Bioorganic & Medicinal Chemistry 21 (2013) 5518–5531

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

# Investigations on synthesis and structure elucidation of novel [1,2,4]triazolo[1,2-*a*]pyridazine-1-thiones and their inhibitory activity against inducible nitric oxide synthase ☆

Ulrike Schulz, Antje Grossmann, Manja Witetschek, Christian Lemmerhirt, Marcus Polzin, Beate Haertel, Heike Wanka, Olaf Morgenstern *

*Institute of Pharmacy, Ernst Moritz Arndt University Greifswald, Friedrich-Ludwig-Jahn-Straße 17, 17487 Greifswald, Germany*

ARTICLE INFO

*Article history:*
Received 24 March 2013
Revised 26 May 2013
Accepted 28 May 2013
Available online 12 June 2013

*Keywords:*
Hexahydropyridazine-1-carbothioamide
[1,2,4]Triazolo[1,2-*a*]pyridazine-1-thiones
Inducible nitric oxide synthase
RINm5F

ABSTRACT

The inducible nitric oxide synthase (iNOS) is a target of great research interest due to its importance in a number of diseases, for example, septic shock and inflammatory lung diseases. A variety of 3-substituted [1,2,4]triazolo[1,2-*a*]pyridazine derivatives was synthesized by ring closure with hexahydropyridazine-1-carbothioamide by using aliphatic and aromatic aldehydes. The activity of the new substances was tested on the insulin-secreting rat insulinoma cell line RINm5F. iNOS was expressed through exposure to interleukin-1β (IL-1β) and interferon-γ (IFN-γ). A number of the investigated compounds were more active than the reference inhibitor aminoguanidine (AG). Structure–activity relationships showed that a phenyl substituent in position 3 is apparently essential for inhibition.



## 1. Introduction

In the 1980s was discovered that the endothelium-derived relaxing factor (EDRF) is actually nitric oxide (NO).[1] Since then an understanding of its role in physiology has dramatically increased. This radical substance is involved in numerous processes, from cardiovascular regulation to neuronal signaling and immune response.[2–6]

NO is synthesized by a family of enzymes called nitric oxide synthases. These enzymes are homodimers possessing a bidomain structure. The oxygenase domain contains binding sites for the cofactors haem, tetrahydrobiopterine ($BH_4$) and the substrate L-arginine. The reductase domain located on the other side of the enzyme reveals binding regions for cofactors FAD, FMN and NADPH. These domains are linked by a calmodulin recognition site.[7–9] There are three isoforms known: endothelial (eNOS, NOS3), neuronal (nNOS, NOS1) and inducible nitric oxide synthase (iNOS, NOS2). They all generate NO by oxidation of L-arginine to L-citrulline via $N^{\omega}$-hydroxy-L-arginine but have different localization, responsibilities and regulation. eNOS and nNOS are constitutively expressed, $Ca^{2+}$/calmodulin-regulated and produce a rather small amount of NO. These two isoforms are of basic physiological importance. On the other hand, the iNOS is a part of the primary immune defense. It is expressed by the influence of proinflammatory cytokines or bacterial lipopolysaccharides (LPS), $Ca^{2+}$/calmodulin-independent and creates a high nitric oxide concentration.[5,10,11]

Although nitric oxide is needed for physiological processes, an excess can be fatal. In fact overproduction of NO is discussed in relation to the pathogenesis of many diseases, for example, septic shock,[12,13] multiple sclerosis/Alzheimer's disease[6,14] and COPD/asthma.[15,16] In most of the cases the inducible isoform seems to be responsible for the massive NO production that leads to pathological changes.

As the number of diseases connected with an overexpression of iNOS has increased, so too has the number of inhibitors, however, to date there is no drug available to treat these clinical conditions. Potency and especially selectivity are central problems of the drug design because it is obvious that an exclusive inhibition of iNOS is required to maintain the physiological function of eNOS and nNOS. Although an inhibition of iNOS mediated NO production is also possible through pteridine analogues that block the cofactor tetrahydrobiopterine[17,18] as well as by substances that hinder the dimerization of iNOS[19,20], most of the reported iNOS inhibitors interact with the arginine binding site of the oxygenase domain. Such inhibitors can be divided into two groups: amino acid-based and non-amino acid-based substances.[21] Amino acid-based

☆ *N,N*-Coupled heterobicycles from cyclic hydrazine derivatives. Part 14. Part 13: Morgenstern, O.; Wanka, H.; Röser, I.; Steveling, A.; Kuttler, B. *Bioorg. Med. Chem.* **2004**, *12*, 1071.

* Corresponding author. Tel.: +49 3834864838.
*E-mail address:* omorgens@uni-greifswald.de (O. Morgenstern).

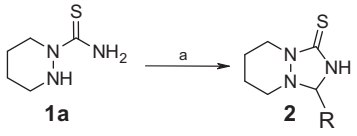
0968-0896/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2013.05.064

inhibitors, as L-NIL, L-NIO[22] or L-NNA,[23] usually show $IC_{50}$ values in the low micromolar range but have poor selectivities.[10,21] Exceptions are GW273629 and GW274150, which show high selectivity.[24–26] Non-amino acid-based inhibitors also mimic L-arginine but with a wider structural variety. There are guanidines, for example, aminoguanidine, which was discovered as one of the first iNOS inhibitors. Due to the high concentrations required for inhibition, severe side effects in vivo (mouse) and the fact that selectivity is reduced with increasing concentrations aminoguanidine is a poor drug candidate.[27–29] Isothioureas such as *S*-ethylisothiourea and AMT showed very good activities in the lower nanomolar range but were at most only partially selective.[30,31] One of the most potent iNOS inhibitors, ONO-1714, is a representative of the amidine derivatives, but also showed only low selectivity.[32,33] On the other hand, another amidine, 1400W, was identified as a highly selective and very potent inhibitor.[34] In the non-amino acid-based group also are spirocyclic analogues[35] and 2-aminopyridines.[36–38] Some of the newer studies investigated sulfur and nitrogen containing heterocyclic structures not unlike those described here.[39,40]

Here we report on a series of novel [1,2,4]triazolo[1,2-*a*]pyridazine-1-thiones as promising iNOS inhibitors, whose hitherto unknown substructure might also be interesting with regards to other bioactivities; the 1,2,4-triazolo partial structure has been investigated in respect of antimicrobial, antifungal[41,42] and analgesic properties[43] as well as HSP90[44] and TACE inhibition.[45] Some time ago our group published the synthesis and biological activity of semicyclic aminoguanidines including their carbothioamide synthetic precursors.[46] Unfortunately, the iNOS inhibitory ability of both of these substance classes was not improved compared to the lead structure.

For this reason our attention focused on the related bicyclic derivatives since these substances possess a more rigid molecular skeletal structure, which could result in a higher efficacy and selectivity. The ring closure with C1 reactants, such as carbonyl compounds, offered an excellent preparative access to a variety of 3-substituted [1,2,4]triazolo[1,2-*a*]pyridazine derivatives. First, the synthesis of the hitherto unknown [1,2,4]triazolo[1,2-*a*]pyridazine-1-thiones **2** (Scheme 1) and their iNOS inhibitory ability was investigated. The known representatives of this heterocyclic system possess $sp^2$-hybridized carbon atoms in the triazol ring such as [1,2,4]triazolo[1,2-*a*]pyridazin-1,3-diones,[47,48]-1,3-dithiones,[47,49] 1-oxo-[47,50] and 1-arylimino-[51,52] [1,2,4]triazolo[1,2-*a*]pyridazin-3-thiones, and 3-oxo-[1,2,4]triazolo[1,2-*a*]pyridazin-1-carboxylic acids (and their esters), respectively.[53,54] In contrast, only very few derivatives with only one $sp^3$-hybridized carbon atom in this part of the condensed heterocyclic system, as in the title compounds, have been described previously.[55,56] With two exceptions[57,58] the hitherto known [1,2,4]triazolo[1,2-*a*]pyridazines without substituents at the pyridazine ring are always substituted at the N(2) atom.

At first, the substitution pattern in the 3-position was varied in order to gain insight into structure–activity relationships. The iNOS inhibitory potential was investigated by using a cell culture assay based on the ability to express iNOS in rat insulinoma cells by addition of proinflammatory cytokines.[59,60]




**Scheme 1.** (a) Different aldehydes; different reaction conditions.

## 2. Results and discussion

### 2.1. Synthesis

To obtain the 3-substituted heterocycles **2,** the starting compound **1a** was reacted with various aliphatic and aromatic aldehydes (Scheme 1). Aliphatic and araliphatic reagents gave the compounds **2b**,**c** (Scheme 3) and **2d**,**e** (Scheme 4). The carbothioamide **1a** was found to be easily accessible from hexahydropyridazine hydrochloride **4** and potassium thiocyanate.[46]

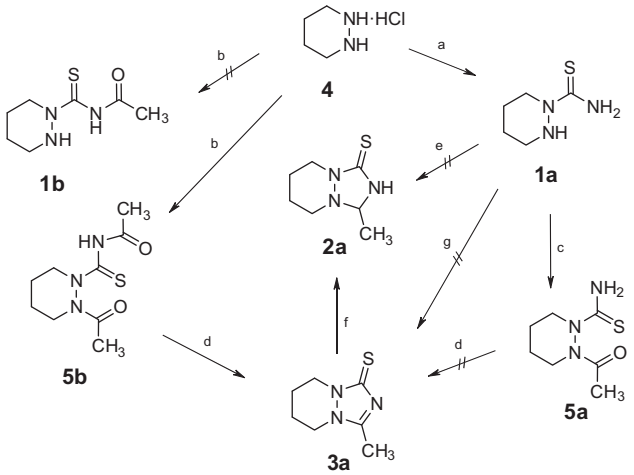
However, **2a** (Scheme 2) was not directly accessible via this route.

#### 2.1.1. 3-Methyl and 3-ethyl derivatives 2a,b

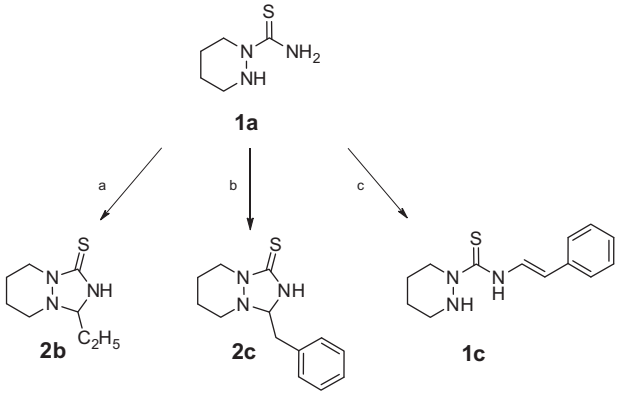
The first attempts to obtain the 3-methyl derivative **2a** by acid-catalyzed reaction of **1a** with acetaldehyde or acetaldehyde dimethyl acetal applying conventional synthesic methods were just as unsuccessful as by using a microwave-assisted synthesis of this compound with acetaldehyde diethyl acetal. Regarding these results it was investigated whether **2a** could be synthesized by reacting **1a** with acetic acid to give **3a** followed by hydrogenation (Scheme 2). However, all attempts to obtain **3a** by this route failed. Therefore, the synthesis of **3a** was attempted by intramolecular cyclocondensation of **1b**. To get to this intermediate stage, hexahydropyridazine hydrochloride **4**[17] was reacted with acetyl isothiocyanate. Nevertheless, even with modification of the reaction conditions, compound **5b**, the 2-acetyl derivative of **1b**, was always isolated. However, when **5b** was heated in methanol the compound rapidly cyclized to **3a**. This reaction is similar to the desired formation of a 2-thioxo-2,3-dihydro-quinazoline-4(1*H*)-one derivative.[61]

The treatment of **1a** with acetic anhydride provided the precursor **5a** in excellent yield. This seemed to offer an additional facile method to obtain **3a**. However, all attempts failed to cyclize **5a** utilizing the same reaction conditions as for **5b**. It seems likely that the formation of **3a** from **5b** is favored because the nascent acetic acid is removed immediately from the reaction by formation of methyl acetate. The reduction of **3a** to **2a** succeeded by using sodium borohydride under mild conditions (Scheme 2).

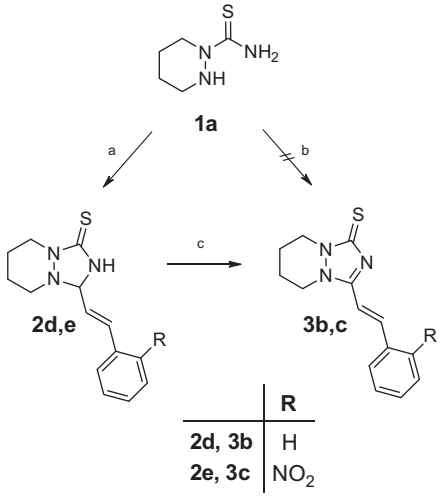
Heating of **1a** with propionaldehyde provided the 2-ethyl derivative **2b**, which was isolated after chromatographic separation of the resulting product mixture, but only in poor yield (Scheme 3).




**Scheme 2.** (a) KSCN, melt;[46] (b) $CH_3CO$-NCS, $(C_2H_5)_3N$, $CH_2Cl_2$, room temperature, 30 min; (c) $(CH_3CO)_2O$, room temperature, 3 weeks; (d) $CH_3OH$, $H^+$, reflux, 24 h; (e) $CH_3CHO$ or $CH_3CH(OCH_3)_2$, $H^+$, different conditions; (g) $CH_3COOH$, 130 °C, 12 h; (f) $NaBH_4$, $C_3H_7OH$, room temperature, 24 h.




**Scheme 3.** (a) $C_2H_5$–CHO, $H^+$, $CH_2Cl_2$, reflux, 9 h; (b) $C_6H_5$–$CH_2$–CHO, $H^+$, 125–130 °C, 4.5 h; (c) $C_6H_5$–$CH_2$–CHO, $CH_2Cl_2$, 60 °C, 5 min.




**Scheme 4.** (a) Compound **2d**: $C_6H_5$–CH=CH–CHO, $CH_2Cl_2$, room temperature, 15 h; Compound **2e**: 2-$O_2N$–$C_6H_5$–CH=CH–CHO, $H^+$, 100–105 °C, 20 min; (b) Compound **3b**: $C_6H_5$–CH=CH–COOH, **3c**:2-$NO_2$–$C_6H_5$–CH=CH–COOH, $H^+$,180 °C, melt; (c) from **1a** via **2d,e**–**3b**: $C_6H_5$–CH=CH–CHO, $H^+$, room temperature, 2 h; **3c**: 2-$O_2N$–$C_6H_5$–CH=CH–CHO, $H^+$, 125–130 °C, 20 min.

### 2.1.2. 3-Araliphatic substituted compounds 2c–e

Phenyl acetaldehyde reacted with **1a** under mild conditions without proton catalysis to give exclusively the *N*-styryl carbothioamide **1c**. In contrast, the desired **2c** was formed in the proton catalyzed reaction (Scheme 3).

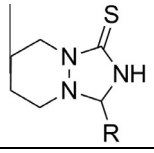
Reaction of **1a** and cinnamaldehyde in dichloromethane under mild conditions yielded the 3-styryl derivative **2d**, but without solvent its oxidation product **3b** was isolated instead (Scheme 4). The latter was also formed by prolonged heating (4 h) at 125–130 °C but in much lower yield.

The reaction with 2′-nitrocinnamaldehyde proceeded much faster and yielded **3c** as the main product, **2e** as a byproduct, respectively. The ease of such oxidation process has already been observed with 1,2,4-triazolidine-3-thiones and utilized for targeted synthesis by other authors.[62] Compound **2e** was formed within a short time and in good yield at 100–105 °C (Scheme 4). However, all attempts to obtain **3b** or **3c** failed by directly melting (180 °C) **1a** with the corresponding cinnamic acids.

