ACS Medicinal Chemistry Letters



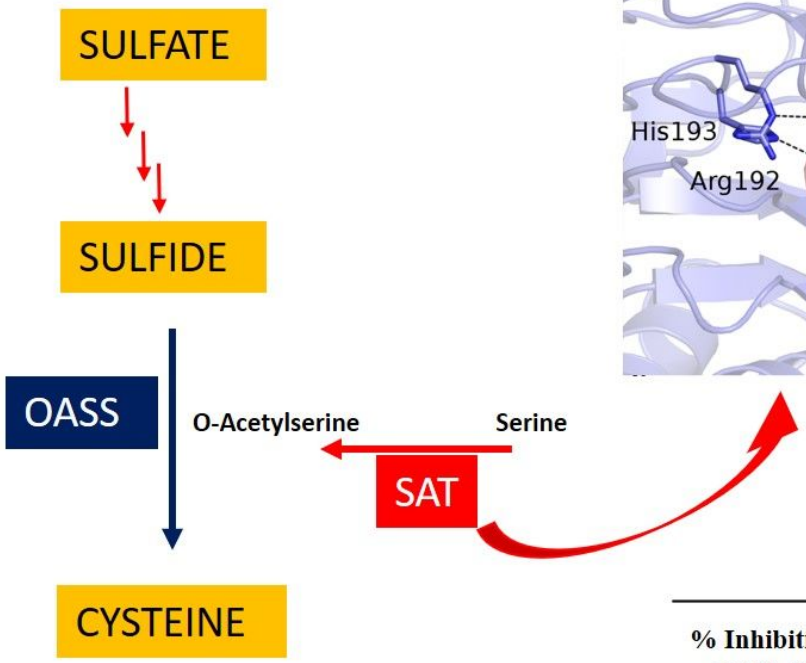
Letter

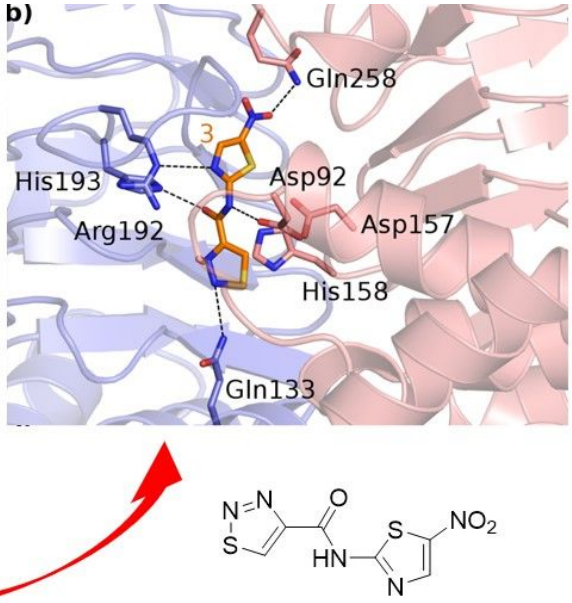
# Inhibition of Non-Essential Bacterial Targets: Discovery of a Novel Serine O-acetyltransferase Inhibitor

Joana Magalhaes, Nina Franko, Samanta Raboni, Giannamaria Annunziato, Päivi Tammela, Agostino Bruno, Stefano Bettati, Andrea Mozzarelli, Marco Pieroni, Barbara Campanini, and Gabriele Costantino

*ACS Med. Chem. Lett.*, **Just Accepted Manuscript** • DOI: 10.1021/acsmedchemlett.9b00627 • Publication Date (Web): 13 Feb 2020

**Table of contents**







| % Inhibition @100µM | $IC_{50}$ (µM) | MIC (µg/mL) in LB 20% | MIC (µg/mL) in MHB |
|---|---|---|---|
| 68 | 48.6±8 | 64 | >128 |

# Inhibition of Non-Essential Bacterial Targets: Discovery of a Novel Serine *O*-acetyltransferase Inhibitor

Joana Magalhães,[a] Nina Franko,[b] Samanta Raboni,[b,f] Giannamaria Annunziato, [a] Päivi Tammela,[c] Agostino Bruno,[a,†] Stefano Bettati,[d,e,f] Andrea Mozzarelli,[b,e,f] Marco Pieroni, [a,g,*] Barbara Campanini, [b] Gabriele Costantino [a,g,h]

[a]*P4T group, and* [b]*Laboratory of Biochemistry and Molecular Biology Department of Food and Drug, University of Parma, 43124 Parma, Italy;* [c]*Drug Research Program, Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, P.O. Box 56 (Viikinkaari 5 E), Helsinki, FI-00014, Finland;* [d]*Department of Medicine and Surgery, University of Parma, 43125 Parma, Italy;* [e]*National Institute of Biostructures and Biosystems, Rome, Italy;* [f]*Institute of Biophysics, CNR, Pisa, Italy;* [g]*Centro Interdipartimentale "Biopharmanet-tec", Università degli Studi di Parma, Parma, Italy;* [h]*Centro Interdipartimentale Misure (CIM) 'G. Casnati', University of Parma, Parma, Italy.*

*†Present address: Experimental Therapeutics Program, IFOM – The FIRC Institute for Molecular Oncology Foundation, Via Adamello 16-20139, Milano, Italy.*

This article is dedicated to the truly brilliant memory of Prof Maurizio Botta, who has inspired generations of researchers with his vision and sympathy.

**Abstract**

In ϒ-proteobacteria and actinomycetales, cysteine biosynthetic enzymes are indispensable during persistence, and become dispensable during growth or acute infection. The biosynthetic machinery required to convert inorganic sulfur into cysteine is absent in mammals, therefore it is a suitable drug target. We searched for inhibitors of *Salmonella* serine acetyltransferase (SAT), the enzyme that catalyzes the rate-limiting step of L-cysteine biosynthesis. The virtual screening of three ChemDiv focused libraries containing 91,243 compounds was performed to identify potential SAT inhibitors. Scaffold similarity and analysis of the overall physico-chemical properties allowed the selection of 73 compounds that were purchased and evaluated on the recombinant enzyme. Six compounds displaying an $IC_{50}$ below 100 μM were identified *via* an indirect assay using Ellman's reagent and then tested on a Gram-negative model organism, with one of them being able to interfere with bacterial growth *via* SAT inhibition.

