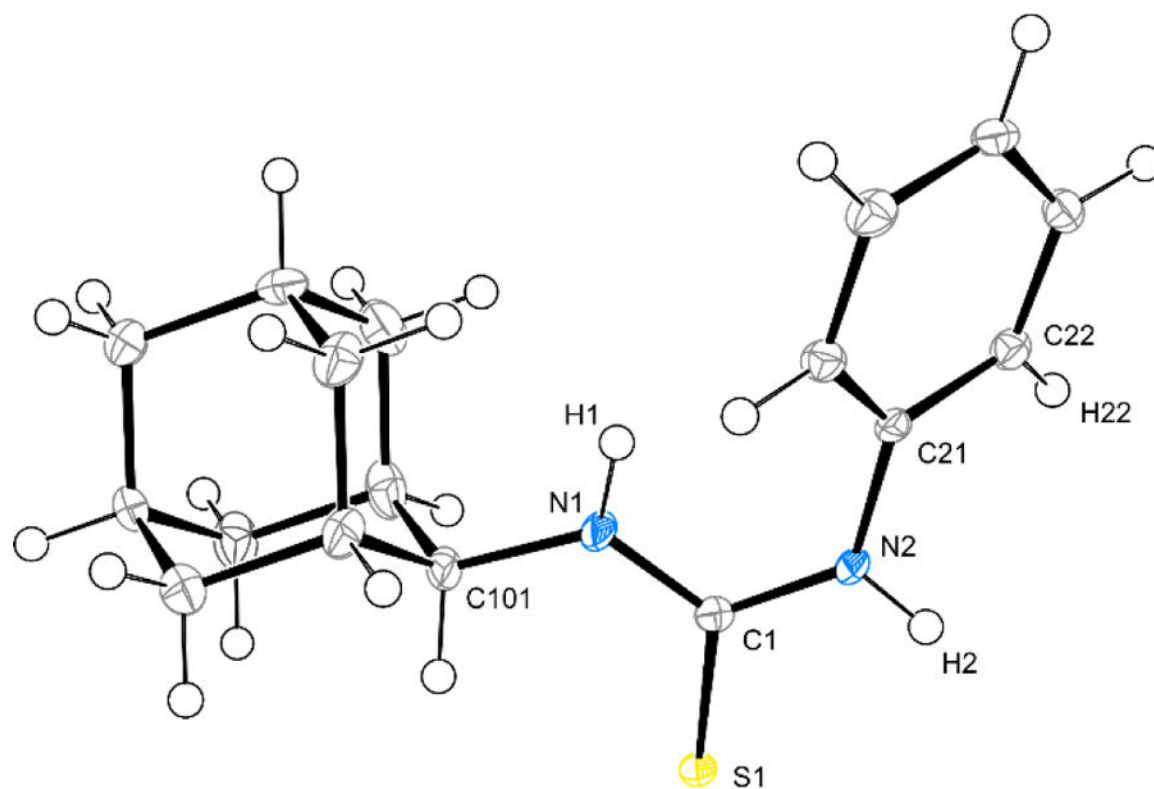


Figure 4. ORTEP diagram showing 50% probability anisotropic displacement ellipsoids of non-hydrogen atoms for compound **2a** according to single crystal XRD data collected at 100(2) K.