### 2.1.3. 3-Aryl derivatives 2f–u

In contrast to the observations described above, the cyclization reaction with aromatic aldehydes proceeded rapidly and without significant problems. Compounds **2f**–**u** (Table 1) were obtained

**Table 1**
3-Substituted [1,2,4]triazolo[1,2-*a*]pyridazine-1-thiones **2**



| **2** | R | Reaction time [min] (Yield [%]) conventional[a] | Reaction time [min] (Yield [%]) microwave assisted[b] |
|---|---|---|---|
| **f** | $C_6H_5$ | 75 (76) | 3 (63) |
| **g** | 2-$CH_3$–$C_6H_4$ | 30 (74) | |
| **h** | 3-$CH_3$–$C_6H_4$ | 25 (68) | |
| **i** | 4-$CH_3$–$C_6H_4$ | 25 (54) | |
| **j** | 2-Cl–$C_6H_4$ | 85 (71) | |
| **k** | 3-Cl–$C_6H_4$ | 15 (70) | |
| **l** | 4-Cl–$C_6H_4$ | 110 (53) | 6 (50) |
| **m** | 2-$NO_2$–$C_6H_4$ | 20 (69) | |
| **n** | 3-$NO_2$–$C_6H_4$ | 20 (73) | |
| **o** | 4-$NO_2$–$C_6H_4$ | 20 (84) | 6 (64) |
| **p** | 2-OH–$C_6H_4$ | 15 (57) | |
| **q** | 3-OH–$C_6H_4$ | 15 (64) | |
| **r** | 4-OH–$C_6H_4$ | 15 (53) | 3 (77) |
| **s** | 4-$CH_3O$–$C_6H_4$ | 75 (35) | 3 (69) |
| **t** | 3-($C_6H_5O$)–$C_6H_4$ | 15 (74) | 3 (84) |
| **u** | 4-[$(CH_3)_2N$]–$C_6H_4$ | 20 (70) | |

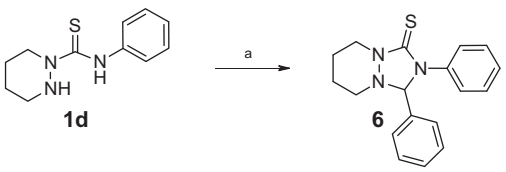
[a] Heating the educts at 150–155 °C (**2l**: 130–135 °C; **2u**: 100–105 °C).
[b] Additional data are given in the experimental part.

in good yields, usually after a general work-up method, by using a mixture of **1a** and the corresponding substituted benzaldehyde (excess of 10%; exception: methylbenzaldehydes 50%) in the presence of catalytic amounts of *p*-toluenesulfonic acid monohydrate. The reaction mixture was heated to 150–155 °C (Scheme 1). In most cases the reaction of **1a** with the aldehydes was already completed within a period of 15–30 min. However, benzaldehyde (**2f**), *o*- and *p*-chlorobenzaldehyde (**2j,l**), and *p*-methoxybenzaldehyde (**2s**) reacted significantly slower than the other aldehydes. To avoid undesirable side reactions, the synthesis of *p*-chlorophenyl and *p*-dimethylamino derivative was done at lower temperatures (Table 1).

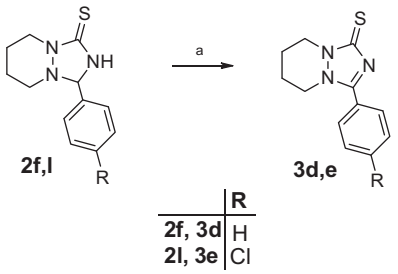
Compound **1d** showed a higher reactivity towards benzaldehyde than did **1a**; thus, the 2,3-diphenyl-2,3,5,6,7,8-hexahydro-1*H*-[1,2,4]triazolo[1,2-*a*]pyridazine-1-thione **6** was already formed by reacting the mixture at room temperature in good yield (Scheme 5). Especially with the 3-arylsubstituted [1,2,4]triazolo[1,2-*a*]-pyridazine-1-thione derivatives **2**, whose preparation took an extended period of time by conventional synthesis, the use of microwave technology was advantageous.

In all the cases studied microwave methods, very significant reduction in reaction times (Table 1) and in some cases (**2r**,**s**,**t**) an improvement in yields were observed. An exception was the reaction with *p*-dimethylaminobenzaldehyde, which under similar conditions resulted in decomposition with strong gas generation. A product was not isolable.

Again, oxidation of the resulting 3-aryl derivatives **2** led to the formation of the 2,3-didehydro compounds **3**, but this was much




**Scheme 5.** (a) $C_6H_5$–CHO, room temperature, 2 weeks.




**Scheme 6.** (a) Compound **3d**: atmospheric $O_2$; refluxing $CH_3OH$ or $CDCl_3$ at room temperature, beginning after 2 h; **3e**: atmospheric $O_2$, DMSO, room temperature, several days (not definable, see experimental data).

less pronounced than with **2d,e**. In some cases, the corresponding **3** could be isolated. Considerable amounts of **3d** could be separated after recrystallization of **2f** from acetone. It precipitated also within few hours from a solution of **2f** in deuterochloroform. Similarly, **3e** was formed when a solution of **2l** in DMSO was allowed to stand for several days (Scheme 6). Worth mentioning is that only the solutions of **2** were sensitive to oxidation. The solids were demonstrably stable, which was proven by NMR. Most likely all compounds of type **2** are sensitive to oxidation in solution as there was precipitation from DMSO stock solutions (**3** are obviously even worse soluble than **2**) observed for all biologically tested samples after several days of storage. Only the yields differed and only for the above mentioned compounds **3** were the structures proven.

## 2.2. Structural investigations

For the verification of the assigned structures of the obtained compounds of type **2,** the exclusion of alternative monocyclic or bicyclic structures was of particular interest. In particular, proton and carbon NMR spectroscopy complemented by related 2D methods provided conclusive information. One of the important criteria for the existence of the assigned bicyclic thione structure **2** is the presence of a proton at the N(2) in a typical thioamide range of 6 to 9 ppm, as well as the absence of a proton at the N(4) [corresponding to the N(2)H of the starting compound **1a**, there at about 3.35 ppm][17] in the proton NMR spectra (solvent: deuterochloroform). In the $^{13}C$ NMR spectra the related signals for the tertiary C(3) atoms were found between 75 and 82 ppm and for the C(1)=S within the expected range of 175–177 ppm.

The NMR data of all characterized **2** were found to be consistent with the known values for the respective partial structures. For all 3-arylsubstituted [1,2,4]triazolo[1,2-*a*]-pyridazine-1-thiones the magnetic non-equivalence of the chemically equivalent protons at C(8)$H_2$ and C(5)$H_2$ is characteristic. The different signal positions of these geminal protons result from the spatial arrangement of the aromatic ring at C(3). Figure 1 exemplary shows the geometry of compound **2f**.

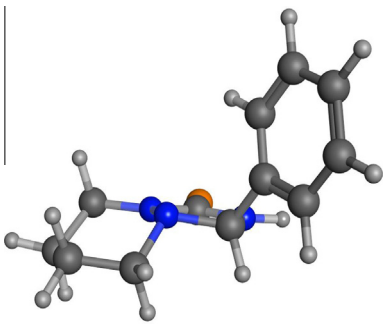
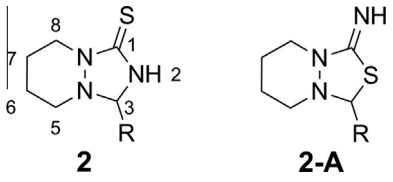


**Figure 1.** Spatial conditions at compound **2f**.

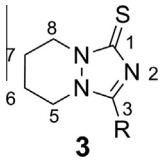
For none of the compounds was the expected signal splitting observed for the proton at N(2) because the protons at the thioamide N(2) and C(3) occupy an angle of nearly 90° to each other (Fig. 1). The signal position influenced by the aromatic ring and the signal splitting of the proton at C(3) of the compounds **2f**–**u** [R = (un)substituted) aryl] differed from those of the spectra of **2a**–**e**. For the C(3) proton, coupling with an adjacent hydrogen atom of the aliphatic or araliphatic 3-substituent was observed, and vice versa the corresponding doublets due to the adjacent proton(s) of the respective 3-substituent.

It was intended to investigate the conformation of the ethenylene part of the compounds **2d** and **2e** but the $^1H$ NMR provided no evidence for the coexistence of isomers because only the expected number of signals was observed. The high coupling constants between the C(α)H and the C(β)H of the ethenylene moiety with both compounds indicated the sole presence of the *E*-isomer.

The electron impact mass spectra (EI-MS) of the 3-arylsubstituted compounds **2f**–**u** supported the structural assignment and showed nearly always the same fragmentation pattern of the molecular ions, which appeared intensive. The fragmentation behavior resembled that reported for 2-thioxo-quinazoline-4-ones.[63] The first fragmentation step was usually the extrusion of [·$CH_2NS$] from the five-membered ring of the molecular ion. Moreover, the loss of the phenyl moiety together with its substituents was always observed. Except for **2u**, the fragmentation of the molecular ions led to the formation of the hexahydropyridazine cation [$C_4H_9N_2^+$].




None of the analyzed spectra (NMR, IR, MS) of the compounds **2** obtained from **1a** showed any indications for the existence of alternative [1,3,4]thiadiazolo[3,4-*a*]pyridazine-1-imines of type **2-A**. Thus, the IR spectra gave no evidence of C=N stretching vibration bands, which would be expected for structure **2-A**. In accordance with the carbon NMR data of structurally related 1,2,4-triazoline-3-thiones and isomeric 1,3,4-thiazolidine-2-imines[64] the spectra of the title compounds showed the expected carbon signals of the C=S group and did not indicate the presence of an alternative C=N structure. The carbon signals for C=N would have been expected at higher field (140–165 ppm).Moreover, the independent route to **2a** through reduction of **3a** and, vice versa, the observed formation of the didehydro compounds **3** under mild oxidizing conditions supports the proposed structure of **2**. In contrast to the compounds **2**, in the $^1H$ NMR spectra of the didehydro analogues **3** the presence of the C(3)=N(2) double bond is evident by absence of N(2)H and C(3)H proton signals. Consequently, due to the absence of a C(3) proton at the compounds **3a**–**c**, no coupling with protons of the 3-substituents are observed.



In the carbon NMR spectra of the compounds **3** the signals for the related C(3) atoms, which in contrast to that of the analog **2** are $sp^2$-hybridized, are strongly shifted to low field (>100 ppm), additionally supporting structure **3**. Moreover, the described magnetic non-equivalence of the proton on $C(8)H_2$ and $C(5)H_2$ did not appear in the $^1H$ NMR spectra of **3**.

### 2.3. Biological results

The screening for iNOS inhibitory effects of the title compounds was performed by using the insulin-secreting rat insulinoma cell line RINm5F.[65,66] Human interleukin-1β (IL-1β) and rat interferon-γ (IFN-γ) were added to induce NO production.[59,60] This test system is often used (with variations especially concerning the cytokine concentrations) to investigate the influence of substances on the cytokine mediated iNOS expression and has its origin in diabetes research.[67–70] The choice of this assay was made with respect to our former investigations, which studied the prevention of NO mediated β-cell damage as a possible approach to treatment of autoimmune insulin-dependent type-1 diabetes.[46] At that time the very similar RIN5AH cell line was used for these studies. In preparation to the screening experiments, the optimization of the NO production of the RIN5F cells was investigated by varying cytokine concentrations and incubation time pre and post cytokine addition (data not shown).

The efficacy of the substances was determined by the inhibitory potency on iNOS in comparison to aminoguanidine (AG). Possible cytotoxicity was also tested. For selected compounds, that inhibited the NO production in both test concentrations significantly stronger than AG, the potency was quantified by $IC_{50}$ value. All compounds listed in Table 1 and substances **2a**,**c**–**e** were tested. Because of very poor yields, the iNOS inhibitory effect of compound **2b** could not be investigated. Due to the observations of chemical instability, the DMSO stock solutions were freshly made before every run.

Considering the solubility of the majority of the test compounds, 39 and 78 μM were chosen as the test concentration (giving ca. 20% inhibition for the reference inhibitor AG). Additionally, there is still a large fraction of uninhibited NO production with either 39 or 78 μM AG, thus these concentrations serve to compare the possible inhibitory effect of our newly designed substances relative to AG.

**Table 2**
$IC_{50}$ values of selected compounds **2**

| Compound | $IC_{50}$ [μM][a] |
|---|---|
| AG | 132.01 ± 14.96 |
| **2d** | 29.89 ± 3.72 |
| **2e** | 9.81 ± 2.12 |
| **2g** | 60.26 ± 20.64 |
| **2i** | 53.23 ± 13.78 |
| **2j** | 62.97 ± 13.14 |
| **2l** | 57.59 ± 17.94 |
| **2m** | 24.16 ± 2.74 |
| **2n** | 16.20 ± 2.90 |
| **2o** | 13.20 ± 3.94 |
| **2p** | 70.03 ± 8.36 |
| **2t** | 40.19 ± 5.07 |
| **2u** | 101.08 ± 18.37 |

[a] $n = 6$ for compounds **2**; $n = 7$ for AG; mean ± SD.

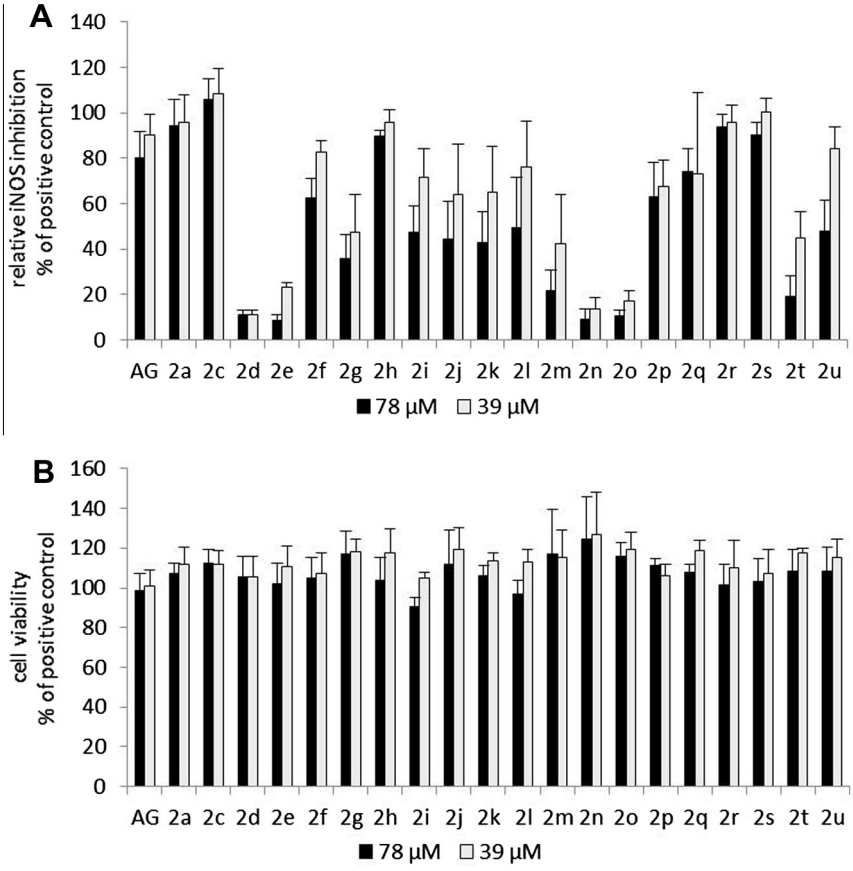



**Figure 2.** Concentration-dependent inhibition of iNOS through aminoguanidine and thiones **2** (**A**). NO production was determined by using griess reaction. Cell viability was tested on substance treated RINm5F cells using MTT assay (B). Both data sets are displayed as percent of positive control (RIN cells treated with IL and IFN but without substance). Aminoguanidine (AG) as the reference inhibitor was tested in every concentration as well. Mean ± SD; $n = 5–8$ (>30 for AG, as control on every plate).

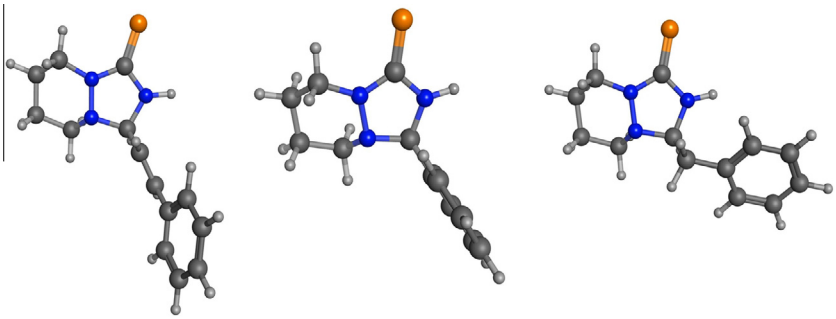


**Figure 3.** 3D structures of compounds **2d**, **2f**, **2c** (in order of decreasing activity towards iNOS from left to right).

None of the compounds shown in Figure 2 had a noticeable cytotoxic effect on RINm5F cells. The aliphatic or araliphatic substituted compounds **2a** and **2c** showed nearly no effect on NO production whereas all other tested substances had at least some impact on the nitrite level or were even more active than AG. Twelve substances were selected for $IC_{50}$ determination together with AG, again for comparison (Table 2).

The *p*-dimethylaminophenyl substituted derivative **2u** showed a slightly lower $IC_{50}$ value than AG whereas most of the other compound showed ca. two- to three-fold better activity compared to AG. The nitro containing **2e**, **2m**, **2n** and **2o** were the most potent compounds tested with an $IC_{50}$ value of 10 to 24 μM. In fact, the inhibitory efficiency increased from *o*- over *m*- to *p*-substitution. Compound **2e**, which has an ethenylene spacer between the phenyl ring and the bicyclic base structure, showed the best results and possessed an $IC_{50}$ value that was about tenfold lower than that of AG.

The oxidized products **3** were not yet tested by themselves because they were obtained only occasionally and incidentally. However, some observations, made during the biological tests, indicate an inactivity of these substances. Although the DMSO stock solutions for the each screening run were freshly prepared, they were stored to monitor for instability in solution. For almost every compound in group **2**, a precipitate was observed after several days as mentioned above (period of time to first signs varied from 7 days for, e.g., **2d** and **2e** to several weeks). Thus the activity of the aged stocked solutions were retested after dissolving the precipitate; and indeed the $IC_{50}$ values for the aged solutions were greater than the freshly prepared ones. For example, after only 2 weeks of storage the $IC_{50}$ of **2e** increased so much that it could no longer be determined. For **2l** comparable results were observed, and for this compound the formation of the oxidation product **3e** could be confirmed. Therefore, it can be assumed that the **3** representing the didehydro products of the related **2** show little or no biologically activity towards the iNOS under comparable conditions.