**Keywords**

Adjuvant therapies; Antimicrobial resistance; Cysteine biosynthesis; Non-essential targets; Serine acetyltransferase.

## Introduction

Antimicrobial resistance (AMR) is one of the most threatening insults to public health worldwide. Features related to this upset are worrying, both in terms of loss of human lives and costs for hospitalization.[1] Experts estimate that 10 million people will die every year due to antimicrobial resistance by 2050, unless proper measures are adopted to contain the emergence.[2] AMR refers to the ability of microorganisms (not necessarily bacteria, but also fungi, viruses, or protozoa) to nullify the therapeutic effects of antimicrobials, and it can affect anyone, at any age, in any country. Generally, misuse of prescribed medicines, incorrect prescriptions and poor prevention and control measures account for the development of AMR.[3–5] The search for novel druggable molecular targets different from those already exploited by the current antibacterial arsenal is crucial to prevent the onset of cross-resistance for the newest molecules under investigation. Along with this conventional approach, adjuvant strategies aimed at engaging non-essential targets, such as those related to bacterial virulence and/or persistence after colonization of the host, are gaining growing interest.[6–9] It has been speculated that targeting those enzymes, which are dispensable during the normal bacterial life cycle, but that become essential during host invasion, might have a lower impact on AMR because they facilitate the clearing action of the immune system without stimulating the rise of resistance. In addition, by decreasing bacterial fitness, adjuvants act as antibiotic activity enhancers. The majority of pathogens spend part of their life cycle in extremely challenging environments like macrophages or the gastric mucosa, where survival and persistence require a fine metabolic adaptation. In many cases, this adaptation is made possible through a series of sulfur-containing biomolecules such as Fe-S clusters, thiamine, glutathione and biotin, which ensure bacterial survival especially due to their detoxifying properties and reducing power.[10–12] In this regard, blocking the synthesis of cysteine, which is the most relevant building block for the above-mentioned biomolecules, holds great promise as an adjuvant strategy to undermine bacterial fitness and promote eradication of the infection. Cysteine metabolism has been indicated as a promising drug target in ϒ-proteobacteria (like *Salmonella enterica serovar* Typhimurium) and actinomycetales (*Mycobacterium tuberculosis*),[10,13] as it has been shown that suppression or reduction of the cysteine biosynthesis led to a decreased bacterial fitness and infectivity.[14,15] In both vegetative and swarm cell populations, inhibition of cysteine biosynthesis is associated with an unpaired oxidative stress response, enhancing the antibacterial activity of common antibiotics and in some cases restoring the activity of obsolete antibacterials.[14,15] Finally, whereas mammals lack the biosynthetic

machinery that leads to the *de novo* synthesis of cysteine from inorganic sulphur, bacteria and plants possess highly conserved enzymes enabling its biosynthesis through the reductive sulphate assimilation pathway (Figure 1).[10,16]

**[Insert Figure 1 here]**

Sulphate and thiosulfate, the most abundant forms of extracellular sulphur, enter the cell through specific transporters. Sulphate is then activated to be reduced and the entire process requires the consumption of one mole of GTP, two moles of ATP and three moles of NADPH for each mole of cysteine produced. In *E. coli* and *Salmonella*, after activation of sulphate by ATP with formation of adenosine 5′-phosphosulphate (APS), 3'-phosphoadenosine-5'-phosphosulfate (PAPS) is synthesized. Afterwards, PAPS is reduced to bisulfide that is incorporated into cysteine by *O*-acetylserine sulfhydrylase (OASS) in the last step of cysteine biosynthesis.[17,18] This enzyme, present in two isoforms, OASS-A and OASS-B, depends on pyridoxal 5'-phosphate and catalyses a β-replacement reaction on *O*-acetylserine (OAS), synthesized by serine acetyltransferase (SAT).[19] It was demonstrated that inhibition of OASS was able to revert the bacterial resistance to fluroquinolones and tetracycline.[14,15] This has prompted us to undertake a medicinal chemistry campaign aimed at discovering potent and selective OASS inhibitors, which culminated with the release of the most potent inhibitor of *St*OASS known so far for both the isoforms.[20] Despite the remarkable inhibitory activity, it was impossible to evaluate the efficacy for bacterial cells likely due to the scarce permeability of the compound through the bacterial cell wall. After thorough investigation of the Structure-Activity Relationships for these OASS inhibitors,[21–24] we have deemed of interest to investigate the effects of SAT inhibition, which is the upstream enzyme of the cysteine synthase complex.[25–28] Of notice, inhibition of SAT is expected to exert partially distinct effects with respect to those brought about by OASS inhibition. Indeed, inhibition of OASS is expected to induce accumulation of OAS, which is converted to N-acetylserine (NAS), a natural inducer of the biosynthetic operon,[29] and cysteine depletion. Conversely, inhibition of SAT is expected to deplete the cell not only of cysteine, but also of the natural inducer. SAT catalyses the rate-limiting step of L-cysteine biosynthesis, where the acetyl group of acetyl-CoA is transferred to the hydroxyl group of L-serine to generate OAS. From the structural point of view, SAT is a dimer of trimers, with an α-helical N-terminal domain and a carboxyl-terminal left-handed β-helix domain ending with a flexible, disordered C-terminal sequence that is fundamental for its function and regulation. The C-terminal peptide binds OASS-A

at the active site and it is responsible for its competitive inhibition. Even though exploitation of SAT inhibition holds promise as antibacterial adjuvant strategy, only a limited amount of literature about potential inhibitors is reported. In one case, after a virtual screening based on *E. coli* SAT crystal structure, a compound with an $IC_{SAT50}$ of 72 μM, (figure 2) was identified.[30] However, in the following studies, the compound was found to inhibit *E. histolytica* proliferation at a concentration of 0.61 μM, ruling out the possibility that inhibition of SAT was the main mechanism of action. In another case, a small library of natural products was screened for their inhibitory activity toward methicillin-resistant *Staphylococcus aureus* (MRSA) SAT, and six compounds inhibiting SAT activity ($IC_{50}$ ranging from 29.83 μM to 203.13 μM) were found.[26] Interestingly, in this case two of the hits (Figure 2, compounds **1** and **2**) showed an inhibitory effect on MRSA growth (MIC at 12.5 μg/ml and 25 μg/ml). Inspired by these encouraging preliminary data, we herein report the virtual screening of a library of compounds in order to find potential inhibitors of SAT. As the most relevant result, we were able to identify a hit compound showing weak antibacterial properties, consistent with inhibition of SAT.