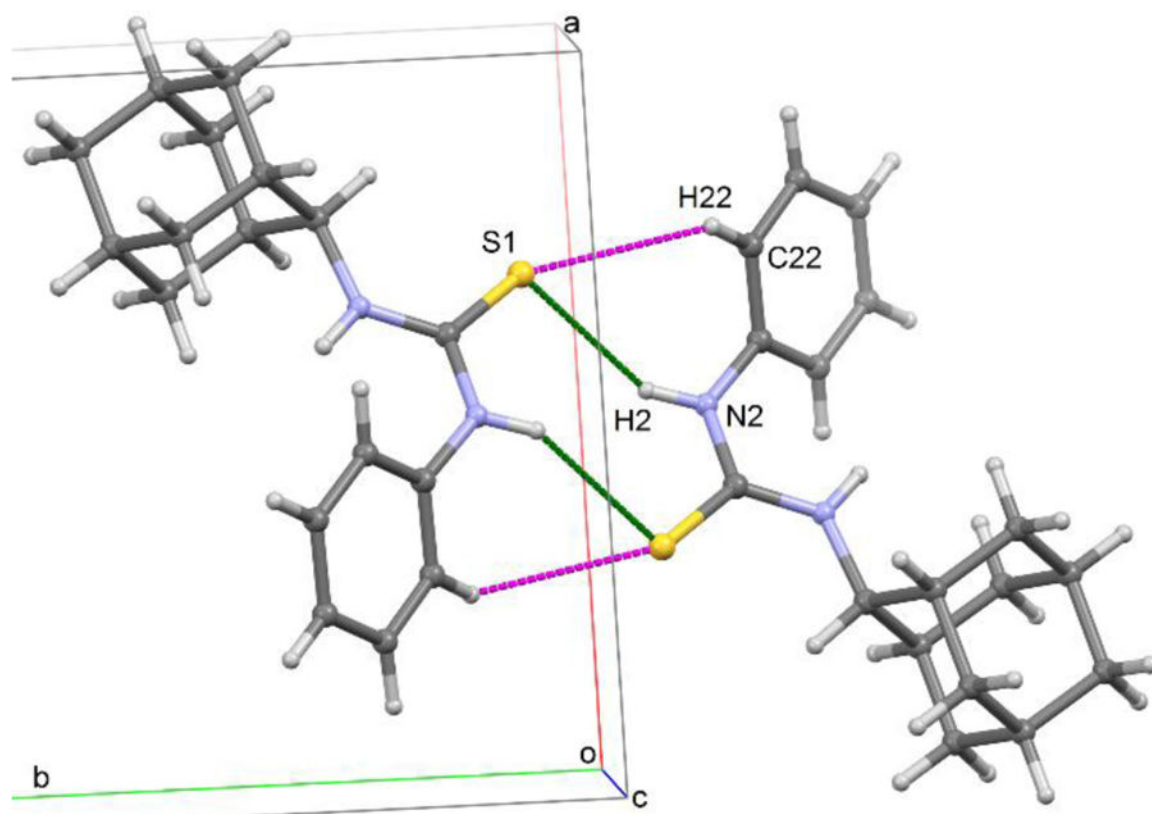


Figure 5. Fragment of the molecular packing in the crystals **2a** viewed along the axis Oc according to XRD. Intermolecular interactions $N-H \cdots S$ and $C-H \cdots S$ are marked in green and magenta dotted lines, respectively.

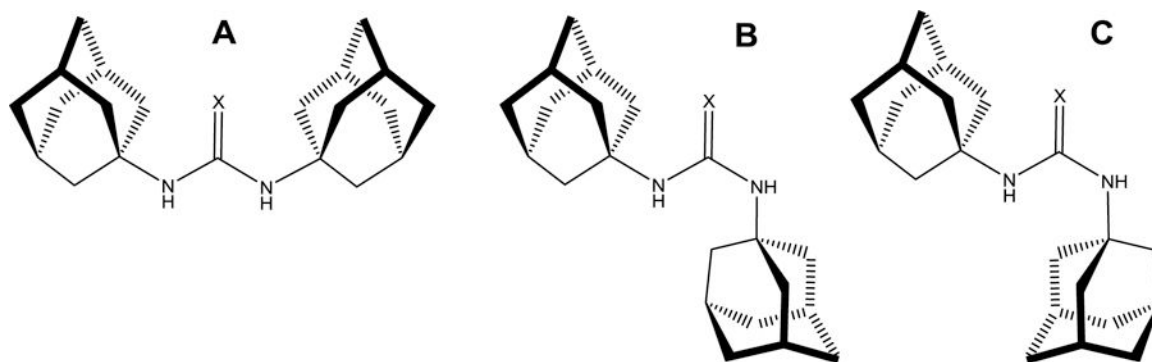


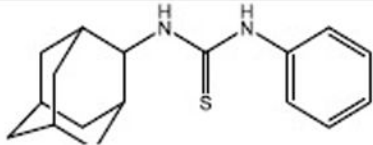
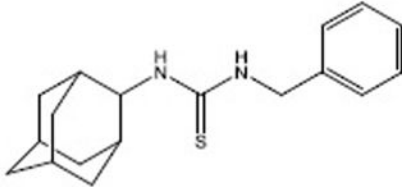
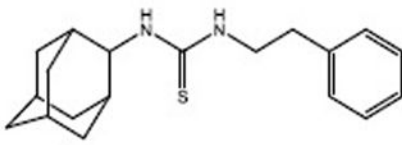
Figure 6.
The conformers of the di(adamantan-1-yl)urea and thiourea used in quantum chemical calculations.