Resulting from the primary and secondary screening, the following structure activity relationships were concurred: firstly, a phenyl moiety in position 3 appears important for an inhibitory effect on iNOS but not all aromatic structures are active, as in the case of **2c** with a benzyl group. The higher conformational restriction of the $sp^2$ hybridized ethenylene spacer of **2e** in comparison to the methylene group of **2c** appears advantageous. The methylene group allows the phenyl ring in **2e** to only to rotate around the axis and not to alter its position relative to the heterocycle as it is possible in **2c**. The same applies to **2f**, which is slightly more active than **2c**. The additional activity of **2e** could be explained with the insertion of a spacer (Fig. 3).

Thus, it can be postulated that a substance of type **2** with an inhibitory activity towards iNOS is characterized by a phenyl ring in position 3. A spacer group between heterocyclic and phenyl ring is useful. Additionally, a nitro substitution on the phenyl ring is also advantageous given the observation that these derivatives were the most effective inhibitors.

## 3. Conclusions

A variety of 3-substituted [1,2,4]triazolo[1,2-*a*]pyridazine-1-thiones has been successfully prepared and tested as inhibitors of inducible nitric oxide. A number of the tested compounds showed activity and the $IC_{50}$ value of 9.8 ± 2.1 μM for the most active phenylethenyl derivative **2d** demonstrates the usefulness of this substance class as iNOS inhibitors. Although much more potent substances have been found in other classes of compounds, the high ligand efficiency and the novelty of this class offers excellent chances for development. Of course, further investigations concerning the selectivity of the substances towards the constitutively expressed eNOS and nNOS are still required. The presented biological investigations are to be understood as a proof of concept for the adequacy of this new substance class for iNOS inhibition.

## 4. Experimental

### 4.1. General

The reported melting temperatures were determined on a Kofler–Boëtius apparatus type PHMK 81/3035 (VEB Wägetechnik Rapido) and are uncorrected. Elemental analyses were done with the 2400 CHN Elemental Analyzer (Perkin–Elmer). For the microwave assisted syntheses the Discover LabMate with the IntelliVent™ Pressure Control System, circular, single-mode-self-tuning-system, frequency 2.45 GHz, and CEM's SynergyT software (CEM Kamp-Lintfort) was used. The spectra were recorded with the following instruments and conditions: IR spectra: FT-IR 1600 (Perkin–Elmer), transmission technique (KBr pressed disks) and for the compounds **2a**, **5a**, **3a**,**d**,**e** only IR 200 FT-IR (Thermo Electron Corporation Nicolet), ATR technique (diamond).; $^1H$, $^1H$, $^1H$ COSY, DEPT-135, $^{13}C$ NMR spectra: AVANCE DPX 200 and for compounds **2a**, **5a**, **3a**,**d**,**e** only the above named and additionally HSQC-und HMBC spectra: FT-NMR-Spektrometer Avance III™ 400 (both spectrometers from Bruker Analytische Messtechnik GmbH); temperature 25 °C, solvents used are specified in the data of the related compound; internal standard tetramethylsilane; chemical

shift $\delta$ in ppm. Mass spectra: EI for **2f**, **g**, **h**, **i**, **j**, **k**, **m**, **n**, **o**, **p**, **q**, **r**, **s**, **u**; **6**: M 40 AMD (Intectra GmbH), electron impact, energy 70 eV, (with the exception of the molecular ion peaks normally only peaks >10% are listed); ESI for **2a**, **e**, **t**, **5a**, **3d**, **e**: Shimadzu High Performance Liquid Chromatograph/Mass Spectrometer LCMS-IT-TOF with the system characterized as following: solvent delivery module LC-20AD Prominence, autosampler SIL-20AC HT Prominence, column oven CTO-20A Prominence, system controller CBM-20A Prominence, UV/vis photodiode array detector SPD-M20A Prominence, evaporative light scattering detector ESD-LT II, spectrometric detector RF-10A XL, LCMS-IT-TOF workstation, software LCMS solution Version 3.41; column Chromolith® SpeedRod RP-18 endcapped, 50 mm; mobile phases (m. ph.) given in the data of the compounds:

m. ph. VI: acetonitrile/water 7:3 (0.1% HCOOH in $H_2O$) (v/v), flow rate 0.2 mL $min^{-1}$ (compound **2t**);
m. ph. VII: methanol/water 1:1 (0.1% HCOOH in $H_2O$) (v/v), flow rate: 0.4 mL $min^{-1}$ (compound **2a**);
m. ph. XI: methanol/water 6:4 (0.1% HCOOH) (v/v), flow rate: 0.4 mL $min^{-1}$ (compounds **2e**, **5a**).

*ESI for compounds **2f**, **3a**:* Bruker Daltonics MicroOTOF-LC (ESI-TOF); external calibration (Agilent ESI TuneMix); mobile phase (m. ph. VIII): gradient propan-2-ol/water 20% Ba' 50% B; flow rate: 0.3 mL $min^{-1}$; column Zorbax RP-C18; 2.1 × 30 mm; 3.5 μm. The gas chromatographic investigations were done for compounds **2c**, **f**, **l** with a FISON Instruments GC 8065 coupled with a mass spectrometric detector MD 800 (70 eV); column: Macherey–Nagel MN FS Hydrodex β-PM (25 m × 0.25 mm); column system pressure: 10 psi (70 kP); column temperature 220 °C. For TLC aluminum foil covered with silica gel 60 $F_{254}$ (Merck) was utilized as stationary phase. The running distance for the front of the mobile phase was 6.5 cm. The following mixtures were used as mobile phases:

m. ph. I: *n*-hexane/ethyl acetate 1:1 (v/v);
m. ph. II: *n*-hexane/ethyl acetate 2:8 (v/v);
m. ph. III: dichloromethane/acetone 1:1 (v/v);
m. ph. IX: cyclohexane/ethyl acetate 1:1 (v/v);
m. ph. XIII: water/acetic acid/*n*-propanol 1:2:8 (v/v)

Each mobile phase (m. ph.) used is specified in the data of the related compound. The substances were detected with UV radiation ($\lambda$ = 254 nm) or munier spray reagent[71] and iodine azide reagent by awe.[72]

The HPLC investigations were done under the following conditions:

*System:* LaChrom (Merck Hitachi) consisting of pump L-7100, autosampler L-7200, column thermostat L-7350, solvent degasser L-7612, interface D-7000, and diode array detector L-7450 ($\lambda$ = 220 or 240 nm);

*Stationary phase:* RP columns (given in the HPLC data of the compounds); the dead time $t_0$ was determined with uracil; each mobile phase (m. ph.) is given in the data for the related compound:

m. ph. IV: acetonitrile/0.02 M $KH_2PO_4$ (pH 2.85) 1:1 (v/v);
m. ph. V: acetonitrile/0.02 M $KH_2PO_4$ (pH 2.85) 3:7 (v/v);
m. ph. X: acetonitrile/water 4:6 (v/v);
m. ph. XII: acetonitrile/0.02 M $KH_2PO_4$ (pH 2.85) 4:6 (v/v)

The values for the dead time $t_0$ and the retention times $t_R$ are given in minutes.

## 4.2. Chemistry

### 4.2.1. Correction

Unfortunately, the signal assignment in the $^1$H NMR of the hexahydropyridazine hydrochloride **4** (starting substance for the synthesis of the hexahydropyridazin-1-carbothioamides **1**, Scheme 2) in a previous publication[17] was partly incorrect. With a new and more powerful NMR instrument the detection of a very broad singlet at 5.87 ppm was found for this substance. Further investigations revealed that this signal should be assigned to the NH. The signal at 10.26 ppm corresponds to the two protons on the quaternary nitrogen.

*Correction of the signal assignment in $^1$HNMR:* $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 1.67 (s, 4H, –C(4)H2– and -C(5)H2–); 2.99 (s, 4H, –C(3)H2– and –C(6)H2–); 5.87 (very broad and flat singlet, 1H, NH); 10.26 (very broad and flat singlet, 2H, $NH_2^+$).

**4.2.1.1. Hexahydropyridazine-1-carbothioamides 1 and 5.** *4.2.1.1.1. Hexahydropyridazine-1-carbothioamides **1a**, **1d**.* The syntheses and the experimental data were described before.[17]

*4.2.1.1.2. N-(2-Phenylethenyl)-1,2,3,4,5,6-hexahydropyridazine-1-carbothioamide **1c**.* A mixture of hexahydropyridazine-1-carbothioamide **1a** (0.7 mmol, 0.102 g)) and phenylacetaldehyde (0.7 mmol, 0.841 g) in dichloromethane (3 mL) was warmed at 60 °C for 5 min. After the solvent was removed, the resulting residue was taken up in a minimum of acetone. The product crystallized when the solution was evaporated off. The formed crystals were collected, washed with a little methanol and dried in vacuo. Yield 17%. Colorless crystals. Mp. 136–141 °C (acetone, mp of the hemihydrate).

IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1168 (C=S); 1435, 1445 (–CH–); 1474 (S=C–N–); 1582, 1645 (–CH=CH–); 2854, 2924, 2953, 2964 (–CH–); 3052, 3075, 3134, 3254 (–NH–). $^1$H NMR ($CDCl_3$, ppm): $\delta$ = 1.58–1.93 (m, 4H, C(4)$H_2$/C(5)$H_2$); 3.20–3.47 (m, 3H, C(3)H/C(6)$H_2$); 5.22 (d, 1H, $^3J$ = 14 Hz, C(3)H); 5.63 (d, 1H, $^3J$ = 16 Hz; –C*H*=CH-ar); 6.09 (br s, 1H, –NH–); 6.45 (d, 1H; $^3J$ = 14 Hz, –CH=C*H*-ar); 6.81 (br s, 1H, –NH–); 7.13–7.32 (m, 5H, arom. ring). $^{13}$C NMR ($CDCl_3$, ppm): $\delta$ = 21.43 (C5); 23.75 (C4); 42.30 (C3); 52.28 (C6); 108.64 (–CH=*C*H-ar); 125.01 (2C, arom. C2 u. C6); 125.96 (arom. C4); 128.73 (2C, arom. C3 u. C5); 132.94 (C1 ar); 136.39 (–*C*H=CH-ar); 181.55 (C(1)=S). Elemental analysis [$C_{13}H_{17}N_3S$. 0.5 $H_2O$ (256.4), %]: Calcd C, 60.91; H, 7.08; N, 16.39. Found C, 60.64; H, 5.90; N, 14.85.

*4.2.1.1.3. 2-Acetyl-1,2,3,4,5,6-hexahydropyridazine-1-carbothioamide **5a**.* A solution of hexahydropyridazin-1-carbothioamide **1a** (1 mmol, 0.145 g) in acetic anhydride (2 mL) was stored at room temperature for 3 weeks. The solution was evaporated to dryness and the crystals formed were collected and washed with a little ethanol. Yield 70%. Colorless needles. Mp 196–200 °C (methanol).

TLC (m. ph. III): $R_f$ = 0.71. **IR** ($cm^{-1}$): $\tilde{\nu}$ = 1159 (C=S); 1457; 1627 ($NH_2$); 1658 (C=O); 2943, 2971, 2998 ($CH_2$, $CH_3$); 3158; 3264, 3306 ($NH_2$). $^1$H NMR ($CDCl_3$, ppm): $\delta$ = 1.73 (m, 3H, C(4)$H_2$ u. C(5)H); 1.88 (m, 1H, C(5)H); 2.81 (dt, 1H, $^3J$ = 4 Hz, $^3J$ = 12 Hz, C(3)H); 3.01 (dt, 1H, $^3J$ = 4 Hz, $^3J$ = 12 Hz, C(6)H); 4.48 (d, 1H, $^3J$ = 16 Hz, C(3)H); 5.41 (d, 1H, $^3J$ = 16 Hz, C(6)H); 6.50 (br s, 1H, NH); 6.79 (br s, 1H, NH). $^{13}$C NMR ($CDCl_3$, ppm): $\delta$ = 20.5 (C3); 22.9 (C4); 23.0 (C5); 43.1 (C3); 49.9 (C6); 173,6 (C=O); 182.8 (C=S). HRMS [(ESI) *m*/*z*]: calcd for $C_7H_{13}N_3OS+H^+$: 188.0852; found [M+$H^+$]: 188.0857. Elemental analysis [$C_7H_{13}N_3OS$ (187.3),%]: Calcd C, 44.90; H, 7.00; N, 22.44. Found: C, 44.89; H, 6.90; N, 22.85.

*N-[(2-Acetyl-1,2,3,4,5,6-hexahydropyridazine-1-yl)thiocarbonyl] ethanamide **5b**. Method 1–from 4 and acetyl isothiocyanate:* To a mixture of dichloromethane (10 mL), hexahydropyridazine-hydrochloride **4** (2 mmol, 0.245 g), and triethylamine (2 mmol, 0.202 g) a

solution of acetyl isothiocyanate (2 mmol, 0.202 g) in dichloromethane (5 mL) was added dropwise within 10 min with continuous stirring. The mixture was stirred further 20 min. Subsequently, the solvent was removed, the residue was crystallized from acetone, and dried. Yield: 45%. Colorless crystals. Mp 139–143 °C (acetone).

TLC (m. ph. I): $R_f$ = 0.34. HPLC (m. ph. XII): RP-select B, $t_0$ = 1.95; $t_R$ = 2.97; $k'$ = 0.52. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1186 (C=S); 1523 (NH); 1647 (NH-*CO*$CH_3$), 1724 (N(2)-*CO*-$CH_3$); 2496, 2623, 2856, 2946, 2978, 3017 ($CH_2$, $CH_3$); 3179 (NH). $^1$H NMR ($CDCl_3$, ppm): $\delta$ = 1.76 (m, 4H, C(4)$H_2$ u. C(5)$H_2$); 2.04 (s, 3H, N(2)-CO*$CH_3$*); 2.64 (s, 3H, CS-NH-CO-*$CH_3$*); 2.79 (br s, 1H, C(3)H); 3.95 (m, 1H, C(6)H); 4.53 (d, 1H, $^3J$ = 7 Hz, C(3)H); 5.54 (d, 1H, $^3J$ = 14 Hz, C(6)H); 8.92 (s, 1H, NH). $^{13}$C NMR ($CDCl_3$, ppm): $\delta$ = 20.84 (–N(2)-CO-*C*$H_3$); 22.79 (C4); 22.98 (–CS-NH-CO-*C*$H_3$); 26.55 (C5); 44.86 (C3); 49.20 (C6); 172.45 (C=O); 173.88 (C=O); 178.12 (C=S). MS (70 eV, 200 °C) *m*/*z* (%): 228.8 (22) [$M^+$]; 186.8 (2) [$M^+$–$H_2C$=C=O]; 128.0 (33) [$M^+$–$CH_3$CO-N=C=S]; 127.0 (10); 111.1 (12); 86.0 (31); 85.0 (96) [$C_4H_9N_2^+$]; 70.9 (26); 57.4 (17); 56.4 (31); 43.1 (100) [$CH_3CO^+$]; 30.1 (42); 28.0 (18). Elemental analysis [$C_9H_{15}N_3O_2S$ (229.3), %]: Calcd C, 47.14; H, 6.59; N, 18.33. Found: C, 47.07; H, 6.61; N, 18.08.

*Method 2–from hexahydropyridazine-1-carbothioamide* ***1a*** *and acetic anhydride:* A mixture of hexahydropyridazine-1-carbothioamide **1a** (1 mmol, 0.145 g) and acetic anhydride (1 mL) was refluxed for 10 min. Subsequently, methanol (2 mL) was added and then the solvent removed in vacuo. The remaining crystals were washed with ethanol and dried. Yield: 10%.

#### 4.2.1.2. 2,3,5,6,7,8-Hexahydro-1*H*-[1,2,4]triazolo-[1,2-*a*]pyridazine-1-thiones 2.

*4.2.1.2.1. 3-Methyl-2,3,5,6,7,8-hexahydro-1**H**-[1,2,4]-triazolo-[1,2-a]-pyridazine-1-thione* ***2a****.* A mixture of propan-2-ol (8 mL), 3-methyl-5,6,7,8-tetrahydro-1*H*-[1,2,4]triazolo [1,2-*a*]pyridazine-1-thione **3a** (2 mmol, 0.338 g), and sodium tetrahydroborate (10 mmol, 0.757 g) was stirred at room temperature for 24 h. Subsequently, water (10 mL) was added and the stirring was continued until the batch became clear. The solution was then extracted three times with dichloromethane (12 mL), the organic phase was dried with sodium sulphate and evaporated to dryness. In order to remove the non-reacted compound **3a**, the resulting residue was purified by column chromatography (Ø 1.5 cm, silica gel H Merck, mobile phase ethyl acetate). Yield: 17%. Colorless crystals. Mp 118–123 °C (ethyl acetate).