**[insert Figure 2 here]**

## Results and discussion

### Virtual Screening

A crucial step of a VS is the selection of the chemical library. In general, to cross bacterial membrane, antibiotics must possess a series of peculiar physico-chemical characteristics, such as molecular weight (MW), LogD and polar surface area (PSA), which are poorly represented in commercial chemical libraries.[31–33] Therefore, compounds structures were retrieved from the ChemDiv Anti-infective (8,523), anti-bacterial (5,460), and antiviral (77,260) focused library collections, for a total of 91,243 compounds. Ligand 3D structures were prepared along the initial stage of the Schrödinger virtual screening workflow (vsw), where compounds were (*i*) filtered according to the Lipinsky's rule of five, (*ii*) removed when the stereochemistry was undefined, (*iii*) protonated at pH 7.4 using "epik", and (*iv*) clustered into conformers generated using the enhanced sampling methods. The structure of *Ec*SAT in complex with cysteine (1T3D) and the structure of *Hi*SAT in complex with CoA (1SSM) were used as protein structures for the VS (see Experimental section). The VS identified a pool of 1,409 compounds, which was reduced to 1,391 hits after visual inspection. The analysis of commercial antibacterials with activity towards Gram-negative pathogens suggests an average

ClogD of -2.8 and a polar surface area of 165 Å as preferred characteristics.[32] Therefore, 37 molecules with PSA lower than 200 and ClogD lower than 2 were initially cherry-picked to undergo activity assays.

**[Insert Figure 2 here]**

The analysis of the remaining 1.354 compounds was carried out using Chemgps software,[34,35] in order to evaluate scaffold similarity and purchase scaffolds as diverse as possible. Principal component (PC) analysis was carried out and in Figure 3 the representation of the 1.354 molecules is depicted using three PCs: PC1 (shape, size and polarizability), PC2 (aromaticity and conjugation) and PC3 (lipophilicity, polarity and H-bond capacity). This process allowed for the selection of 36 compounds with diverse chemical structures. At the end of the VS process, 73 compounds were purchased and evaluated for their ability to inhibit SAT in biochemical assay.

**Determination of *St*SAT inhibition**

While *St*SAT three-dimensional structure has not been solved yet, our group has a long-standing expertise in the characterization of cysteine biosynthetic enzymes from Salmonella, as above reported.[11,17,23] Since our ultimate goal is to develop molecules effective in blocking cysteine biosynthesis in *Salmonella*, we choose to test the compounds identified in the VS on *St*SAT. The very high overall sequence identity between the two proteins (i.e. *Ec*SAT and *St*SAT, with 100% identity of the active site residues) supports the use of *St*SAT in the activity assays. SAT catalyses the transfer of the acetyl group of acetyl-CoA to serine, with release of CoASH. SAT activity can be determined by measuring the decrease in absorbance at 232 nm due to hydrolysis of the thioester bond of acetyl-CoA.[36,37] However, this methodology is not suitable for screening of compound libraries since most organic molecules display high absorbance at 232 nm.

**[Insert Figure 4 here]**

**[Insert Figure 5 here]**

**[Insert Scheme 1 here]**

Since the transfer of the acetyl group of acetyl-CoA to serine occurs with release of the free thiol CoASH, we used an indirect assay for the determination of SAT activity. 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, also known as Ellman's reagent)) has a highly oxidizing disulfide bond that is stoichiometrically reduced by free thiols originating a mixed disulfide and a molecule of 5-thio-2-nitrobenzoic acid (TNB) (Scheme 1). The product TNB at pH higher than 7 absorbs strongly ($\varepsilon$ = 14,000 $M^{-1}cm^{-1}$)[38] and was thus used to follow the

catalytic activity of the enzyme.[16] The method was optimized and validated under our experimental conditions through the determination of the kinetic constants for L-Ser and acetyl-CoA and the $IC_{50}$ for the known inhibitor glycine (Figure 4). The results obtained agree with data in literature determined using the direct assay.[39] In particular, the $IC_{50}$ for glycine is in very good agreement with the $K_i$ 13 ± 4 mM previously reported.[25] The method was adapted and validated to a 96-well plate format to allow higher throughput. At the primary screening phase, the inhibitory activity of the 73 compounds from VS was determined at 100 μM (Figure 5). The obtained values for assay quality parameters, Z', SW and S/B, were robust enough to conclude that this assay can be used for screening procedures.[40,41] Inhibitory effects were first assessed by kinetic measurements with 27s intervals, since the total number of compounds allowed such measurements. Nevertheless, the 180s time-point was selected as the optimal endpoint measurement for calculation of %inhibition since it provided higher robustness of the parameters Z', SW and S/B. 13 compounds, displaying a percentage of inhibition ≥ 40% in the primary screen, were re-tested at 100 μM and, after the confirmatory testing, dose-response experiments were performed for 6 compounds displaying a percentage of inhibition higher than 50% at 100 μM concentration. These 6 compounds (Table 1) displayed micromolar $IC_{50}$ values towards *St*SAT with compound **7** being the most potent inhibitor with an $IC_{50}$ of 14 μM (Table 1). Being aware that many Gram negative antibacterial drug discovery programs fail because of the scarce aptitude for numerous small molecules to cross the cellular membrane, before establishing the most promising hit compound to more forward we decided to investigate the in-cell antimicrobial activity of compounds **3-8.**