Table 1IC₅₀ values and some physico-chemical properties for adamantyl-aryl sEH inhibitors 1a-q

#	Structure	mp (°C)	Solubility (μM) ^a	Human sEH IC ₅₀ (nM) ^b
1a		146-147	40-60	1224
1b		174-175	-	2592
1c		148-149	-	50248
1d		117-118	10-15	58
1e		167-168	40-50	234
1f		75-76	8-12	216
1g		138-139	25-30	133
1h		126-127	20-30	345
1i		67-68	60-80	52
1j		147-148	50-60	58
1k		153-154	30-40	281
1l		161-162	35-45	451
1m		69-70	80-90	160
1n		140-141	175-200	551
1o		136-137	150-175	159
1p		190-191	75-100	1084
1q		146-147	125-150	148

^aSolubilities were measured in sodium phosphate buffer (pH 7.4, 0.1 M) containing 1% of DMSO.^bAs determined via a kinetic fluorescent assay. Results are means of three separate experiments.³⁰

Table 2IC₅₀ values and some physico-chemical properties for adamantyl-aryl sEH inhibitors 2a-c

#	Structure	mp (°C)	Solubility (μM) ^a	IC ₅₀ (nM) ^b
2a		152-153	70-80	229
2b		124-125	65-75	34
2c		57-58	20-30	8.2

^aSolubilities were measured in sodium phosphate buffer (pH 7.4, 0.1 M) containing 1% of DMSO.

^bAs determined via a kinetic fluorescent assay. Results are means of three separate experiments.³⁰

Table 3IC₅₀ values and some physico-chemical properties for adamantyl-aryl sEH inhibitors 3a-j, 4a-f and 5

#	Structure	mp (°C)	Solubility (μM) ^a	IC ₅₀ (nM) ^b
3a		166-167	30-40	11.2
DAU ^c		310-314 ¹⁸	5-10 ¹⁸	1.2 ¹⁸
3b		121-122	30-40	16.1
3c		172-173	70-80	20.8
3d		116-117	-	60.3
3e		136-137	30-40	1248
3f		149-150	70-80	2479
3g		116-117	65-75	5903
3h		229-230	10-15	57393
3i		191-192	100-120	5334
3j		146-147	100-125	15314
4a		181-182	60-70	14.8
4b		117-118	80-90	152
4c		116-117	30-40	902
4d		230-231	30-40	7784
4e		191-192	125-150	259
4f		234-235	175-200	5936
5		250 (sublimes)	50-60	131

^aSolubilities were measured in sodium phosphate buffer (pH 7.4, 0.1 M) containing 1% of DMSO.^bAs determined via a kinetic fluorescent assay. Results are means of three separate experiments.²⁹

^cDAU: 1,3-diadamantyl urea

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Table 4

The relative conformational energies for di(adamantan-1-yl)urea and di(adamantan-1-yl)thiourea compared with the conformer of the lowest energy (kcal/mol)

X	Conformer A	Conformer B	Conformer C
X = O	0	6.2	2.8
X = S	0.3	4.2	0

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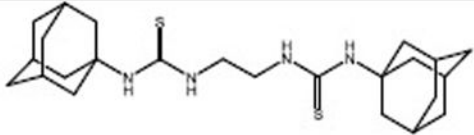
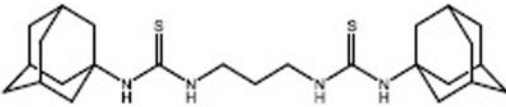
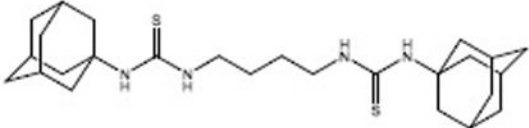
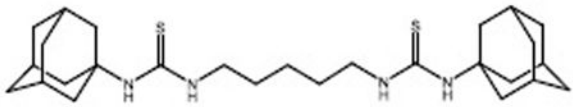
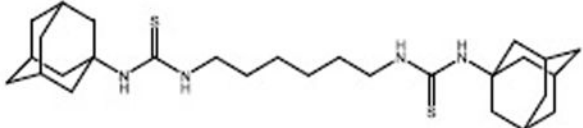
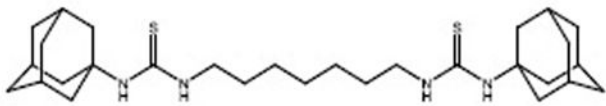
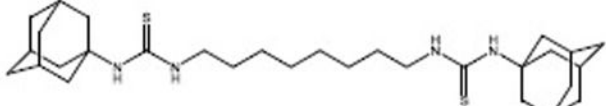
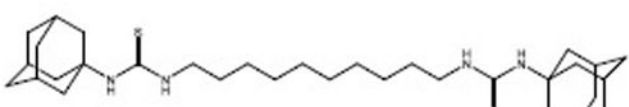
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Table 5