TLC (m. ph. III): $R_f$ = 0.79. IR ($cm^{-1}$): $\tilde{\nu}$ = 1184 (C=S); 1486 (NH); 2849, 2940 u. 2972 (CH); 3175 (NH). $^1$H NMR ($CDCl_3$, ppm): $\delta$ = 1.33 (d, $^3J$ = 6 Hz, 3H, C(3)$CH_3$); 1.61 (m, 1H, C(7)H); 1.70–1.83 (m, 3H, C(6)$H_2$ u. C(7)H); 2.66–2.72 (m, 1H, C(5)H); 2.87–2.90 (m, 1H, C(5)H); 3.11 (br s, 1H, C(8)H); 4.39 (br s, 2H, C(3)H + C(8)H); 6.83 (br s, 1H, N(2)H). $^{13}$C NMR ($CDCl_3$, ppm): $\delta$ = 19.7 (C(3)$CH_3$); 23.4 (C6); 23.4 (C7); 45.1 (C8); 53.5 (C5); 74.6 (C3); 176.1 (C(1)=S). HRMS [(ESI) *m*/*z*]: Calcd for [$C_7H_{13}N_3S$+H]$^+$: 172.0903. Found for [M+H]$^+$: 172.0900.

*4.2.1.2.2. 3-Ethyl-2,3,5,6,7,8-hexahydro-1H-[1,2,4]triazolo[1,2-a]pyrid-azine-1-thione* ***2b****.* A mixture of hexahydropyridazine-1-carbothioamide **1a** (1 mmol, 0.145 g), dichloromethane (5 mL), freshly distilled propionaldehyde (2 mmol, 0.116 g), and *p*-toluenesulfonic acid monohydrate (0.05 mmol, 0.010 g) was refluxed for 9 h. After complete evaporation the residue was dried in vacuo. Then a column chromatographic purification was done (mobile phase petroleum ether/ethyl acetate 2:1 (v/v), column length 10 cm, ∅ = 1 cm) and the isolated crystals were dried in vacuo. Yield 15%. Colorless crystals. Mp 115–118 °C [petroleum ether/ethyl acetate 2:1 (v/v)].

TLC (m. ph. II): $R_f$ = 0.70. HPLC (m. ph. V): RP-select B, $t_0$ = 2.11 min; $t_R$ = 6.48 min; $k'$ = 2.07. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1184 (C=S); 2832, 2854, 2926, 2946 (–$CH_2$–, $CH_3$–); 3170 (–NH–). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 0.85 (m, 3H, (–$CH_3$)); 1.61 (m, 7H, C(6)$H_2$ u. C(7)$H_2$ u. C(5)$H_2$ u. C(8)H); 2.84 (m; 2H C(3)-*C*$H_2$–); 4.16 (m, 2H, C(3)H u. C(8)H); 8.92 (s; 1H; –NH). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 8.33 (C(3)-$CH_2$-*C*$H_3$); 23.12 (C6); 23.19 (C7); 26.02 (C(3)-*C*$H_2$–); 44.02 (C8); 53.11 (C5); 78.59 (C3); 175.77 (C(1)=S). HRMS (ESI) *m*/*z* (Intens. 100): 369.185336 [2M+H]$^+$. Elemental analysis [$C_8H_{15}N_3S$ (185.3), %]: Calcd C, 51.86; H, 8.16; N, 22.68; S, 17.30. Found: C, 52.20; H, 8.21; N, 22.83; S, 17.42.

*4.2.1.2.3. 3-Phenylmethyl-2,3,5,6,7,8-hexahydro-1H-[1,2,4]triazolo[1,2-a]-pyridazine-1-thione* ***2c****.* Hexahydropyridazine-1-carbothioamide **1a** (1 mmol, 0.145 g), phenylacetaldehyde (1 mmol, 0.120 g), and *p*-toluenesulfonic acid monohydrate (0.033 mmol, 0.006 g) were completely mixed. Subsequently, the mixture was heated between 125 and 130 °C for 4, 5 h. The cooled melt was taken up in hot acetone and the product crystallized by careful removal of the solvent. The resulting crystals were filtered off, washed twice with a little methanol and dried in vacuo. Yield: 46%. Colorless crystals. Mp 133.5–137.5 °C (acetone).

TLC (m. ph. I): $R_f$ = 0.72. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1180 (C=S); 1434, 1457 (–CH–);1481 (S=C–N–); 2846, 2943 (–CH–); 3180 (–NH–). $^1$H NMR ($CDCl_3$, ppm): $\delta$ = 1.61–1.87 (m, 4H, C(6)$H_2$/C(7)$H_2$); 2.73–3,00 (m, 4H, –C(α)$H_2$-/C(8)$H_2$); 3.20 (br t, 1H, C(5)H); 4.38–4.49 (m, 2H, C(3)H/C(5)H); 6.00 (s, 1H, –NH–); 7.19–7.38 (m, 5H, arom.). $^{13}$C NMR ($CDCl_3$, ppm): $\delta$ = 23.47 and 23.54 (2C, C6 + C7); 40.40 (–$CH_2$–); 44.99 (C8); 53.85 (C5); 79.07 (C3); 127.25 (arom. C4); 128.91 (2C, arom. C2 + C6); 129.46 (2C, arom. C3 + C5); 135.29 (arom. C1); 176.96 (C=S). GC/MS (220 °C): *m*/*z* (%): 247.5 (12) [$M^+$]; 173.2 (13) [$M^+$−$CH_2NS$]; 156.0 (17) [$M^+$−$C_6H_5$]; 148.1 (10) [$M^+$−$C_4H_9N_2$]; 85.3 (100) [$C_4H_9N_2^+$]; 77.2 (14). Elemental analysis [$C_{13}H_{17}N_3S$ (247.4), %]: Calcd C, 63.12; H, 6.93; N, 16.99. Found: C, 61.32; H, 6.61; N, 16.71.

*4.2.1.2.4. 3-(2-Phenylethenyl)-2,3,5,6,7,8-hexahydro-1H-[1,2,4]triazolo-[1,2-a]pyridazine-1-thione* ***2d****.* A solution of hexahydropyridazine-1-carbothioamide **1a** (0.5 mmol, 0.073 g) and of cinnamaldehyde (0.5 mmol, 0.066 g) in dichloromethane (2.5 mL) were allowed to stand at room temperature for 15 h. Subsequently, the solvent was removed and the resulting residue was treated with a little acetone. The resulting solid was recrystallized with a minimum of methanol and the formed crystals were collected, washed with a little methanol, and dried in vacuo. By concentration of the methanolic mother liquor additional amounts of compound **2d** were obtained. Yield: 49%. Yellowish crystals. Mp 144–146 °C (methanol).

TLC (m. ph. I): $R_f$ = 0.62. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1183 (C=S); 1433, 1452 (–CH–); 1477 (S=C–N–); 1657 (C=C); 2849, 2924, 2946 (–CH–); 3133 (–NH–). $^1$H NMR ($CDCl_3$, ppm): $\delta$ = 1.57–1.84 (m, 4H, C(6)$H_2$/C(7)$H_2$); 2.63–2.73 (m, 1H, C(5)H); 2.99–3.04 (m, 1H, C(5)H); 3.19 (br s, 1H, C(8)H); 4.36 (br d, 1H, $^3J$ = 8 Hz, C(8)H); 4.80 (d, 1H, $^3J_{C(3)H\text{-}C(\alpha)H}$ = 8 Hz, C(3)H); 6.16 (dd, 1H, $^3J_{C(\alpha)H\text{-}C(3)H}$ = 8 Hz, $^3J_{C(\alpha)H\text{-}C(\beta)H}$ = 16 Hz, –C(α)*H*=C(β)*H*-ar); 6.39 (s, 1H, –NH–); 6.68 (d, 1H, $^3J_{C(\beta)H\text{-}C(\alpha)H}$ = 16 Hz, –C(α)H=C(β)*H*-$C_6H_5$); 7.29–7.39 (m, 5H, arom.). $^{13}$C NMR ($CDCl_3$, ppm): $\delta$ = 23.20 and 23.24 (2C, C6 + C7); 45.52 (C8); 52.67 (C5); 80.00 (C3); 123.54 (–*C*H=CH-ar); 127.01 (arom. C4); 128.74 (2 C, arom. C3 + C5); 128.80 (2C, arom. C2 + C6); 135.26 (arom. C1); 135.91 (–CH=*C*H-ar); 176.87 (C=S). Elemental analysis [$C_{14}H_{17}N_3S$ (259.4), %]: Calcd C, 64.83; H, 6.61; N, 16.20. Found: C, 64.25; H, 6.73; N, 15.82.

*4.2.1.2.5. 3-[2-(2-Nitrophenyl)ethenyl]-2,3,5,6,7,8-hexahydro-1H-[1,2,4]-triazolo[1,2-a]pyridazine-1-thione* ***2e****. Variant 1–preparation as the main product:* Hexahydropyridazine-1-carbothioamide **1a** (0.3 mmol, 0.044 g), 2′-nitrocinnamaldehyde (0.33 mmol, 0.058 g), and *p*-toluenesulfonic acid monohydrate (0.03 mmol, 0.006 g) were intensively powdered together. Then the mixture was heated to between 100 °C and 105 °C for 20 min. After the mixture was cooled, the solidified product was suspended in hot acetone. The crystals that formed were collected by suction

filtration, washed with a little methanol and dried in vacuo. Yield: 61%. Yellow-brown crystals. Mp 163–166.5 °C (acetone).

TLC (m. ph. I): $R_f$ = 0.45. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1187 (C=S); 1433, 1453 (–CH–); 1491 (S=C–N–); 1521 ($–NO_2$); 1606 (C=C); 2850, 2949 (–CH–); 3183 (–NH–). $^1$H NMR ($CDCl_3$, ppm): $\delta$ = 1.66–1.89 (m, 4H, C(6)$H_2$/C(7)$H_2$); 2.70–2.82 (m, 1H, C(5)H); 3.02–3.19 (m, 2H, C(5)H/C(8)H); 4.43 (br d, 1H, $^3J$ = 16 Hz, C(8)H); 4.85 (d, 1H, $^3J_{C(3)H\text{-}C(\alpha)H}$ = 8 Hz, C(3)H); 6.12 (s, 1H, –NH); 6.13 (dd, 1H, $^3J_{C(\alpha)H\text{-}C(3)H}$ = 8 Hz, $^3J_{C(\alpha)H\text{-}C(\beta)H}$ = 16 Hz, –C(α)*H*=C(β)H-ar); 7.22 (d, 1H, $^3J_{C(\beta)H\text{-}C(\alpha)H}$ = 16 Hz, –C(α)H=C(β)*H*-ar); 7.43–7.52 (m, 1H, arom. C(4)H); 7.60 (m, 2H, arom. C(5)H + C(6)H); 8.00 (d, 1H, $^3J$ = 6 Hz, arom. C(3)H). $^{13}$C NMR ($CDCl_3$, ppm): $\delta$ = 23.18 and 23.37 (2 C, C6 u. C7); 45.53 (C8); 52.93 (C5); 79.37 (C3); 124.80 (2 C, –*C*H=CH-ar, arom. C3); 128.91 (arom. C4); 129.09 (arom. C6); 129.24 (arom. C5); 131.26 (arom. C1); 131.33 (–CH=*C*H-ar); 133.46 (arom. C2); 176.68 (C=S)). HRMS [(ESI) *m*/*z*]: Calcd for $[C_{14}H_{16}N_4O_2S+H]^+$: 305,1067. Found for $[M+H]^+$: 305,1072. Elemental analysis [$C_{14}H_{16}N_4O_2S$ (304.4), %]: Calcd C, 55.25; H, 5.30; N, 18.41. Found: C, 55.25; H, 5.26; N, 18.38.

*Variant 2–isolation as by-product:* Hexahydropyridazine-1-carbothioamide **1a** (0.5 mmol, 0.073 g), 2′-nitrocinnamaldehyde (0.55 mmol, 0.097 g), and *p*-toluenesulfonic acid monohydrate (0.035 mmol, 0.007 g) were intensively powdered together. Then the mixture was heated to between 125 and 130 °C for 20 min. After the mixture was cooled, the solidified product was suspended in hot acetone. The formed crystals (compound **3c**) were collected by suction filtration and washed with a little methanol. The filtrate was evaporated to dryness and the residue was taken up in a small volume of absolute ethanol. The crystallization was initiated by friction with a glass stick. Yield 8%. Yellow-brown crystals. Mp 158–160 °C (ethanol).

*4.2.1.2.6. 3-Arylsubstituted 2,3,5,6,7,8-hexahydro-1H-[1,2,4]triazolo-[1,2-a]pyridazine-1-thiones **2f**–**u**. Preparation by heating of 1a with benzaldehydes in a melt–general procedure:* A well prepared mixture of hexahydropyridazine-1-carbothioamide **1a** (1 mmol, 0.145 g), the appropriate aromatic aldehyde (1.1 mmol), and *p*-toluenesulfonic acid monohydrate (0.025 mmol, 0.005 g) was heated between 150 and 155 °C, whereby the course of the reaction was observed using TLC. After the starting compound **1a** was completely reacted the cooled down melt was recrystallized from acetone. The formed crystals were collected, washed with a little methanol and dried in vacuo.

In this manner the following compounds were obtained:

*4.2.1.2.7. 3-Phenyl-2,3,5,6,7,8-hexahydro-1H-[1,2,4]triazolo[1,2-a]pyridazine-1-thione **2f**.* With benzaldehyde (0.117 g). Reaction time 75 min. Yield: 76%. Colorless, bright crystals. Mp 152.5–155 °C (acetone).

TLC (m. ph. I): $R_f$ = 0.64. HPLC (m. ph. IV): RP-Select B, $t_0$ = 1.98; $t_R$ = 4.78; $k'$ = 1.41. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1184 (C=S); 1429, 1458 ($–CH_3$); 1482 (S=C–N–); 2808, 2850, 2919, 2949, 2977 ($–CH_3$); 3040 (aryl-H); 3185 (–NH–). $^1$H NMR ($CDCl_3$, ppm): $\delta$ = 1.56–1.79 (m, 4H; C(6)$H_2$/C(7)$H_2$); 2.59–2.71 (m, 1H, C(5)H); 2.94 (m, 1H, C(5)H); 3.22 (br s, 1H, C(8)H); 4.36 (d, 1H, $^3J$ = 12 Hz, C(8)H); 5.15 (s, 1H, C(3)H); 6.45 (s, 1H, –NH–); 7.36–7.46 (m, 5H, arom.). $^{13}$C NMR ($CDCl_3$, ppm): $\delta$ = 23.21 and 23.23 (2C, C6 + C7); 45.53 (C8); 52.91 (C5); 80.86 (C3); 127.53 (arom. C4); 128.76 (2C, arom. C2 + C6); 129.80 (2 C, arom. C3 u. C5); 136.26 (arom. C1); 176.60 (C=S). GC/MS (220 °C): *m*/*z* (%): 233.2 (31) [$M^+$]; 173.2 (13) [$M^+$–$CH_2NS$]; 156.0 (17) [$M^+$–$C_6H_5$]; 148.1 (10) [$M^+$-$C_4H_9N_2^·$]; 85.3 (100) [$C_4H_9N_2^+$]; 77.2 (14). MS (70 eV, 25 °C) *m*/*z* (%): 232.8 (76) [$M^+$]; 184.9 (98); 169.9 (100); 155.9 (10) [$M^+$–C6H5]; 84.9 (88) [C4H9N2$^+$]; 70.8 (15); 27.9 (37). Elemental analysis [$C_{12}H_{15}N_3S$ (233.3), %]: Calcd C, 61.77; H, 6.48; N, 18.01. Found: C, 61.61; H, 6.23; N, 17.93.

*4.2.1.2.8. 3-(2-Methylphenyl)-2,3,5,6,7,8-hexahydro-1H-[1,2,4]triazolo-[1,2-a]pyridazine-1-thione **2g**.* The synthesis differed from the general method by using 1.5 mmol *o*-methylbenzaldehyde (0.180 g). Reaction time 30 min. Yield 74%. Colorless crystals. Mp 201–206 °C (acetone).

TLC (m. ph. I): $R_f$ = 0.69. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1185 (C=S); 1452 ($–CH_3$); 1478 (S=C–N–); 2933, 2951 ($–CH_3$); 3175 (–NH–). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 1.34–1.49 and 1.57–1.72 (m + m, 4H, C(6)$H_2$/C(7)$H_2$); 2.34 (s, 3H, $CH_3$); 2.50 (m, 1H, C(5)H → beneath solvent signal); 2.61–3.02 (m, 2H, C(5)H/C(8)H); 4.18–4.23 (m, 1H, C(8)H); 5.48 (s, 1H, C(3)H); 7.18–7.30 (m, 4H, arom.); 9.32 (s, 1H, –NH–). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 19.16 (arom. *C*$H_3$); 23.56 and 23.72 (2C, C6 u. C7); 44.79 (C8); 53.05 (C5); 76.58 (C3); 126.22 and 126.78 (2C, arom. C3 u. C4), 128.95 (arom. C6); 131.05 (arom. C5); 135.86 (arom. C1); 137.06 (arom. C2); 177.27 (C=S). MS (70 eV, 205 °C): *m*/*z* (%): 247.1 (100) [$M^+$]; 187.0 (12) [$M^+$–$CH_2NS$]; 162.0 (11) [$M^+$-$C_4H_9N_2$]; 156.0 (25) [$M^+$–$C_6H_4$–$CH_3$]; 118.0 (10); 91.0 (11); 85.1 (98) [$C_4H_9N_2^+$]; 77.3 (10); 56.3 (23); 41.0 (12); 30.1 (35); 28.1 (10). Elemental analysis [$C_{13}H_{17}N_3S$ (247.3), %]: Calcd C, 63.12; H, 6.93; N, 16.99. Found: C, 62.36; H, 6.81; N, 17.02.