**[Insert Table 1 here]**

**Inhibitory effects on *E. coli* growth**

The inhibitory activity of compounds **1-6** against the Gram-negative *E.coli* ATCC25922 was determined by using broth microdilution assays. As mentioned, testing SAT inhibitors in rich medium would be of poor significance for the purpose of this study. In fact, the cysteine present in the growth medium would be transported inside the cells thus reversing the inhibition of its biosynthesis. Therefore, minimum inhibitory concentrations (MICs) were evaluated both in conventional Muller-Hinton broth (MHB) and in minimal media (LB 20%) with limited amount of nutrients, where cysteine is absent and thus the effect of inhibiting its biosynthesis would lead to growth inhibiting effects. Compound **3** was able to interfere with bacterial growth in LB20% (Table 1), although at a fairly high concentration of 64 μg/mL. In addition, it can be speculated that

SAT inhibition is indeed accountable for the activity of compound **3,** as in rich medium, MHB, the MIC was not reached even at the highest concentration tested (128 μg/mL). Results were confirmed upon re-synthesis of compound **3**, previously used from a commercial source.

**Binding modes of compound 3**

The binding modes of the substrate cysteine and compound **3** are reported in Figure 6. Cysteine is bound inside *Ec*SAT active site through a network of H-bonds with Asp92, Asp157, His158, Arg192, and His193 (Figure 6a, dashed black lines). Interestingly, **3** establishes: (*i*) similar interaction with Asp92, Asp157, Arg192, and His193; (*ii*) a hydrophobic interaction with His158; (*iii*) further H-bond interaction with Gln133 and Gln258 (Figure 6b). The comparison of the binding modes of both cysteine and **3** reveals how compound **3** is able to mimic the interactions observed for cysteine (Figure 6c, d).

**[Insert Figure 6 here]**

In summary, antimicrobial resistance (AMR) is currently one of the most serious threats to public health, and developing strategies for its containment is a crucial pharmaceutical challenge. Adjuvant strategies, such as targeting the biosynthesis of molecular building blocks that are crucial for bacterial adaptation upon host invasion, represent an intriguing approach to face the onset of bacterial resistance. Among these strategies, inhibition of the biosynthesis of cysteine, an amino acid involved in the synthesis of detoxifying biomolecules such as glutathione, holds promise for further investigation. The VS of three focused libraries from ChemDiv containing 91,243 compounds was carried out in order to deliver an inhibitor of bacterial SAT, an enzyme responsible for the penultimate step of cysteine biosynthesis. The process led to the disclosure of 73 virtual hit compounds that were purchased and tested against *St*SAT. A screening methodology using Ellman's reagent to indirectly measure SAT activity was developed in 96-well plate format, yielding 6 compounds displaying an $IC_{50}$ below 100 μM that were further evaluated for growth inhibitory effects against *E. coli*. To rule out off-target effects, MIC assays were carried out in both complete and minimal media, and we were pleased to notice that compound **3** retained an encouraging inhibitory activity towards Gram-negative *E. coli* in cell-based assay.

Although aware that 2-aminothiazoles may cause a false positive, and taking into consideration the call made by some editors of the ACS journals with regard to the early identification of PAINS, we would like to point out that derivatives **3-8** were not recognized as PAINS using the software “False Positive Remover”

(http://www.cbligand.org/PAINS/) nor as aggregator according to the software "Aggregator Advisor" (http://advisor.bkslab.org/).

**Supporting Information** The Supporting Information is available free of charge athttps://pubs.acs.org/. Description of methods used to set up the virtual screening, protein expression and purification, determination of *St*SAT inhibition, and evaluation of MIC are reported in the supporting information.

**Corresponding Author information** marco.pieroni@unipr.it

**Author Addresses** Food and Drug Department, University of Parma, Parco Area delle Scienze 27, 43124, Parma

**Author Contributions** JM: synthesis, virtual screening, set up of the *St*SAT assay and preliminary microbiological investigation; NF: data collection, data analysis, SR: data collection and analysis, manuscript revision; GA: data analysis, manuscript revision; PT: Supervision of *St*SAT screening and MIC determination, manuscript revision; AB: computational analysis, manuscript revision; SB: study design, resources, manuscript revision; AM: resources, study design and supervision, manuscript revision; MP: supervision, manuscript writing and revision; BC: study design, supervision, manuscript writing and revision; GC: resources, supervision, manuscript revision.

**Acknowledgments**: This work was funded under the MSCA-ITN-2014-ETN project INTEGRATE [grant number 642620]. PT thanks the Academy of Finland (Grant. no. 277001) for financial support. The "Centro Interdipartimentale Misure "G. Casnati" is kindly acknowledged for the contribution in the analytical determination of the molecules synthesized.

**Abbreviations** AMR, antimicrobial resistance; CDI, 1,1'-Carbonyldiimidazole; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); MHB, Muller-Hinton broth; MRSA, methicillin-resistant *S. aureus*; NAS, N-acetylserine; OASS, *O*-acetylserine sulfhydrylase; PSA, polar surface area; SAT, serine acetyltransferase; VS, virtual screening.

**References**

(1) Antimicrobial resistance https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance (accessed Jan 16, 2020).

(2) Kraker, M. E. A. de; Stewardson, A. J.; Harbarth, S. Will 10 Million People Die a Year Due to Antimicrobial Resistance by 2050? *PLOS Med.* **2016**, *13* (11), e1002184. https://doi.org/10.1371/journal.pmed.1002184.

(3) O'Connell, K. M. G.; Hodgkinson, J. T.; Sore, H. F.; Welch, M.; Salmond, G. P. C.; Spring, D. R. Combating Multidrug-Resistant Bacteria: Current Strategies for the Discovery of Novel Antibacterials. *Angew. Chem. Int. Ed Engl.* **2013**, *52* (41), 10706–10733. https://doi.org/10.1002/anie.201209979.

(4) Blair, J. M. A.; Webber, M. A.; Baylay, A. J.; Ogbolu, D. O.; Piddock, L. J. V. Molecular Mechanisms of Antibiotic Resistance. *Nat. Rev. Microbiol.* **2015**, *13* (1), 42–51. https://doi.org/10.1038/nrmicro3380.