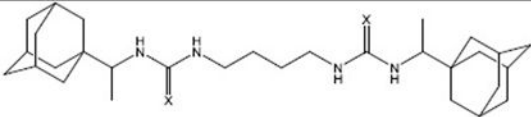
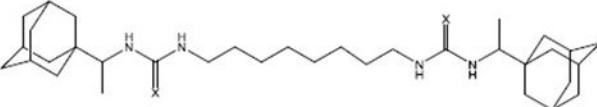
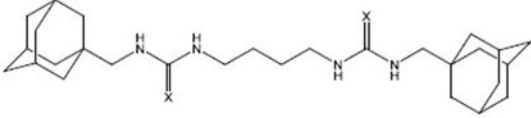
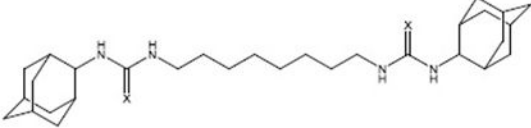
IC₅₀ values and some physico-chemical properties for adamantyl-aryl sEH inhibitors 6a-h

#	Structure	mp (°C)	Solubility (μM) ^a	IC ₅₀ (nM) ^b
6a		185-186	100-110	987.4
6b		134-135	90-100	97.6
6c		107-108	90-100	90.8
6d		83-84	80-90	21.1
6e		88-89	70-80	7.7
6f		60-61	70-80	7.2
6g		72-73	40-50	9.8
6h		71-72	35-45	15.3

^aSolubilities were measured in sodium phosphate buffer (pH 7.4, 0.1 M) containing 1% of DMSO.^bAs determined via a kinetic fluorescent assay. Results are means of three separate experiments.³⁰

Table 6

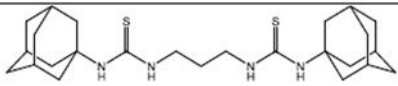
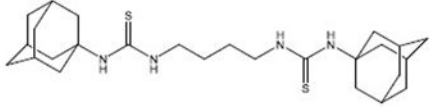
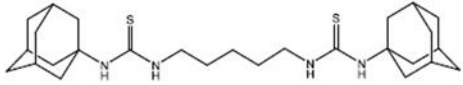
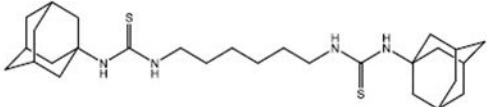
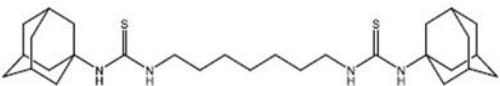
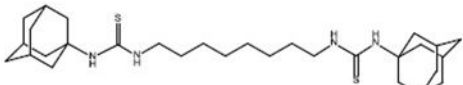
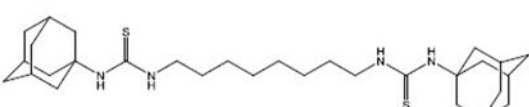
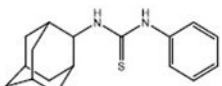
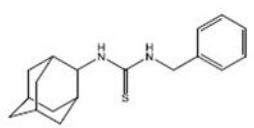
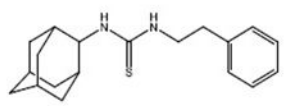
Water solubility of thioureas compared to ureas of similar structure

#	Structure	Solubility (μM) ^a		cLogP	
		X = O ¹⁷	X = S	X = O	X = S
6i		10-25	120-130	6.26	7.34
6j		10-25	120-130	8.25	8.86
6k		50-75	140-160	5.60	6.68
6l		5-10	25-35	7.29	8.31

^aSolubilities were measured in sodium phosphate buffer (pH 7.4, 0.1 M) containing 1% of DMSO.

Table 7

Inhibitory potency of thioureas 2a, 2c, 2e, 6b-6h against human (hsEH), rat (rsEH) and murine (msEH) soluble epoxide hydrolases

№	Structure	sEH IC ₅₀ (nM) ^a		
		Human	Rat	Mouse
6b		97.6	43.3	74.9
6c		90.8	122.7	130.4
6d		21.1	46.1	36.2
6e		7.7	61.6	50.6
6f		7.2	35.3	47.3
6g		9.8	374.0	1166.7
6h		15.3	44.4	59.5
2a		229	355.6	408.0
2b		34	60.3	61.0
2c		8.2	78.3	108.1

^aAs determined via a kinetic fluorescent assay. Results are means of three separate experiments.³⁰