*4.2.1.2.9. 3-(3-Methylphenyl)-2,3,5,6,7,8-hexahydro-1H-[1,2,4]triazolo-[1,2-a]pyridazine-1-thione **2h**.* The synthesis differed from the general method by using 1.5 mmol *m*-methylbenzaldehyde (0.180 g). Reaction time 25 min. Yield 68%. Colorless crystals. Mp 175–177 °C (acetone).

TLC (m. ph. I): $R_f$ = 0.66. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1183 (C=S); 1453 ($–CH_3$); 1474 (S=C–N–); 2930, 2946 ($–CH_3$); 3168 (–NH–). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 1.38–1.50 and 1.58–1.76 (m + m, 4H, C(6)$H_2$/C(7)$H_2$); 2.31 (s, 3H, $CH_3$); 2.49–2.63 (m, 1H, C(8)H → beneath solvent signal); 2.83–2.98 (m, 2H, C(5)$H_2$); 4.18 (d, 1H, $^3J$ = 14 Hz, C(8)H); 5.18 (s, 1H, C(3)H); 7.16–7.32 (m, 4H, arom.); 9.31 (s, 1H, –NH–). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 19.16 (arom. *C*$H_3$); 23.56 and 23.72 (2C, C6 + C7); 44.79 (C8); 53.05 (C5); 76.58 (C3); 126.22 (arom. C6); 126.78 (arom. C4); 128.95 (arom. C5); 131.05 (arom. C6); 135.86 (arom. C1); 137.06 (arom. C3); 177.27 (C=S). MS (70 eV, 180 °C): *m*/*z* (%): 247.1 (100) [$M^+$]; 187.0 (20) [$M^+$–$CH_2NS$]; 161.9 (12) [$M^+$-$C_4H_9N_2^·$]; 156.0 (20) [$M^+$–$C_6H_4$-$CH_3$]; 117.9 (16); 91.0 (10); 85.0 (82) [$C_4H_9N_2^+$]; 56.4 (16); 41.0 (10); 30.0 (26). Elemental analysis [$C_{13}H_{17}N_3S$ (247.3), %]: Calcd C, 63.12; H, 6.93; N, 16.99. Found: C, 62.97; H, 7.09; N, 17.05.

*4.2.1.2.10. 3-(4-Methylphenyl)-2,3,5,6,7,8-hexahydro-1H-[1,2,4]triazolo-[1,2-a]pyridazine-1-thione **2i**.* The synthesis differed from the general method by using 1.5 mmol *p*-methylbenzaldehyde (0.180 g). Reaction time 25 min. Yield 54%. Colorless crystals. Mp 160.5–164 °C (acetone).

TLC (m. ph. I): $R_f$ = 0.68. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1181 (C=S); 1438 ($–CH_3$); 1469 (S=C–N–); 2926, 2958 ($–CH_3$); 3164 (NH–). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 1.38–1.72 (m, 4H, C(6)$H_2$/C(7)$H_2$); 2.30 (s, 3H, $CH_3$); 2.49–2.61 (m, 1H, C(5)H → beneath solvent signal); 2.78–3.03 (m, 2H, C(5)H); 4.16 (br d, 1H, $^3J$ = 12 Hz, C(8)H); 5.17 (s, 1H, C(3)H); 7.19 (d, 2H, $^3J$ = 8 Hz, arom. C(3)H + C(8)H); 7.28 (d, 2H, $^3J$ = 8 Hz, arom. C(2)H + C(6)H); 9.30 (s, 1H, –NH–). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 21.27 (C7); 23.50 (C6); 40.32 (arom. *C*$H_3$); 45.11 (C8); 52.71 (C5); 79.38 (C3); 127.66 (2C, arom. C2 + C6); 129.37 (2C, arom. C3 + C5); 135.32 (arom. C1); 138.73 (arom. C4); 176.99 (C=S). MS (70 eV, 160 °C): *m*/*z* (%): 247.0 (100) [$M^+$]; 187.0 (20) [$M^+$–$CH_2NS$]; 161.9 (15) [$M^+$-$C_4H_9N_2^·$]; 156.0 (12) [$M^+$–$C_6H_4$-$CH_3$]; 117.9 (13); 104.5 (12); 91.0 (12); 85.0 (96) [$C_4H_9N_2^+$]; 56.3 (20); 41.0 (12); 30.0 (33). Elemental analysis [$C_{13}H_{17}N_3S$ (247.3), %]: Calcd C, 63.12; H, 6.93; N, 16.99. Found: C, 62.18; H, 6.75; N, 17.13.

*4.2.1.2.11. 3-(2-Chlorophenyl)-2,3,5,6,7,8-hexahydro-1H-[1,2,4]triazolo-[1,2-a]pyridazine-1-thione **2j**.* The synthesis differed from the general method by using 1.5 mmol *o*-chlorobenzaldehyde (0.211 g). Reaction time 85 min. Yield 71%. Light beige crystals. Mp 215–217 °C (acetone).

TLC (m. ph. I): $R_f$ = 0.78. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 756 (Ar-Cl); 1081 (–Cl); 1177 (C=S); 1475 (S=C–N–); 2931, 2952 (–CH–), 3161

(–NH–). $^1$H NMR ($CDCl_3$, ppm): $\delta$ = 1.66–1.95 (m, 4H, C(6)$H_2$/C(7)$H_2$); 2.87–3.16 (m, 2H, C(5)H/C(8)H); 3.24 (br t, 1H, C(5)H); 4.49 (d, 1H, $^3J$ = 12 Hz, C(8)H); 5.64 (s, 1H, C(3)H); 6.48 (s, 1H, –NH–); 7.28–7.42 (m, 3H, arom. C(4)H/C(5)H/ C(6)H); 7.55–7.61 (m, 1H, arom. C(3)H). $^{13}$C NMR ($CDCl_3$, ppm): $\delta$ = 23.28 and 23.63 (2 C, C6 + C7); 45.12 (C8); 53.88 (C5); 77.68 (C3); 127.44 (arom. C5); 128.07 (arom. C4); 129.82 (arom. C3); 130.38 (arom. C6); 132.94 (arom. C1); 134.55 (arom. C2); 175.97 (C(1)=S). MS (70 eV, 230 °C): *m*/*z* (%): 266.8 (84) [$M^+$]; 231.9 (17) [$M^+$-Cl]; 206.8 (14) [$M^+$–$CH_2NS$]; 155.9 (37) [$M^+$–$C_6H_4N$-Cl]; 124.9 (11); 101.8 (11); 85.0 (88) [$C_4H_9N_2^+$]; 56.3 (22); 55.3 (11); 41.0 (15); 32.0 (21); 30.1 (28); 28.0 (100). Elemental analysis [$C_{12}H_{14}ClN_3S$ (267.8), %]: Calcd C, 53.82; H, 5.27; N, 15.76. Found: C, 53.49; H, 5.51; N, 15.83.

*4.2.1.2.12. 3-(3-Chlorophenyl)-2,3,5,6,7,8-hexahydro-1H-[1,2,4] triazolo-[1,2-a]pyridazine-1-thione* ***2k***. With *m*-chlorobenzaldehyde (0.211 g). Reaction time 15 min. Yield 70%. Yellowish crystals. Mp 190–199 °C (acetone).

TLC (m. ph. I): $R_f$ = 0.63. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1076 (–Cl); 1183 (C=S); 1472 (S=C–N–); 2929, 2950 (–CH–); 3169 (–NH–). $^1$H NMR ($CDCl_3$, ppm): $\delta$ = 1.60–1.82 (m, 4H, C(6)$H_2$/C(7)$H_2$); 2.63–2.75 (m, 1H, C(5)H); 2.95 (m, 1H, C(8)H); 3.18 (br t, 1H, C(5)H); 4.38 (d, 1H, $^3J$ = 6 Hz, C(8)H); 5.13 (s, 1H, C(3)H); 6.85 (s, 1H, –NH–); 7.31–7.37 and 7.47 (m + s, 4H, arom.). $^{13}$C NMR ($CDCl_3$, ppm): $\delta$ = 23.17 and 23.25 (2C, C6 + C7); 45.47 (C8); 53.16 (C5); 79.98 (C3); 125.68 (arom. C4); 127.70 (arom. C2); 129.87 (arom. C5); 130.12 (arom. C6); 134.85 (arom. C1); 138.67 (arom. C3); 176.30 (C=S). MS (70 eV, 205 °C): *m*/*z* (%): 268.9 (37) [$M^+$]; 206.9 (24) [$M^+$–$CH_2NS$]; 156.0 (22) [$M^+$–$C_6H_4$-Cl]; 88.9 (10); 85.0 (57) [$C_4H_9N_2^+$]; 56.3 (13); 41.0 (13); 30.1 (17); 28.0 (29). Elemental analysis [$C_{12}H_{14}ClN_3S$ (267.8), %]: Calcd C, 53.82; H, 5.27; N, 15.76. Found: C, 53.82; H, 5.35; N, 15.69.

*4.2.1.2.13. 3-(4-Chlorophenyl)-2,3,5,6,7,8-hexahydro-1H-[1,2,4] triazolo-[1,2-a]pyridazine-1-thione* ***2l***. With *p*-chlorobenzaldehyde (0.155 g). Differing from the given prescription it was heated between 125 and 130 °C. Reaction time 110 min. Yield 53%. Light beige crystals. Mp 178–179 °C (acetone).

TLC (m. ph. I): $R_f$ = 0.89. HPLC (m. ph. IV): RP-Select B, $t_0$ = 1.99; $t_R$ = 6.19; $k'$ = 2.11. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1088 (–Cl); 1184 (C=S); 1479 (S=C–N–); 2930, 2955 (–CH–); 3186 (–NH–). $^1$H NMR ($CDCl_3$, ppm): $\delta$ = 1.58–1.80 (m, 4H, C(6)$H_2$/C(7)$H_2$); 2.62–2.72 (m, 1H, C(5)H); 2.93 (m, 1H, C(5)H); 3.18 (br t, 1H, C(8)H); 4.39 (br d, 1H, $^3J$ = 6 Hz, C(8)H); 5.12 (s, 1H, C(3)H); 6.49 (s, 1H, –NH–); 7.34–7.43 (m, 4H, arom.). $^{13}$C NMR ($CDCl_3$, ppm): $\delta$ = 23.16 and 23.23 (2C, C6 + C7); 45.56 (C8); 52.99 (C5); 80.14 (C3); 128.99 (2 C, arom. C3 + C5); 129.11 (2 C, arom. C2 + C6); 134.92 (arom. C4); 135.80 (arom. C1); 176.52 (C=S). GC/MS (220 °C): *m*/*z* (%): 156.3 (15) [$M^+$–$C_6H_4$-Cl]; 125.3 (20); 111.2 (10) [$^\bullet C_6H_{10}N_3S$]; 85.4 (100) [$C_4H_9N_2^+$]; 70.4 (20); 56.4 (81). Elemental analysis [$C_{12}H_{14}ClN_3S$ (267.8), %]: Calcd C, 53.82; H, 5.27; N, 15.76. Found: C, 52.33; H, 4.35; N, 15.12.

*4.2.1.2.14. 3-(2-Nitrophenyl)-2,3,5,6,7,8-hexahydro-1H-[1,2,4] triazolo-[1,2-a]pyridazine-1-thione* ***2m***. With *o*-nitrobenzaldehyde (0.166 g). Reaction time 20 min. Yield 69%. Yellow-beige crystals. Mp 171–172 °C (acetone).

TLC (m. ph. I): $R_f$ = 0.62. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1186 (C=S); 1477 (S=C–N–); 1356, 1529 (–$NO_2$); 2924, 2952 (–CH–); 3144 (–NH–).$^1$H NMR ($CDCl_3$, ppm): $\delta$ = 1.63–1.88 (m, 4H, C(6)$H_2$/C(7)$H_2$); 2.79 (m, 1H, C(5)H); 2.98 (m, 1H, C(5)H); 3.17 (bt, 1H, $^3J$ = 12 Hz, C(8)H); 4.44 (d, 1H, $^3J$ = 14 Hz, C(8)H); 5.28 (s, 1H, C(3)H); 6.84 (s, 1H, –NH–); 7.60 (t, 1H, $^3J$ = 12 Hz, arom. C(4)H); 7.83 (m, 1H, arom. C(6)H); 8.23–8.28 (m, 2H, arom. C(3)H + C(5)H). $^{13}$C NMR ($CDCl_3$, ppm): $\delta$ = 23.13 and 23.29 (2 C, C6 + C7); 45.49 (C8); 53.44 (C5); 79.40 (C3); 122.62 (arom. C3); 124.56 (arom. C4); 129.99 (arom. C6); 133.32 (arom. C5); 139.23 (arom. C1); 148.54 (arom. C2); 176.26 (C(1)=S). MS (70 eV, 300 °C): *m*/*z* (%): 277.8 (38) [$M^+$]; 275.8 (22); 155.9 (12) [$M^+$–$C_6H_4$-$NO_2$]; 134.9 (26); 85.0 (25) [$C_4H_9N_2^+$]; 77.3 (11); 41.0 (11); 28.0 (30). Elemental analysis [$C_{12}H_{14}N_4O_2S$ (278.3), %]: Calcd C, 51.78; H, 5.07; N, 20.13. Found: C, 51.50; H, 5.27; N, 20.09.

*4.2.1.2.15. 3-(3-Nitrophenyl)-2,3,5,6,7,8-hexahydro-1H-[1,2,4] triazolo[1,2-a]pyridazine-1-thione* ***2n***. With *m*-nitrobenzaldehyde (0.166 g). Reaction time 20 min. Yield 73%. Yellow crystals. Mp 203–205.5 °C (acetone).

TLC (m. ph. I): $R_f$ = 0.42. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1186 (C=S); 1475 (S=C–N–); 1354, 1528 (-$NO_2$); 2923, 2951 (–CH–); 3145 (–NH–). $^1$H NMR ($CDCl_3$, ppm): $\delta$ = 1.58–1.86 (m, 4H, C(6)$H_2$/C(7)$H_2$); 2.73–2.83 (m, 1H, C(5)H); 2.96 (m, 1H, C(5)H); 3.18 (br t, 1H, $^3J$ = 10 Hz, C(8)H); 4.41 (d, 1H, $^3J$ = 14 Hz, C(8)H); 5.29 (s, 1H, C(3)H); 7.33 (s, 1H, –NH–); 7.58 (t, 1H, $^3J$ = 8 Hz, arom. C(4)H); 7.82 (d, 1H, $^3J$ = 8 Hz, arom. C(6)H); 8.24 (m, 2H, arom. C(5)H + C(3)H). $^{13}$C NMR ($CDCl_3$, ppm): $\delta$ = 23.16 and 23.31 (2 C, C6 + C7); 45.42 (C8); 53.45 (C5); 79.32 (C3); 122.58 (arom. C4); 124.49 (arom. C2); 129.95 (arom. C5); 133.35 (arom. C6); 139.32 (arom. C1); 148.52 (arom. C2); 176.11 (C=S). MS (70 eV, 210 °C): *m*/*z* (%): 277.9 (100) [$M^+$]; 217.8 (27) [$M^+$–$CH_2NS$]; 156.0 (53) [$M^+$–$C_6H_4$-$NO_2$]; 88.9 (12); 85.0 (100) [$C_4H_9N_2^+$]; 56.3 (25); 55.3 (14); 41.0 (19); 30.0 (34); 28.0 (17). Elemental analysis [$C_{12}H_{14}N_4O_2S$ (278.3), %]: Calcd C, 51.78; H, 5.07; N, 20.13. Found: C, 51.52; H, 4.85; N, 19.62.

*4.2.1.2.16. 3-(4-Nitrophenyl)-2,3,5,6,7,8-hexahydro-1H-[1,2,4]-[1,2-a] pyridazine-1-thione* ***2o***. With *p*-nitrobenzaldehyde (0.166 g). Reaction time 20 min. Yield 84%. Red-brown crystals. Mp 205–210 °C (acetone).