(5) Antibiotics: mode of action and mechanisms of resistance: art science https://www.scribd.com/document/202262261/Antibiotic-Action-and-Resistance (accessed Sep 3, 2019).

(6) Taylor, P. L.; Wright, G. D. Novel Approaches to Discovery of Antibacterial Agents. *Anim. Health Res. Rev.* **2008**, *9* (2), 237–246. https://doi.org/10.1017/S1466252308001527.

(7) Alekshun, M. N.; Levy, S. B. Targeting Virulence to Prevent Infection: To Kill or Not to Kill? *Drug Discov. Today Ther. Strateg.* **2004**, *1* (4), 483–489. https://doi.org/10.1016/j.ddstr.2004.10.006.

(8) Shallcross, L. J.; Howard, S. J.; Fowler, T.; Davies, S. C. Tackling the Threat of Antimicrobial Resistance: From Policy to Sustainable Action. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **2015**, *370* (1670), 20140082. https://doi.org/10.1098/rstb.2014.0082.

(9) Annunziato, G. Strategies to Overcome Antimicrobial Resistance (AMR) Making Use of Non-Essential Target Inhibitors: A Review. *Int. J. Mol. Sci.* **2019**, *20* (23), 5844. https://doi.org/10.3390/ijms20235844.

(10) Campanini, B.; Pieroni, M.; Raboni, S.; Bettati, S.; Benoni, R.; Pecchini, C.; Costantino, G.; Mozzarelli, A. Inhibitors of the Sulfur Assimilation Pathway in Bacterial Pathogens as Enhancers of Antibiotic

Therapy. *Curr. Med. Chem.* **2015**, *22* (2), 187–213. https://doi.org/10.2174/0929867321666141112122553.

(11) Spyrakis, F.; Singh, R.; Cozzini, P.; Campanini, B.; Salsi, E.; Felici, P.; Raboni, S.; Benedetti, P.; Cruciani, G.; Kellogg, G.; et al. Isozyme-Specific Ligands for O-Acetylserine Sulfhydrylase, a Novel Antibiotic Target. *PloS One* **2013**, *8*, e77558. https://doi.org/10.1371/journal.pone.0077558.

(12) Noji, M.; Takagi, Y.; Kimura, N.; Inoue, K.; Saito, M.; Horikoshi, M.; Saito, F.; Takahashi, H.; Saito, K. Serine Acetyltransferase Involved in Cysteine Biosynthesis from Spinach: Molecular Cloning, Characterization and Expression Analysis of CDNA Encoding a Plastidic Isoform. *Plant Cell Physiol.* **2001**, *42* (6), 627–634. https://doi.org/10.1093/pcp/pce078.

(13) Bhave, D. P.; Muse, W. B.; Carroll, K. S. Drug Targets in Mycobacterial Sulfur Metabolism. *Infect. Disord. Drug Targets* **2007**, *7* (2), 140–158.

(14) Turnbull, A. L.; Surette, M. G. L-Cysteine Is Required for Induced Antibiotic Resistance in Actively Swarming Salmonella Enterica Serovar Typhimurium. *Microbiol. Read. Engl.* **2008**, *154* (Pt 11), 3410–3419. https://doi.org/10.1099/mic.0.2008/020347-0.

(15) Turnbull, A. L.; Surette, M. G. Cysteine Biosynthesis, Oxidative Stress and Antibiotic Resistance in Salmonella Typhimurium. *Res. Microbiol.* **2010**, *161* (8), 643–650. https://doi.org/10.1016/j.resmic.2010.06.004.

(16) Kredich, N. M.; Tomkins, G. M. The Enzymic Synthesis of L-Cysteine in Escherichia Coli and Salmonella Typhimurium. *J. Biol. Chem.* **1966**, *241* (21), 4955–4965.

(17) Spyrakis, F.; Felici, P.; Bayden, A. S.; Salsi, E.; Miggiano, R.; Kellogg, G. E.; Cozzini, P.; Cook, P. F.; Mozzarelli, A.; Campanini, B. Fine Tuning of the Active Site Modulates Specificity in the Interaction of O-Acetylserine Sulfhydrylase Isozymes with Serine Acetyltransferase. *Biochim. Biophys. Acta* **2013**, *1834* (1), 169–181. https://doi.org/10.1016/j.bbapap.2012.09.009.

(18) Mozzarelli, A.; Bettati, S.; Campanini, B.; Salsi, E.; Raboni, S.; Singh, R.; Spyrakis, F.; Kumar, V. P.; Cook, P. F. The Multifaceted Pyridoxal 5'-Phosphate-Dependent O-Acetylserine Sulfhydrylase. *Biochim. Biophys. Acta* **2011**, *1814* (11), 1497–1510. https://doi.org/10.1016/j.bbapap.2011.04.011.

(19) Kredich, N. M.; Becker, M. A.; Tomkins, G. M. Purification and Characterization of Cysteine Synthetase, a Bifunctional Protein Complex, from Salmonella Typhimurium. *J. Biol. Chem.* **1969**, *244* (9), 2428–2439.

(20) Pieroni, M.; Annunziato, G.; Beato, C.; Wouters, R.; Benoni, R.; Campanini, B.; Pertinhez, T. A.; Bettati, S.; Mozzarelli, A.; Costantino, G. Rational Design, Synthesis, and Preliminary Structure-Activity Relationships of α-Substituted-2-Phenylcyclopropane Carboxylic Acids as Inhibitors of Salmonella Typhimurium O-Acetylserine Sulfhydrylase. *J. Med. Chem.* **2016**, *59* (6), 2567–2578. https://doi.org/10.1021/acs.jmedchem.5b01775.

(21) Annunziato, G.; Pieroni, M.; Benoni, R.; Campanini, B.; Pertinhez, T. A.; Pecchini, C.; Bruno, A.; Magalhães, J.; Bettati, S.; Franko, N.; et al. Cyclopropane-1,2-Dicarboxylic Acids as New Tools for the Biophysical Investigation of O-Acetylserine Sulfhydrylases by Fluorimetric Methods and Saturation Transfer Difference (STD) NMR. *J. Enzyme Inhib. Med. Chem.* **2016**, *31* (sup4), 78–87. https://doi.org/10.1080/14756366.2016.1218486.

(22) Magalhães, J.; Annunziato, G.; Franko, N.; Pieroni, M.; Campanini, B.; Bruno, A.; Costantino, G. Integration of Enhanced Sampling Methods with Saturation Transfer Difference Experiments to Identify Protein Druggable Pockets. *J. Chem. Inf. Model.* **2018**, *58* (3), 710–723. https://doi.org/10.1021/acs.jcim.7b00733.

(23) Magalhães, J.; Franko, N.; Annunziato, G.; Welch, M.; Dolan, S. K.; Bruno, A.; Mozzarelli, A.; Armao, S.; Jirgensons, A.; Pieroni, M.; et al. Discovery of Novel Fragments Inhibiting O-Acetylserine Sulphhydrylase by Combining Scaffold Hopping and Ligand-Based Drug Design. *J. Enzyme Inhib. Med. Chem.* **2018**, *33* (1), 1444–1452. https://doi.org/10.1080/14756366.2018.1512596.

(24) Magalhães, J.; Franko, N.; Annunziato, G.; Pieroni, M.; Benoni, R.; Nikitjuka, A.; Mozzarelli, A.; Bettati, S.; Karawajczyk, A.; Jirgensons, A.; et al. Refining the Structure-Activity Relationships of 2-Phenylcyclopropane Carboxylic Acids as Inhibitors of O-Acetylserine Sulfhydrylase Isoforms. *J. Enzyme Inhib. Med. Chem.* **2019**, *34* (1), 31–43. https://doi.org/10.1080/14756366.2018.1518959.

(25) Leu, L. S.; Cook, P. F. Kinetic Mechanism of Serine Transacetylase from Salmonella Typhimurium. *Biochemistry* **1994**, *33* (9), 2667–2671. https://doi.org/10.1021/bi00175a040.

(26) Chen, C.; Yan, Q.; Tao, M.; Shi, H.; Han, X.; Jia, L.; Huang, Y.; Zhao, L.; Wang, C.; Ma, X.; et al. Characterization of Serine Acetyltransferase (CysE) from Methicillin-Resistant Staphylococcus Aureus and Inhibitory Effect of Two Natural Products on CysE. *Microb. Pathog.* **2019**, *131*, 218–226. https://doi.org/10.1016/j.micpath.2019.04.002.

(27) Johnson, C. M.; Huang, B.; Roderick, S. L.; Cook, P. F. Kinetic Mechanism of the Serine Acetyltransferase from Haemophilus Influenzae. *Arch. Biochem. Biophys.* **2004**, *429* (2), 115–122. https://doi.org/10.1016/j.abb.2004.06.006.

(28) Inoue, K.; Noji, M.; Saito, K. Determination of the Sites Required for the Allosteric Inhibition of Serine Acetyltransferase by L-Cysteine in Plants. *Eur. J. Biochem.* **1999**, *266* (1), 220–227. https://doi.org/10.1046/j.1432-1327.1999.00847.x.

(29) Tyrrell, R.; Verschueren, K. H.; Dodson, E. J.; Murshudov, G. N.; Addy, C.; Wilkinson, A. J. The Structure of the Cofactor-Binding Fragment of the LysR Family Member, CysB: A Familiar Fold with a Surprising Subunit Arrangement. *Structure* **1997**, *5* (8), 1017–1032. https://doi.org/10.1016/S0969-2126(97)00254-2.

(30) Agarwal, S. M.; Jain, R.; Bhattacharya, A.; Azam, A. Inhibitors of Escherichia Coli Serine Acetyltransferase Block Proliferation of Entamoeba Histolytica Trophozoites. *Int. J. Parasitol.* **2008**, *38* (2), 137–141. https://doi.org/10.1016/j.ijpara.2007.09.009.

(31) Tommasi, R.; Brown, D. G.; Walkup, G. K.; Manchester, J. I.; Miller, A. A. ESKAPEing the Labyrinth of Antibacterial Discovery. *Nat. Rev. Drug Discov.* **2015**, *14* (8), 529–542. https://doi.org/10.1038/nrd4572.

(32) Brown, D. G.; May-Dracka, T. L.; Gagnon, M. M.; Tommasi, R. Trends and Exceptions of Physical Properties on Antibacterial Activity for Gram-Positive and Gram-Negative Pathogens. *J. Med. Chem.* **2014**, *57* (23), 10144–10161. https://doi.org/10.1021/jm501552x.

(33) O'Shea, R.; Moser, H. E. Physicochemical Properties of Antibacterial Compounds: Implications for Drug Discovery. *J. Med. Chem.* **2008**, *51* (10), 2871–2878. https://doi.org/10.1021/jm700967e.

(34) Larsson, J.; Gottfries, J.; Muresan, S.; Backlund, A. ChemGPS-NP: Tuned for Navigation in Biologically Relevant Chemical Space. *J. Nat. Prod.* **2007**, *70* (5), 789–794. https://doi.org/10.1021/np070002y.

(35) Rosén, J.; Lövgren, A.; Kogej, T.; Muresan, S.; Gottfries, J.; Backlund, A. ChemGPS-NP(Web): Chemical Space Navigation Online. *J. Comput. Aided Mol. Des.* **2009**, *23* (4), 253–259. https://doi.org/10.1007/s10822-008-9255-y.

(36) Takagi, H.; Yoshioka, K.; Awano, N.; Nakamori, S.; Ono, B. Role of Saccharomyces Cerevisiae Serine O-Acetyltransferase in Cysteine Biosynthesis. *FEMS Microbiol. Lett.* **2003**, *218* (2), 291–297. https://doi.org/10.1111/j.1574-6968.2003.tb11531.x.

(37) Yeon, J. Y.; Yoo, S. J.; Takagi, H.; Kang, H. A. A Novel Mitochondrial Serine O -Acetyltransferase, OpSAT1, Plays a Critical Role in Sulfur Metabolism in the Thermotolerant Methylotrophic Yeast Ogataea Parapolymorpha. *Sci. Rep.* **2018**, *8* (1), 1–12. https://doi.org/10.1038/s41598-018-20630-8.