TLC (m. ph. I): $R_f$ = 0.40. HPLC (m. ph. IV): RP-Select B, $t_0$ = 1.97; $t_R$ = 4.90; $k'$ = 1.49. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1188 (C=S); 1475 (S=C–N–); 1349, 1526 (–$NO_2$); 2927, 2957 (–CH–); 3156 (–NH–). $^1$H NMR ($CDCl_3$, ppm): $\delta$ = 1.66–1.88 (m, 4H, C(6)$H_2$/C(7)$H_2$); 2.73–2.83 (m, 1H, C(5)H); 2.95 (m, 1H, C(5)H); 3.17 (br t, 1H, $^3J$ = 10 Hz, C(8)H); 4.43 (d, 1H, $^3J$ = 12 Hz, C(8)H); 5.28 (s, 1H, C(3)H); 6.98 (s, 1H, –NH–); 7.61–7.68 (m, 2H, arom. C(2)H + C(6)H); 8.22–8.28 (m, 2H, arom C(3)H + C(5)H). $^{13}$C NMR ($CDCl_3$, ppm): $\delta$ = 23.15 and 23.34 (2 C, C6 + C7); 45.46 (C8); 53.53 (C5); 79.29 (C3); 124.05 (2C, arom. C3 + C5); 128.38 (2 C, arom. C2 + C6); 143.71 (arom. C1); 148.79 (arom. C4); 176.25 (C=S). MS (70 eV, 205 °C): *m*/*z* (%): 277.9 (30) [$M^+$]; 155.9 (13) [$M^+$–$C_6H_4$-$NO_2$]; 85.0 (29) [$C_4H_9N_2^+$]; 32.0 (22); 30.0 (14); 28.0 (100). Elemental analysis [$C_{12}H_{14}N_4O_2S$ (278.3), %]: Calcd C, 51.78; H, 5.07; N, 20.13. Found: C, 51.77; H, 4.69; N, 19.06.

*4.2.1.2.17. 3-(2-Hydroxyphenyl)-2,3,5,6,7,8-hexahydro-1H-[1,2,4] triazolo-[1,2-a]pyridazine-1-thione* ***2p***. With *o*-hydroxybenzaldehyde (0.134 g). Reaction time 15 min. Yield 57%. Yellowish crystals. Mp. 193–195.5 °C (acetone).

TLC (m. ph. I): $R_f$ = 0.44. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1186 (C=S); 1428, 1460 (–OH); 1475 (S=C–N–); 2945, 2958 (–CH–); 3040 (–NH–); 3409 (–OH). $^1$H NMR ($CDCl_3$, ppm): $\delta$ = 1.60–1.87 (m, 4H, C(6)$H_2$/C(7)$H_2$); 2.72 (dt, 1H, $^3J$ = 4 Hz, $^3J$ = 12 Hz, C(5)H); 3.13 (m, 2H, C(8)H); 4.47 (d, 1H, $^3J$ = 12 Hz, C(5)H); 5.26 (s, 1H, C(3)H); 6.58 (s, 1H, –NH–); 6.88 (?, 2H, arom. C(3)H + C(5)H); 7.14 (dd, 1H, $^3J$ = 2 Hz, $^3J$ = 8 Hz, arom. C(6)H); 7.27 (dt, 1H, $^3J$ = 2 Hz, $^3J$ = 8 Hz, arom. C(4)H); 8.9 (br s, 1H, –OH). $^{13}$C NMR ($CDCl_3$, ppm): $\delta$ = 22.91 and 23.02 (2 C, C6 + C7); 46.18 (C8); 52.59 (C5); 80.17 (C3); 117.50 (arom. C3); 118.05 (arom. C5); 120.07 (arom. C4); 129.22 (arom. C6); 131.33 (arom. C1); 156.42 (arom. C2); 178.54 (C=S). MS (70 eV, 190 °C): *m*/*z* (%): 249.0 (100) [$M^+$]; 247.0 (20); 156.0 (12) [$M^+$–$C_6H_4$-OH]; 120.0 (12); 85.0 (77) [$C_4H_9N_2^+$]; 77.3 (19); 70.9 (17); 56.3 (14); 41.0 (16); 30.1 (21); 28.1 (43). Elemental analysis [$C_{12}H_{15}N_3OS$ (249.3), %]: Calcd C, 57.80; H, 6.06; N, 16.93. Found: C, 57.08; H, 6.03; N, 16.41.

*4.2.1.2.18. 3-(3-Hydroxyphenyl)-2,3,5,6,7,8-hexahydro-1H-[1,2,4] triazolo-[1,2-a]pyridazine-1-thione* ***2q***. With *m*-hydroxybenzaldehyde (0.134 g). Reaction time 15 min. Yield 64%. Pale gray crystals. Mp 198–205 °C (acetone).

TLC (m. ph. I): $R_f$ = 0.36. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1182 (C=S) 1416, 1445 (–OH); 1475 (S=C–N–); 2933, 2954 (–CH–); 3172 (–OH/–NH–). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 1.37–1.68 (m, 4H, C(6)$H_2$/C(7)$H_2$); 2.49–2.63 (m, 1H, C(5)H → beneath solvent signal); 2.83–2.97 (m, 2H, C(5)H/C(8)H); 4.18 (d, 1H, $^3J$ = 12 Hz, C(8)H); 5.14 (s, 1H, C(3)H); 6.72–6.83 (m, 3H, arom. C(2)H + C(3)H + C(4)H); 7.17 (t, 1H, $^3J$ = 8 Hz, arom. C(6)H); 9.26 (s, 1H, –NH–); 9.42 (s, 1H, –OH). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 23.50 and 23.53 (2 C, C6 + C7); 45.06 (C8); 52.93 (C5); 79.39 (C3); 114.35 (arom. C2); 116.29 (arom. C4); 118.36 (arom. C6); 129.83 (arom. C5); 139.78 (arom. C1); 157.87 (arom. C3); 176.807 (C=S). MS (70 eV, 205 °C): *m*/*z* (%): 249.0 (8) [$M^+$]; 206.9 (20); 156.0 (62) [$M^+$–$C_6H_4$-OH]; 88.9 (16); 85.0 (100) [$C_4H_9N_2^+$]; (23); 55.4 (12); 41.0 (18); 30.1 (33); 28.0 (21). Elemental analysis [$C_{12}H_{15}N_3OS$ (249.3),%]: Calcd C, 57.80; H, 6.06; N, 16.93. Found: C, 57.42; H, 5.56; N, 16.85.

*4.2.1.2.19. 3-(4-Hydroxyphenyl)-2,3,5,6,7,8-hexahydro-1H-[1,2,4] triazolo-[1,2-a]pyridazine-1-thione* ***2r***. With *p*-hydroxybenzaldehyde (0.134 g). Reaction time 15 min. Yield 53%. Yellowish crystals. Mp 210–214 °C (acetone).

TLC (m. ph. I): $R_f$ = 0.35. HPLC (m. ph. IV): RP-Select B, $t_0$ = 1.97; $t_R$ = 3.16; $k'$ = 0.60. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1184 (C=S); 1436, 1456 (–OH); 1494 (S=C–N–); 2855, 2940 (–CH–); 3135 (–OH). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 1.37–1.67 (m, 4H, C(6)$H_2$/C(7)$H_2$); 2.50 (m, 1H, C(5)H → beneath solvent signal); 2.78–2.99 (m, 2H, C(5)H/C(8)H); 4.14 (d, 1H, $^3J$ = 12 Hz, C(8)H); 5.07 (s, 1H, C(3)H); 6.76 (d, 2H, $^3J$ = 8 Hz, arom. C(3)H + C(5)H); 7.20 (d, 2H, $^3J$ = 8 Hz, arom. C(2)H + C(6)H); 9.20 (s, 1H, –NH–); 9.60 (s, 1H, –OH). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 23.44 and 23.48 (2 C, C6 + C7); 45.19 (C8); 52.48 (C5); 79.68 (C3); 115.54 (2 C, arom. C3 + C5); 129.18 (2 C, arom. C2 + C6); 131.22 (arom. C1); 160.78 (arom. C4); 177.02 (C=S). MS (70 eV, 25 °C): *m*/*z* (%): 249.0 (75) [$M^+$]; 189.0 (18) [$M^+$–$CH_2NS$]; 164.0 (23); 120.0 (11); 106.5 (10); 85.0 (100) [$C_4H_9N_2^+$]; 77.3 (11); 56.4 (21); 41.0 (13); 39.1 (10); 30.0 (44); 28.0 (13). Elemental analysis [$C_{12}H_{15}N_3OS$ (249.3), %]: Calcd C, 57.80; H, 6.06; N, 16.93. Found: C, 57.24; H, 5.64; N, 16.73.

*4.2.1.2.20. 3-(4-Methoxyphenyl)-2,3,5,6,7,8-hexahydro-1H-[1,2,4] triazolo-[1,2-a]pyridazine-1-thione* ***2s***. With *p*-methoxybenzaldehyde (0.150 g). Reaction time 75 min. Yield 35%. Pale beige crystals. Mp 136–138 °C (acetone).

TLC (m. ph. I): $R_f$ = 0.59. HPLC (m. ph. IV): RP-Select B, $t_0$ = 1.97; $t_R$ = 4.71; $k'$ = 1.39. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1184 (C=S); 1587; 1611 (arom. ring); 2834, 2927, 2948 (–$CH_2$–); 3164 (–NH–). $^1$H NMR ($CDCl_3$, ppm): $\delta$ = 1.63-1.75 (m, 4H, C(6)$H_2$, + C(7)$H_2$); 2.60 (br t; 1H, C(5)H); 2.94 (br d, 1H, $^3J$ = 10 Hz, C(5)H); 3.16 (br s, 1H, C(8)H); 3.82 (s, 3H, $OCH_3$); 4.37 (bs, 1H, C(8)H); 5.10 (s, 1H; (C3)H); 6.36 (s, 1H, NH); 6.93 (d, 2H, $^3J$ = 10 Hz, arom. C(2)H + arom. C(6)H); 7.39 (d, 2H, $^3J$ = 8 Hz, arom. C(3)H + arom. C(5)H). $^{13}$C NMR ($CDCl_3$, ppm): $\delta$ = 18.51 (C6); 18.75 (C7); 41.03 (C8); 48.07 (C5) 50.85 (-$OCH_3$); 76.17 (C3); 109.57 and 109.79 (arom. C2 + arom. C6); 123.49 (arom C1); 124,45 and 124,67 (arom. C3 + arom. C5); 156.29 (arom. C4); 172.08 (C=S). MS (70 eV, 160 °C) *m*/*z* (%): 263.5 (71) [$M^+$]; 203.5 (13) [$M^+$–NH-C=S]; 178.4 (33) [$M^+$–$C_4H_8N_2$]; 156.2 (4) [$M^+$–$C_6H_4$-$OCH_3$]; 134.3 (12); 85.2 (100) [CH-$N_2$-C=$S^+$]; 56.1 (20) [$C_4H_8^+$]; 30.0 (33) [–N-$CH_2^+$]. HRMS [(ESI) *m*/*z* (Intens. 0.35 × $10^4$)]: 264.114713 [M+H]$^+$. Elemental analysis [$C_{13}H_{17}N_3OS$ (263.4), %]: Calcd C, 59.28; H, 6.51; N, 15.95; S, 12.17; O, 6.07. Found: C, 59.35; H, 6.51; N, 15.97; S, 12.19; O, 6.08.

*4.2.1.2.21. 3-(3-Phenoxyphenyl)-2,3,5,6,7,8-hexahydro-1H-[1,2,4] triazolo-[1,2-a]pyridazine-1-thione* ***2t***. With *m*-phenoxybenzaldehyde (0.218 g). Reaction time 10 min. Yield 74%. Pale beige crystals. Mp 146–148 °C (acetone).

TLC (m. ph. I): $R_f$ = 0.63. HPLC (m. ph. IV): RP-Select B, $t_0$ = 1.97; $t_R$ = 9.49; $k'$ = 3.82. IR ($cm^{-1}$): $\tilde{\nu}$ = 1183 (C=S); 1478 (S=C–N–); 1583 (C=N) 2841, 2959 (–CH–); 3176 (–NH). $^1$H NMR ($CDCl_3$, ppm): $\delta$ = 1.55–1.78 (m, 4H, C(6)$H_2$ + C(7)$H_2$); 2.60–2.69 (m, 1H, C(5)H); 2.96 (d, 1H, $^3J$ = 10 Hz, C(5)H); 3.18 (br s, 1H, C(8)H); 4.38 (br d, 1H,$^3J$ = 12 Hz, C(8)); 5.12 (s, 1H, C(3)H); 6.39 (s, 1H, NH); 6.98–7.39 (m, 9H, arom.). $^{13}$C NMR ($CDCl_3$, ppm): $\delta$ = 23.16 and 23.21 (C6 + C7); 45.49 (C5 + C8); 80.38 (C3); 117.82; 119.67; 122.04; 123.73; 130.24 (C2′, C4′, C5′, C6′, C4″); 119.14 (C2″ + C6″); 129.90 (C3″ + C5″); 138.33 (C1′); 156.66; 157.84 (C3′ + C1″); 176.44 (C=S). MS [(ESI) *m*/*z*]: Calcd for [$C_{18}H_{19}N_3OS$+H]$^+$: 326.1293. Found [M+H$^+$]: 326.129. Elemental analysis [$C_{18}H_{19}N_3OS$ (325.4), %]: Calcd C, 66.43; H, 5.88; N, 12.91. Found: C, 65.96; H, 5.86; N, 12.45.

*4.2.1.2.22. 3-(4-Dimethylaminophenyl)-2,3,5,6,7,8-hexahydro-1H-[1,2,4]tri-azolo[1,2-a]pyridazine-1-thione* ***2u***. With *p*-dimethylaminobenzaldehyde (0.164 g). Differing from the given prescription it was heated between 100 °C and 105 °C. Reaction time 20 min. Yield 70%. Colorless crystals. Mp 196–202 °C (acetone).

TLC (m. ph. I): $R_f$ = 0.53. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 813 (p-subst. aromatic ring); 1182 (C=S); 1436 (–CH–); 1469 (S=C–N–); 1528; 1568; 1614 (-CN-); 2803, 2842, 2910, 2948 (–CH–); 3177 (–NH–). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 1.37–1.73 (m, 4H, C(6)$H_2$/C(7)$H_2$); 2.43–2.55 (m, 1H, C(5)H → beneath solvent signal); 2.76–3.04 (m, 8H, C(5)H/C(8)H/–N($CH_3$)$_2$); 4.11 (br d, 1H, $^3J$ = 12 Hz, C(8)H); 5.03 (s, 1H, C(3)H); 6.70 (d, 2H, $^3J$ = 8 Hz, arom. C(3)H + C(5)H); 7.20 (d, 2H, $^3J$ = 8 Hz, arom. C(2)H + C(6)H); 9.17(s, 1H, –NH–). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 23.43 and 23.49 (2 C, C6 + C7); 40.14 and 40.57 (2 C, arom. N-$CH_3$); 45.28 (C8); 52.30 (C5); 80.04 (C3); 112.38 (2C, arom. C3 + C5); 128.78 (2C, arom. C2 + C6); 130.49 (arom. C1); 151.48 (arom. C4); 177.23 (C=S). MS (70 eV, 205 °C): *m*/*z* (%): 276.0 (39) [$M^+$]; 191.9 (13); 190.9 (100). Elemental analysis [$C_{14}H_{20}N_4S$ (276.4), %]: Calcd C, 60.83; H, 7.29; N, 20.27. Found: C, 60.32; H, 7.14; N, 20.60.

*Preparation by heating of* ***1a*** *with benzaldehydes in a microwave reactor - general procedure:* Hexahydropyridazine-1-carbothioamide **1a** (1 mmol, 0.145 g)) and the appropriate aldehyde (1.1 mmol) were reacted in a microwave reactor under the described conditions (power, temperature, pressure) without or with a solvent and for the time, respectively, which are given below. Subsequently, the mixture was cooled for 24 h at 4 °C. The solvent was evaporated and the residue was suspended in a small amount of acetone. The formed crystalline product was collected, washed careful with acetone and dried.

In this manner the following compounds were obtained:

*4.2.1.2.23. 3-Phenyl-2,3,5,6,7,8-hexahydro-1H-[1,2,4]triazolo[1,2-a] pyridazine-1-thione* ***2f***. With benzaldehyde (0.117 g). Without solvent. 300 W, 130 °C, 15 bar, Reaction time 3 min. Yield 63%.

*4.2.1.2.24. 3-(4-Chlorophenyl)-2,3,5,6,7,8-hexahydro-1H-[1,2,4] triazolo-[1,2-a]pyridazine-1-thione* ***2l***. With *p*-chlorobenzaldehyde (0.155 g). Solvent ethanol (1 mL). Double-stage, stage 1: 200 W, 15 bar, 180 °C, Reaction time 1 min; stage 2: 300 W, 15 bar, 180 °C, Reaction time: 5 min. Yield 50%.

*4.2.1.2.25. 3-(4-Nitrophenyl)-2,3,5,6,7,8-hexahydro-1H-[1,2,4] triazolo-[1,2-a]pyridazine-1-thione* ***2o***. With *p*-nitrobenzaldehyde (0.166 g). Solvent ethanol (1 mL). Double-stage, stage 1: 200 W, 15 bar, 180 °C, Reaction time 1 min; stage 2: 300 W, 15 bar, 180 °C, Reaction time 5 min. Yield 64%.

*4.2.1.2.26. 3-(4-Hydroxyphenyl)-2,3,5,6,7,8-hexahydro-1H-[1,2,4] triazolo-[1,2-a]pyridazine-1-thione* ***2r***. With *p*-hydroxybenzaldehyde (0.134 g). Solvent ethanol (1 mL). 300 W, 130 °C, 15 bar, Reaction time 3 min. Yield 77%.