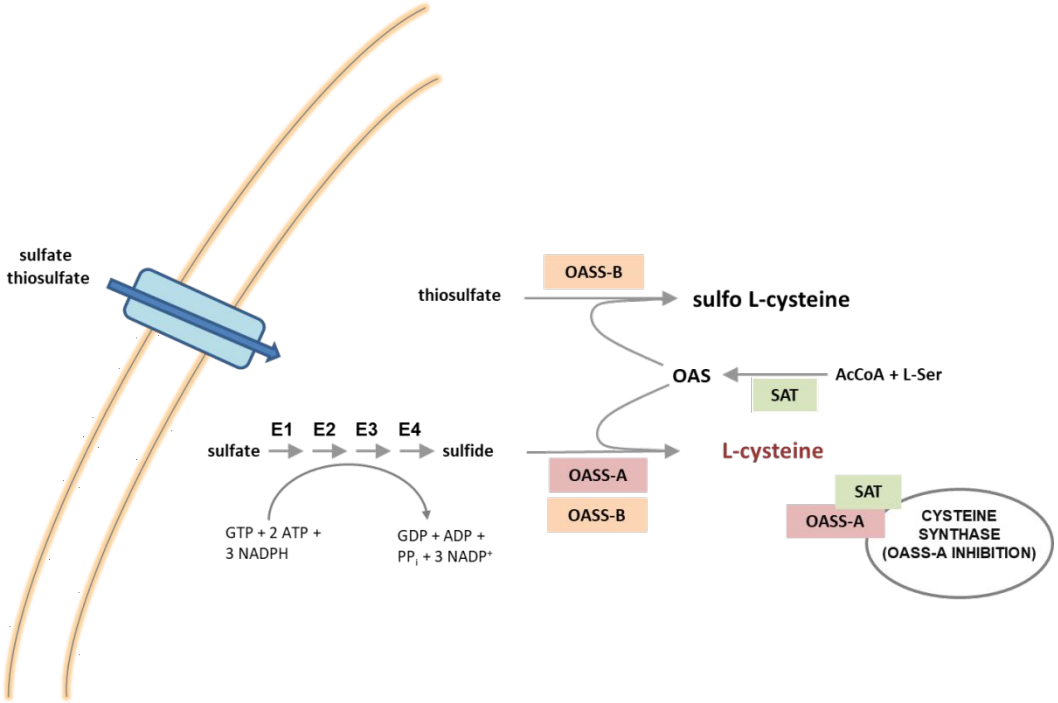
(38) Eyer, P.; Worek, F.; Kiderlen, D.; Sinko, G.; Stuglin, A.; Simeon-Rudolf, V.; Reiner, E. Molar Absorption Coefficients for the Reduced Ellman Reagent: Reassessment. *Anal. Biochem.* **2003**, *312* (2), 224–227. https://doi.org/10.1016/s0003-2697(02)00506-7.

(39) Baecker, P. A.; Wedding, R. T. Purification of Serine Acetyltransferase, a Component of a Multienzyme Complex, by Immunoadsorption and Selective Dissociation of the Complex. *Anal. Biochem.* **1980**, *102* (1), 16–21. https://doi.org/10.1016/0003-2697(80)90310-3.

(40) Inglese, J.; Shamu, C. E.; Guy, R. K. Reporting Data from High-Throughput Screening of Small-Molecule Libraries. *Nat. Chem. Biol.* **2007**, *3* (8), 438–441. https://doi.org/10.1038/nchembio0807-438.

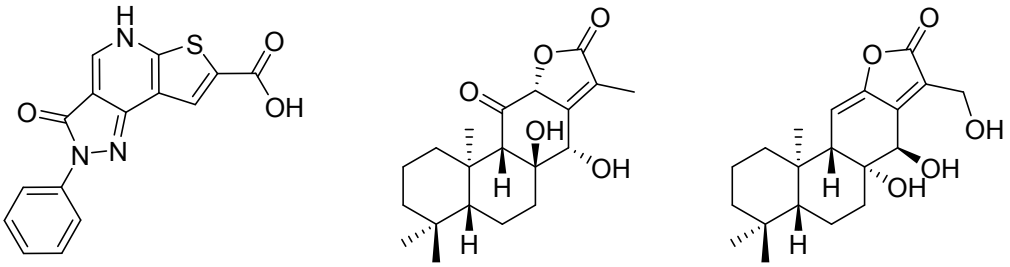
(41) Iversen, P. W.; Eastwood, B. J.; Sittampalam, G. S.; Cox, K. L. A Comparison of Assay Performance Measures in Screening Assays: Signal Window, Z' Factor, and Assay Variability Ratio. *J. Biomol. Screen.* **2006**, *11* (3), 247–252. https://doi.org/10.1177/1087057105285610.