*4.2.1.2.27. 3-(4-Methoxyphenyl)-2,3,5,6,7,8-hexahydro-1H-[1,2,4] triazolo-[1,2-a]pyridazine-1-thione* ***2s***. With *p*-methoxybenzaldehyde (0.150 g). Without solvent. 300 W, 15 bar, 130 °C. Reaction time 3 min. Differing from the given prescription ethyl acetate was used for working up. Yield 69%.

*4.2.1.2.28. 3-(3-Phenoxyphenyl)-2,3,5,6,7,8-hexahydro-1H-[1,2,4] triazolo-[1,2-a]pyridazine-1-thione* ***2t***. With *m*-phenoxybenzaldehyde (0.218 g). Solvent acetone (1 mL). 300 W, 130 °C, 15 bar. Reaction time 3 min. Yield 84%.

**4.2.1.3. 2,3-Diphenyl-2,3,5,6,7,8-hexahydro-1*H*-[1,2,4]triazolo[1,2-*a*]-pyridazine-1-thione 6.** A mixture of *N*-phenylhexahydropyridazine-1-carbothioamide **1d** (2 mmol, 0.443 g) and benzaldehyde (20 mL) was allowed to stand at room temperature for 2 weeks. Then ethanol (15 mL) was added, the precipitated product was isolated, washed with ethanol, and dried in vacuo. Yield 90%. Colorless crystals. Mp 145–148 °C (ethanol).

TLC (m. ph. IX): $R_f$ = 0.23. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 3034 (arom. H); 2948, 2932, 2854, 2830 (–$CH_2$–); 1684; 1596; 1497; 1395; 1260 (C=S). $^1$H NMR ($CDCl_3$, ppm) $\delta$ = 1.80 (br s, 4H, C(6)$H_2$ + C(7)$H_2$); 2.73 (br m, 1H, C(5)H); 3.00 (br d, 1H, $^3J$ = 10 Hz, C(5)H); 3.26 (br t, 1H, C(8)H); 4.61 (br d, 1H, C(8)H); 5.35 (s, 1H, C(3)H); 7.24–7.32 (m, 10H, arom.). $^{13}$C NMR ($CDCl_3$, ppm): $\delta$ = 23.23, 23.63 (C6 + C7); 46.48 (C8); 53.30 (C5); 87.01 (C3); 127.03 (2 arom. C); 127.30 (1 arom. C); 128.36 (2 arom. C); 128.64 (2 arom. C); 128.86 (2 arom. C); 129.70 (1 arom. C); 135.47 (1 arom. C); 138.29 (1 arom. C); 176.73 (C=S). MS (70 eV, 180 °C): *m*/*z* (%): 309.4 (37) [$M^+$]; 308.5 (93) [M–H]$^+$; 261.2 (52); 246.3 (98); 231.7 (50); 192.2 (16); 175.4 (79); 174.3 (100); 134.7 (16); 130.6 (17); 125.8 (21); 124.8 (11); 117.7 (32); 103.9 (22); 90.8 (41); 76.8 (65); 69.9 (46); 68.9 (27); 57.0 (18); 55.0 (25); 51.0 (22); 43.0 (17); 42.0 (17); 41.0 (27). Elemental analysis [$C_{18}H_{19}N_3S$ (309.4), %]: Calcd C, 69.87; H, 6.20; N, 13.58. Found: C, 69.32; H, 6.35; N, 13.35

**4.2.1.4. 5,6,7,8-Tetrahydro-1*H*-[1,2,4]triazolo-[1,2-*a*]pyridazine-1-thiones 3.** *4.2.1.4.1. 3-Methyl-5,6,7,8-tetrahydro-1**H**-[1,2,4]triazolo[1,2-**a**]pyridazine-1-thione **3a**.* A mixture of *N*-[(2-acetyl-1,2,3,4,5,6-hexahydropyridazine-1-yl)thiocarbonyl]ethan-amide **5b** (1 mmol, 0.229 g), methanol (5 mL), and *p*-toluenesulfonic acid monohydrate (0.1 mmol, 0.019 g) was refluxed for 24 h. Subsequently, the solvent was slowly evaporated in vacuo, the resulting crystals were washed with a little ethanol, collected, and dried in vacuo. Yield: 60%. Colorless crystals. Mp 210–213 °C (methanol).

TLC (m. ph. III): $R_f$ = 0.18. HPLC (m. ph. V): RP-select B, $t_0$ = 1.87; $t_R$ = 2.41; $k'$ = 0.29. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1177 (C=S); 1520; 1632 (C=N); 2876 ($CH_3$); 2944, 2960 ($CH_2$). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 1.91 (br s, 4H, C(6)$H_2$, C(7)$H_2$); 2.28 (s, 3H; (C3)$CH_3$); 3.91 (br s, 2H, C(8)$H_2$); 4.03 (br s, 2H, C(5)$H_2$). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 11.45 ($CH_3$); 19.60 (C6); 19.79 (C7); 45.21 (C8); 45.34 (C5); 155.48 (C=N); 174.62 (C=S). Elemental analysis [$C_7H_{11}N_3S$ (169.2), %]: Calcd C, 49.68; H, 6.55; N, 24.83; S, 18.94. Found: C, 49.73; H, 6.56; N, 24.85; S, 18.96.

*4.2.1.4.2. 3-(2-Phenylethenyl)-5,6,7,8-tetrahydro-1H-[1,2,4]triazolo[1,2-a]-pyridazine-1-thione **3b**. Method 1:* A mixture of hexahydropyridazine-1-carbothioamide **1a** (0.1 mmol, 0.145 g), cinnamaldehyde (1 mmol, 0.132 g), and p-toluenesulfonic acid monohydrate (0.03 mmol, 0.006 g) was allowed to stand at room temperature for 2 h. Subsequently, a small amount of acetone was added and the crystallization of the product was initiated by rubbing with a glass stick. The crystals were collected by suction filtration, washed with a little acetone, and dried in vacuo. Yield: 35%. Pale yellow crystals. Mp 255–259 °C (acetone, mp. of the monohydrate).

TLC (m. ph. I): $R_f$ = 0.68. HPLC (m. ph. X): RP-18, $t_0$ = 1.90; $t_R$ = 4.47; $k'$ = 1.35. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1251 (C=S); 1458; 1504; 1578; 1634 (C=N, CH=CH); 2973 (aliph. H) 3023 (olefin. H); 3054 (arom. H); 3443 ($H_2O$). $^1$H NMR ($CDCl_3$, ppm): $\delta$ = 1.61 (s, 2H, $H_2O$); 2.09 (m, 4H, C(6)$H_2$/C(7)$H_2$); 4.15 (bs, 4H, C(5)$H_2$/C(8)$H_2$); 6.68 (d, 1H, $^3J$ = 16 Hz, olefin. C*H*=CH); 7.38–7.41 (m, 3H, arom. C(3′)H, C(4′)H, C(5′)H); 7.52–7.55 (m, 2H, arom. C(2′)H, C(6′)H); 8.06 (d, 1H, $^3J$ = 14 Hz, CH=C*H*). $^{13}$C NMR ($CDCl_3$, ppm): $\delta$ = 20.68 (C6); 20.72 (C7); 45.85 (C5, C8); 108.03 (olefin. *C*H=CH); 128.02 (arom. C3′, C5′); 129.07 (arom. C2′, C6′); 130.51 (C4′); 134.57 (arom. C1′); 143.24 (olefin. CH=*C*H); 155.60 (C=N); 176.82 (C=S). Elemental analysis [$C_{14}H_{15}N_3S$ (257.4) × $H_2O$ (275.4),%]: Calcd C, 61.06; H, 6.22; N, 15.26. Found: C, 59.82; H, 5.55; N, 15.00.

*Method 2:* A mixture of hexahydropyridazine-1-carbothioamide **1a** (1 mmol, 0.145 g), cinnamaldehyde (1 mmol, 0.132 g), and p-toluenesulfonic acid monohydrate (0.03 mmol, 0.006 g) was heated to between 125 °C and 130 °C for 4 h. Subsequently, the cooled mass was taken up in acetone and solvent was removed. The formed crystals were collected, washed with a little methanol, and dried in vacuo. Yield 7%. Pale yellow crystals. Mp 246–250 °C (acetone).

*4.2.1.4.3. 3-[2-(2-Nitrophenyl)ethenyl)]-5,6,7,8-tetrahydro-1H-[1,2,4]tri-azolo[1,2-a]pyridazine-1-thione **3c**.* A well prepared mixture of hexahydropyridazine-1-carbothioamide **1a** (0.5 mmol, 0.073 g), 2′-nitrocinnamaldehyde (0.55 mmol, 0.097 g), and *p*-toluenesulfonic acid monohydrate (0.03 mmol, 0.006 g) was heated to between 125 °C and 130 °C for 20 min. After cooling the mass was treated with hot acetone, the crystals were collected by suction filtration, washed with methanol and dried in vacuo. Yield: 20%. Yellow-orange crystals. Mp 253–256.5 °C (acetone).

TLC (m. ph. I): $R_f$ = 0.83. HPLC (m. ph. X): RP-18, $t_0$ = 1.90; $t_R$ = 4.29; $k'$ = 1.26. IR (KBr, $cm^{-1}$): $\tilde{\nu}$ = 1256 (C=S); 1441; 1501, 1523 ($NO_2$); 1571 (C=N); 1604 (CH=CH); 2868, 2957 (aliph. H); 3011 (olefin. H); 3047 (arom. H). $^1$H NMR (DMSO-$d_6$, ppm): $\delta$ = 1.98 (m, 4H, C(6)$H_2$/C(7)$H_2$); 4.00 (m, 2H, C(8)$H_2$); 4.31 (m, 2H, C(5)$H_2$); 7.30 (d, 1H, $^3J$ = 16 Hz, olefin. C*H*=CH); 7.70 (t, 1H, $^3J$ = 8 Hz, arom. C(4′)H); 7.83 (t, 1H, $^3J$ = 8 Hz, arom. C(5′)H); 8.02 (d, 1H, $^3$ = 16 Hz, olefin. CH=C*H*); 8.04–8.10 (m, 2H, arom. C(3′)H, C(6′)H). $^{13}$C NMR (DMSO-$d_6$, ppm): $\delta$ = 19.35 (C6); 19.79 (C7); 45.43 (C8); 45.66 (C5); 115.09 (olefin. C); 124.60 (arom. C3′); 128.85 (arom. C6); 129.53 (arom. C4′), 130.55 (arom. C1′); 133.56 (arom. C5′); 133.99 (olefin. C); 148.27 (arom. C2′); 153.19 (C=N); 174.70 (C=S). Elemental analysis [$C_{14}H_{14}N_4O_2S$, 302.4),%]: Calcd C, 55.61; H, 4.67; N, 18.53. Found: C, 53.79; H, 3.96; N, 18.00.

*4.2.1.4.4. 3-Phenyl-5,6,7,8-tetrahydro-1H-[1,2,4]triazolo[1,2-a]pyridazine-1-thione **3d**.* Substance was obtained as filter cake from a recrystallization of larger amounts of **2f** from acetone. Yield: 25 %. Colorless crystals. Mp 262–268 °C.

TLC (m. ph. XIII): $R_f$ = 0.71. IR ($cm^{-1}$): $\tilde{\nu}$ = 1189 (C=S); 1421 (CH); 1459 (C=N); 1533, 1604 (C=C, arom.). $^1$H NMR ($CDCl_3$, ppm): $\delta$ = 2.03–2.17 (m, 4H, C(6)$H_2$ u. C(7)$H_2$); 4.18 (t, 2H, $^3$ = 11.6 Hz, C(5)$H_2$); 4,28 (t, 2H, $^3$ = 12.4 Hz, C(8)$H_2$); 7,49–7,58 (m, 3H, arom. C(3)H, C(4)H u. C(5)H); 7,73–7,75 (m, 2H, arom. C(2)H u. C(6)H). $^{13}$C NMR ($CDCl_3$, ppm): $\delta$ = 20.82 u. 21.15 (C6 u. C7); 46.43 (C8); 48.70 (C5); 125.52 (arom. C1); 129.30 u. 129.38 (arom. C2, C3, C5 u. C6); 132.11 (arom. C4); 158.55 (C3); 176.24 (C(1)=S; very weak signal). HRMS [(ESI) *m*/*z*]: Calcd for [$C_{12}H_{15}N_3S$+H]$^+$: 234.1059. Found for [M+H$^+$]: 234.1066.

*4.2.1.4.5. 3-(4-Chlorophenyl)-5,6,7,8-tetrahydro-1H-[1,2,4]triazolo[1,2-a]-pyridazine-1-thione **3e**.* The substance precipitated after several days from a solution of **2l** in DMSO. To obtain the solid the suspension was centrifuged and the supernatant removed. The remaining DMSO was gently gathered in filter paper (as far as possible). Yield: 30 %. Colorless crystals. Mp 205–215 °C (DMSO).

TLC (m. Ph. XIII): $R_f$ = 0.76. IR ($cm^{-1}$): $\tilde{\nu}$ = 1171 (C=S); 1468 (C=N); 1522, 1575, 1595 u. 1656 (C=C, arom.); 2874, 2916, 2964 u. 3060 (CH). $^1$H NMR ($CDCl_3$, ppm): $\delta$ = 2.06–2.17 (m, 4H, C(6)$H_2$) u. C(7)$H_2$); 4.18–4.23 (m, 4H, C(5($H_2$) u. C(8)$H_2$); 7.45–7.49 (m,2H, arom. C(3)H u. C(5)H); 7.58–7.71 (m, 2H, arom. C(2)H u. C(6)H). $^{13}$C NMR ($CDCl_3$, ppm): $\delta$ = 20.62 u. 21.01 (C6 u. C7); 46.39 (C8); 48.64 (C5); 123,95 (arom. C1); 129.58 (arom. C3 u. C5); 130.68 (arom. C2 u. C6); 138.50 (arom. C4); 157.19 (C3); 176.26 (C(1)=S). HRMS [(ESI) *m*/*z*]. Calcd for [$C_{12}H_{11}N_3SCl$+H$^+$]: 266.0513. Found for [M+H$^+$]: 266.0524.

### 4.3. Biology

#### 4.3.1. Cell culture materials

All chemicals (sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride, MTT, dimethyl sulfoxide, aminoguanidine hydrochloride) as well as interleukin-1β, fetal calf serum and trypane blue were from Sigma Aldrich. Cell culture plastics, PBS, trypsin/EDTA and interferon-γ were obtained from Biochrom AG, RPMI1640 and penicillin/streptomycin from Lonza.

#### 4.3.2. Cell line

The insulin-producing rat insulinoma cell line RIN5F (ECACC catalogue No. 95090402) was used. The adherent cells were grown under standard conditions (95% humidity, 37 °C, 5% $CO_2$) in RPMI1640 supplemented with L-glutamine (2 mmol/L), 10% heat-inactivated FCS, penicillin (100 I.E.) and streptomycin (100 I.E.). Cells were trypsinized once in a week, washed with FCS containing media and then counted. Vitality was determined with trypane blue staining.

#### 4.3.3. iNOS expression and inhibition

Tests were performed with $1 \times 10^5$ cells per well in 200 μL culture media (as defined above) in 96-well plates at standard culture conditions. Twenty-four hours after cells had been seeded out, iNOS was induced by adding 1 ng (500 I.E.)/mL IL-1β (human, recombinant) and 10 ng (30 I.E.)/mL IFN-γ (rat, recombinant). For the primary screening, test compounds were prepared as a 62.5 mM stock solution in DMSO and added to the cells at concentrations of 78 and 39 μM with a resulting final concentration of 0.5% DMSO in the well. The DMSO concentration did not affect the NO production and lowered the cell viability only marginally (about 10%) and was for that reason acceptable for the cells. The reference inhibitor aminoguanidine was tested at the same concentrations (added in DMSO) on every plate as well. DMSO was added to the controls at the same concentration. Selected substances with significantly higher inhibition of iNOS than aminoguanidine were chosen for $IC_{50}$ determination. Therefore, concentrations that result in 10 to 90% inhibition of iNOS (plus one to two values under or above) were selected for each individual compound. $IC_{50}$ was calculated from linear regression of logarithmized concentrations versus percentage of inhibition.

#### 4.3.4. NO assay

To determine the nitrite concentration, as a degree for NO production, 50 μL cell-free supernatant per well (two samples from each cavity) were transferred to a new multiwell plate and the Griess[73,74] reaction was performed. To every 50 μL supernatant the same amount of a 0.1% sulfanilamide solution in 5% phosphoric acid was pipetted and incubated for 5 min in the dark at room temperature. With 50 μL of an aqueous 0.01% *N*-(1-naphthyl)ethylenediamine dihydrochloride solution the same procedure was executed. Afterwards, the absorption was measured in a plate reader at a wavelength of 550 nm. For calibration, a dilution series of nitrite (0 to 100 μM) was included on every plate.

#### 4.3.5. MTT assay

The remaining cells in the test plates were used to perform the MTT assay as a test of cytotoxicity.[75] Medium was completely removed and replaced with 100 μL of fresh medium containing 20 μL MTT in PBS (2.5 mg/mL). After 4 h incubation at 37 °C medium was carefully removed (not everything to avoid disturbing the formazan crystals), 100 μL DMSO were added and plates were shortly shaken by hand to fully dissolve the developed violet crystals. The absorption was immediately measured at a wavelength of 570 nm.

#### 4.3.6. Statistics

Normality was tested and if positive *F*- and *t*-test were performed. Otherwise Mann–Whitney-test was executed. Statistical significance was assumed for $p < 0.05$ or lower.

### 4.4. 3D structures

3D structure models of compounds **2c**, **2d** and **2f** were obtained with the program MOE 2011.10[76] by using MMFF94x force field.


### Acknowledgements

The authors gratefully thank Mr. Franz studener, pharmacist and head of the pharmacy, 'Mohren-Apotheke', Neumarkt 8, 99947 Bad Langensalza, Germany, for the financial basis of this work. They also wish to sincerely thank Prof. Dr. Thomas von woedtke (Campus PlasmaMed) and Dr. Kai masur (head of the workgroup 'Cellular effects', Centre of Innovation Competence plasmatis), both of them INP Greifswald e.V., for the generous technical support of the biological works of this project. Finally, the authors are grateful for the scholarship of the 'Studienstiftung des deutschen Volkes', that made the continuative working on this project possible.


### Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.05.064.

### References and notes


1. Ignarro, L. J.; Buga, G. M.; Wood, K. S.; Byrns, R. E.; Chaudhuri, G. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 9265.
2. Gao, Y. *Pflugers Arch.* **2010**, *459*, 829.
3. Kerwin, J. F., Jr.; Lancaster, J. R.; Feldman, P. L. *J. Med. Chem.* **1995**, *38*, 4343.
4. Lowenstein, C. J.; Dinerman, J. L.; Snyder, S. H. *Ann. Intern. Med.* **1994**, *120*, 227.
5. Moncada, S.; Palmer, R. M.; Higgs, E. A. *Pharmacol. Rev.* **1991**, *43*, 109.
6. Steinert, J. R.; Chernova, T.; Forsythe, I. D. *Neuroscientist* **2010**, *16*, 435.
7. Ghosh, D. K.; Stuehr, D. J. *Biochemistry (Mosc.)* **1995**, *34*, 801.
8. Marsden, P. A.; Schappert, K. T.; Chen, H. S.; Flowers, M.; Sundell, C. L.; Wilcox, J. N.; Lamas, S.; Michel, T. *FEBS Lett.* **1992**, *307*, 287.
9. McMillan, K.; Masters, B. S. *Biochemistry (Mosc.)* **1995**, *34*, 3686.
10. Alderton, W. K.; Cooper, C. E.; Knowles, R. G. *Biochem. J.* **2001**, 357.
11. Nathan, C.; Xie, Q. W. *J. Biol. Chem.* **1994**, *269*, 13725.
12. Fortin, C. F.; McDonald, P. P.; Fulop, T.; Lesur, O. *Shock* **2010**, *33*, 344.
13. Titheradge, M. A. *Biochim. Biophys. Acta* **1999**, *1411*, 437.
14. Lassmann, H. *J. Neural Transm.* **2011**, *118*, 747.
15. Barnes, P. J.; Liew, F. Y. *Immunol. Today* **1995**, *16*, 128.
16. Sugiura, H.; Ichinose, M. *Nitric Oxide* **2011**, *25*, 138.
17. Werner, E. R.; Pitters, E.; Schmidt, K.; Wachter, H.; Werner-Felmayer, G.; Mayer, B. *Biochem. J.* **1996**, *320*, 193.
18. Matter, H.; Kumar, H. S.; Fedorov, R.; Frey, A.; Kotsonis, P.; Hartmann, E.; Frohlich, L. G.; Reif, A.; Pfleiderer, W.; Scheurer, P.; Ghosh, D. K.; Schlichting, I.; Schmidt, H. H. *J. Med. Chem.* **2005**, *48*, 4783.
19. Davey, D. D.; Adler, M.; Arnaiz, D.; Eagen, K.; Erickson, S.; Guilford, W.; Kenrick, M.; Morrissey, M. M.; Ohlmeyer, M.; Pan, G.; Paradkar, V. M.; Parkinson, J.; Polokoff, M.; Saionz, K.; Santos, C.; Subramanyam, B.; Vergona, R.; Wei, R. G.; Whitlow, M.; Ye, B.; Zhao, Z. S.; Devlin, J. J.; Phillips, G. *J. Med. Chem.* **2007**, *50*, 1146.
20. Gahman, T. C.; Herbert, M. R.; Lang, H.; Thayer, A.; Symons, K. T.; Nguyen, P. M.; Massari, M. E.; Dozier, S.; Zhang, Y.; Sablad, M.; Rao, T. S.; Noble, S. A.; Shiau, A. K.; Hassig, C. A. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 6888.
21. Salerno, L.; Sorrenti, V.; Di, G. C.; Romeo, G.; Siracusa, M. A. *Curr. Pharm. Des.* **2002**, *8*, 177.
22. Wolff, D. J.; Lubeskie, A.; Gauld, D. S.; Neulander, M. J. *Eur. J. Pharmacol.* **1998**, *350*, 325.
23. Furfine, E. S.; Harmon, M. F.; Paith, J. E.; Garvey, E. P. *Biochemistry (Mosc.)* **1993**, *32*, 8512.
24. Young, R. J.; Beams, R. M.; Carter, K.; Clark, H. A. R.; Coe, D. M.; Chambers, C. L.; Davies, P. I.; Dawson, J.; Drysdale, M. J.; Franzman, K. W.; French, C.; Hodgson, S. T.; Hodson, H. F.; Kleanthous, S.; Rider, P.; Sanders, D.; Sawyer, D. A.; Scott, K. J.; Shearer, B. G.; Stocker, R.; Smith, S.; Tackley, M. C.; Knowles, R. G. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 597.
25. Fedorov, R.; Hartmann, E.; Ghosh, D. K.; Schlichting, I. *J. Biol. Chem.* **2003**, *278*, 45818.

26. Alderton, W. K.; Angell, A. D. R.; Craig, C.; Dawson, J.; Garvey, E.; Moncada, S.; Monkhouse, J.; Rees, D.; Russell, L. J.; Russell, R. J.; Schwartz, S.; Waslidge, N.; Knowles, R. G. *Br. J. Pharmacol.* **2005**, *145*, 301.
27. Misko, T. P.; Moore, W. M.; Kasten, T. P.; Nickols, G. A.; Corbett, J. A.; Tilton, R. G.; McDaniel, M. L.; Williamson, J. R.; Currie, M. G. *Eur. J. Pharmacol.* **1993**, *233*, 119.
28. Wolff, D. J.; Lubeskie, A. *Arch. Biochem. Biophys.* **1995**, *316*, 290.
29. Zhou, X.; Potoka, D. A.; Boyle, P.; Nadler, E. P.; McGinnis, K.; Ford, H. R. *FEMS Microbiol. Lett.* **2002**, *206*, 93.
30. Nakane, M.; Klinghofer, V.; Kuk, J. E.; Donnelly, J. L.; Budzik, G. P.; Pollock, J. S.; Basha, F.; Carter, G. W. *Mol. Pharmacol.* **1995**, *47*, 831.
31. Southan, G. J.; Szabo, C.; Thiemermann, C. *Br. J. Pharmacol.* **1995**, *114*, 510.
32. Naka, M.; Nanbu, T.; Kobayashi, K.; Kamanaka, Y.; Komeno, M.; Yanase, R.; Fukutomi, T.; Fujimura, S.; Seo, H. G.; Fujiwara, N.; Ohuchida, S.; Suzuki, K.; Kondo, K.; Taniguchi, N. *Biochem. Biophys. Res. Commun.* **2000**, *270*, 663.
33. Hallinan, E. A.; Tsymbalov, S.; Dorn, C. R.; Pitzele, B. S.; Hansen, D. W., Jr.; Moore, W. M.; Jerome, G. M.; Connor, J. R.; Branson, L. F.; Widomski, D. L.; Zhang, Y.; Currie, M. G.; Manning, P. T. *J. Med. Chem.* **2002**, *45*, 1686.
34. Garvey, E. P.; Oplinger, J. A.; Furfine, E. S.; Kiff, R. J.; Laszlo, F.; Whittle, B. J.; Knowles, R. G. *J. Biol. Chem.* **1997**, *272*, 4959.
35. Tinker, A. C.; Beaton, H. G.; Boughton-Smith, N.; Cook, T. R.; Cooper, S. L.; Fraser-Rae, L.; Hallam, K.; Hamley, P.; McInally, T.; Nicholls, D. J.; Pimm, A. D.; Wallace, A. V. *J. Med. Chem.* **2003**, *46*, 913.
36. Hagmann, W. K.; Caldwell, C. G.; Chen, P.; Durette, P. L.; Esser, C. K.; Lanza, T. J.; Kopka, I. E.; Guthikonda, R.; Shah, S. K.; MacCoss, M.; Chabin, R. M.; Fletcher, D.; Grant, S. K.; Green, B. G.; Humes, J. L.; Kelly, T. M.; Luell, S.; Meurer, R.; Moore, V.; Pacholok, S. G.; Pavia, T.; Williams, H. R.; Wong, K. K. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1975.
37. Garcin, E. D.; Arvai, A. S.; Rosenfeld, R. J.; Kroeger, M. D.; Crane, B. R.; Andersson, G.; Andrews, G.; Hamley, P. J.; Mallinder, P. R.; Nicholls, D. J.; St-Gallay, S. A.; Tinker, A. C.; Gensmantel, N. P.; Mete, A.; Cheshire, D. R.; Connolly, S.; Stuehr, D. J.; Aberg, A.; Wallace, A. V.; Tainer, J. A.; Getzoff, E. D. *Nat. Chem. Biol.* **2008**, *4*, 700.
38. Connolly, S.; Aberg, A.; Arvai, A.; Beaton, H. G.; Cheshire, D. R.; Cook, A. R.; Cooper, S.; Cox, D.; Hamley, P.; Mallinder, P.; Millichip, I.; Nicholls, D. J.; Rosenfeld, R. J.; St-Gallay, S. A.; Tainer, J.; Tinker, A. C.; Wallace, A. V. *J. Med. Chem.* **2004**, *47*, 3320.
39. Lopez-Cara, L. C.; Carrion, M. D.; Entrena, A.; Gallo, M. A.; Espinosa, A.; Lopez, A.; Escames, G.; Acuna-Castroviejo, D.; Camacho, M. E. *Eur. J. Med. Chem.* **2012**, *50*, 129.
40. Gradler, U.; Fuchss, T.; Ulrich, W. R.; Boer, R.; Strub, A.; Hesslinger, C.; Anezo, C.; Diederichs, K.; Zaliani, A. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 4228.
41. Zoumpoulakis, P.; Camoutsis, C.; Pairas, G.; Sokovic, M.; Glamoclija, J.; Potamitis, C.; Pitsas, A. *Bioorg. Med. Chem.* **2012**, *20*, 1569.
42. Al-Amiery, A. A.; Al-Majedy, Y. K.; Ibrahim, H. H.; Al-Tamimi, A. A. *Org. Med. Chem. Lett.* **2012**, *2*, 4.
43. Girgis, A. S.; Tala, S. R.; Oliferenko, P. V.; Oliferenko, A. A.; Katritzky, A. R. *Eur. J. Med. Chem.* **2012**, *50*, 1.
44. Feldman, R. I.; Mintzer, B.; Zhu, D.; Wu, J. M.; Biroc, S. L.; Yuan, S.; Emayan, K.; Chang, Z.; Chen, D.; Arnaiz, D. O.; Bryant, J.; Ge, X. S.; Whitlow, M.; Adler, M.; Polokoff, M. A.; Li, W.-W.; Ferrer, M.; Sato, T.; Gu, J.-M.; Shen, J.; Tseng, J.-L.; Dinter, H.; Buckman, B. *Chem. Biol. Drug Des.* **2009**, *74*, 43.
45. Gilmore, J. L.; King, B. W.; Asakawa, N.; Harrison, K.; Tebben, A.; Sheppeck, J. E.; Liu, R.-Q.; Covington, M.; Duan, J. J. W. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 4678.
46. Morgenstern, O.; Wanka, H.; Roser, I.; Steveling, A.; Kuttler, B. *Bioorg. Med. Chem.* **2004**, *12*, 1071.
47. Li, B.; Man, Y.; Zhang, Z.; Xu, L.; Hsu, A. C.-T. US 6,251,829 B1; **2001**, 11.
48. Wakabayashi, O.; Matsuya, K.; Ohta, H.; Jikihara, T.; Suzuki, S. DE 2,526,358 A1; **1976**, 87.
49. Iida, T.; Senoo, S.; Sato, Y.; Nicolaus, B.; Wakabayashi, K.; Boeger, P. *Z. Naturforsch., C: Biosci.* **1995**, *50*, 186.
50. Schussler, J. R.; Crosby, K. E.; Backus, P. A.; Tsukamoto, M. WO 2,001,085,907 A2; **2001**, 33.
51. Sato, Y.; Noguchi, N.; Koizumi, K.; Wakabayashi, K. *Tamagawa Daigaku Nogakubu Kenkyu Hokoku* **1992**, *32*, 73.
52. Wakabayashi, O.; Matsutani, K.; Ohta, H.; Naohara, T.; Watanabe, H. JP 52,113,972A; **1977**, 10.
53. Kilama, J. J. WO 9,414,817 A1; **1994**, 105.
54. Kilama, J. J. US 564,3855A; **1997**, 46
55. Alsop, S. H.; Barr, J. J.; Storr, R. C.; Cameron, A. F.; Freer, A. A. *J. Chem. Soc., Chem. Commun.* **1976**, 888.
56. Snyder, J. P.; Heyman, M.; Gundestrup, M. *J. Org. Chem.* **1978**, *43*, 2224.
57. Foldeak, S.; Matkovics, B.; Porszasz, J. *Acta Phys. Chem.* **1962**, *8*, 88.
58. Ogino, C.; Hoshi, T.; Iida, T.; Koura, S.; Ogawa, H.; Kohno, H.; Sato, Y.; Takai, M.; Wakabayashi, K. J. *J. Pestic. Sci. (Int. Ed.)* **1993**, *18*, 369.
59. Ankarcrona, M.; Dypbukt, J. M.; Brune, B.; Nicotera, P. *Exp. Cell Res.* **1994**, *213*, 172.
60. Cetkovic-Cvrlje, M.; Eizirik, D. L. *Cytokine* **1994**, *6*, 399.
61. Neumann, U.; Guetschow, M. *Bioorg. Chem.* **1995**, *23*, 72.
62. Arai, I.; Abe, S.; Hagitani, A. *Bull. Chem. Soc. Jpn.* **1973**, *46*, 677.
63. Leistner, S.; Guetschow, M.; Stach, J. *Arch. Pharm. (Weinheim, Ger.)* **1990**, *323*, 857.
64. Zelenin, K. N.; Alekseyev, V. V.; Kuznetsova, O. B.; Torocheshnikov, V. N.; Khorseeva, L. A. *Tetrahedron* **1993**, *49*, 5327.
65. Gazdar, A. F.; Chick, W. L.; Oie, H. K.; Sims, H. L.; King, D. L.; Weir, G. C.; Lauris, V. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 3519.
66. Chick, W. L.; Warren, S.; Chute, R. N.; Like, A. A.; Lauris, V.; Kitchen, K. C. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 628.
67. Cho, J. M.; Chang, S. Y.; Kim, D. B.; Needs, P. W.; Jo, Y. H.; Kim, M. J. *J. Nutr. Biochem.* **2012**, *23*, 1394.
68. Jeong, G.-S.; Lee, D.-S.; Park, B.-H.; Kwon, K.-B.; Kim, Y.-C. *Toxicol. In Vitro* **2011**, *25*, 505.
69. Kim, E. K.; Kwon, K. B.; Lee, J. H.; Park, B. H.; Park, J. W.; Lee, H. K.; Jhee, E. C.; Yang, J. Y. *Biol. Pharm. Bull.* **2007**, *30*, 242.
70. Tabatabaie, T.; Graham, K. L.; Vasquez, A. M.; Floyd, R. A.; Kotake, Y. *Nitric Oxide* **2000**, *4*, 157.
71. Munier, R. *Bull. Soc. Chim. Biol.* **1953**, *35*, 1225.
72. Awe, W. *Pharmazie* **1948**, *3*, 492.
73. Green, L. C.; Wagner, D. A.; Glogowski, J.; Skipper, P. L.; Wishnok, J. S.; Tannenbaum, S. R. *Anal. Biochem.* **1982**, *126*, 131.
74. Griess, P. *Ber. Dtsch. Chem. Ges.* **1879**, *12*, 426.
75. Mosmann, T. J. *Immunol. Methods* **1983**, *65*, 55.
76. *Molecular Operating Environment (MOE)*, 2011.10; Chemical Computing Group Inc., 1010 Sherbooke St. West, Suite #910, Montreal, QC, Canada, H3A 2R7, **2011**.