**Figure 1**



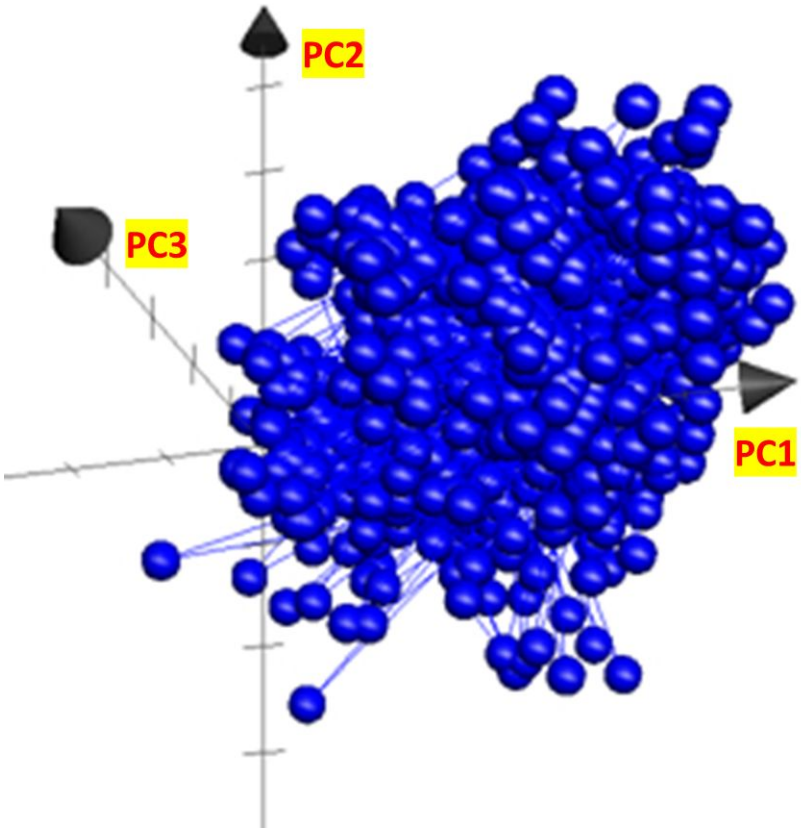

Reductive sulphate assimilation pathway in E. coli and Salmonella. OASS and SAT are highlighted. The enzymes that catalyse sulfate reduction are: E1, ATP sulfurylase; E2, APS kinase; E3, PAPS sulfotransferase; E4, NADPH sulfite reductase.

**Figure 2**



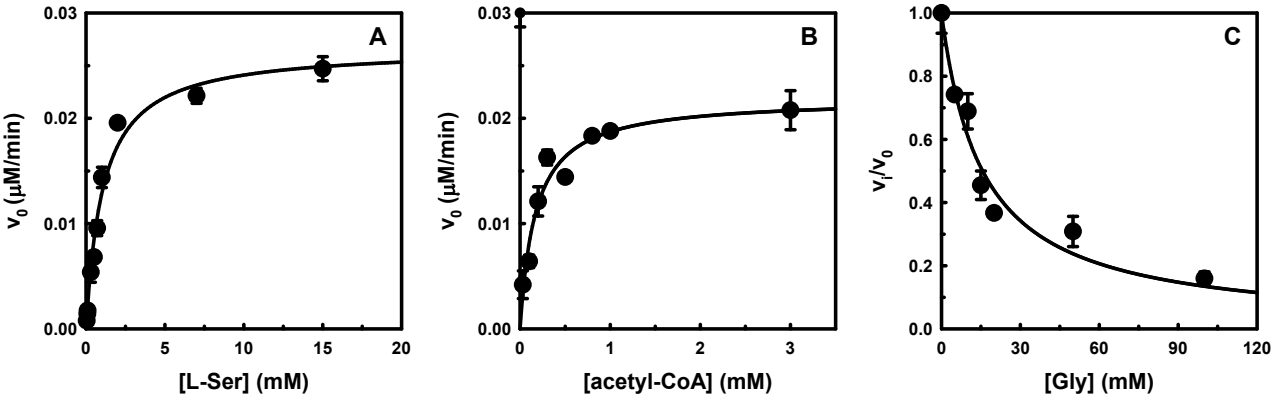

Chemical structure of some SAT inhibitors reported in literature.[26,28]

**Figure 3**




Graphical representation of the principal components of the 1,354 compounds. PC1 (shape, size and polarizability), is represented in the x axis, PC2 (aromaticity and conjugation) is in y axis and PC3 (lipophilicity, polarity and H-bond capacity) is represented in z axis.

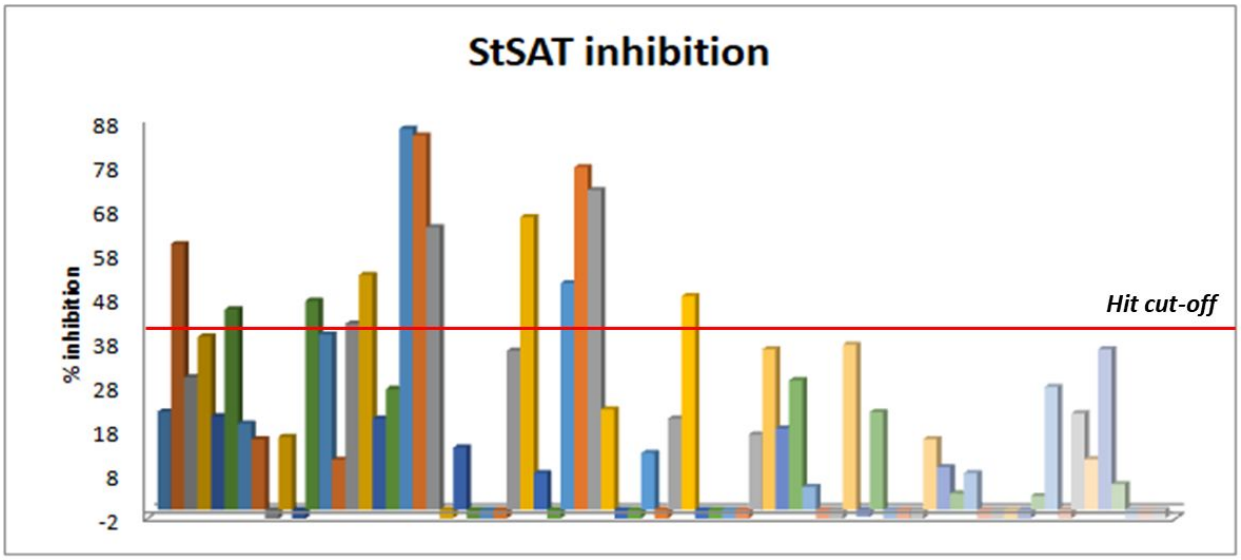
**Figure 4**




Validation of the indirect assay for the screening of potential SAT inhibitors. Panel A: initial reaction rate as a function of L-Ser concentration. The line through data points represents the fitting to the Michaelis-Menten equation with $k_{cat}$ = 3813 ± 169 $min^{-1}$, $K_m$ = 1.07 ± 0.15 mM, $k_{cat}/K_m$ = 3563 $min^{-1}$ $mM^{-1}$. Panel B: initial reaction rate as a function of acetyl-CoA concentration. The line through data points represents the fitting to

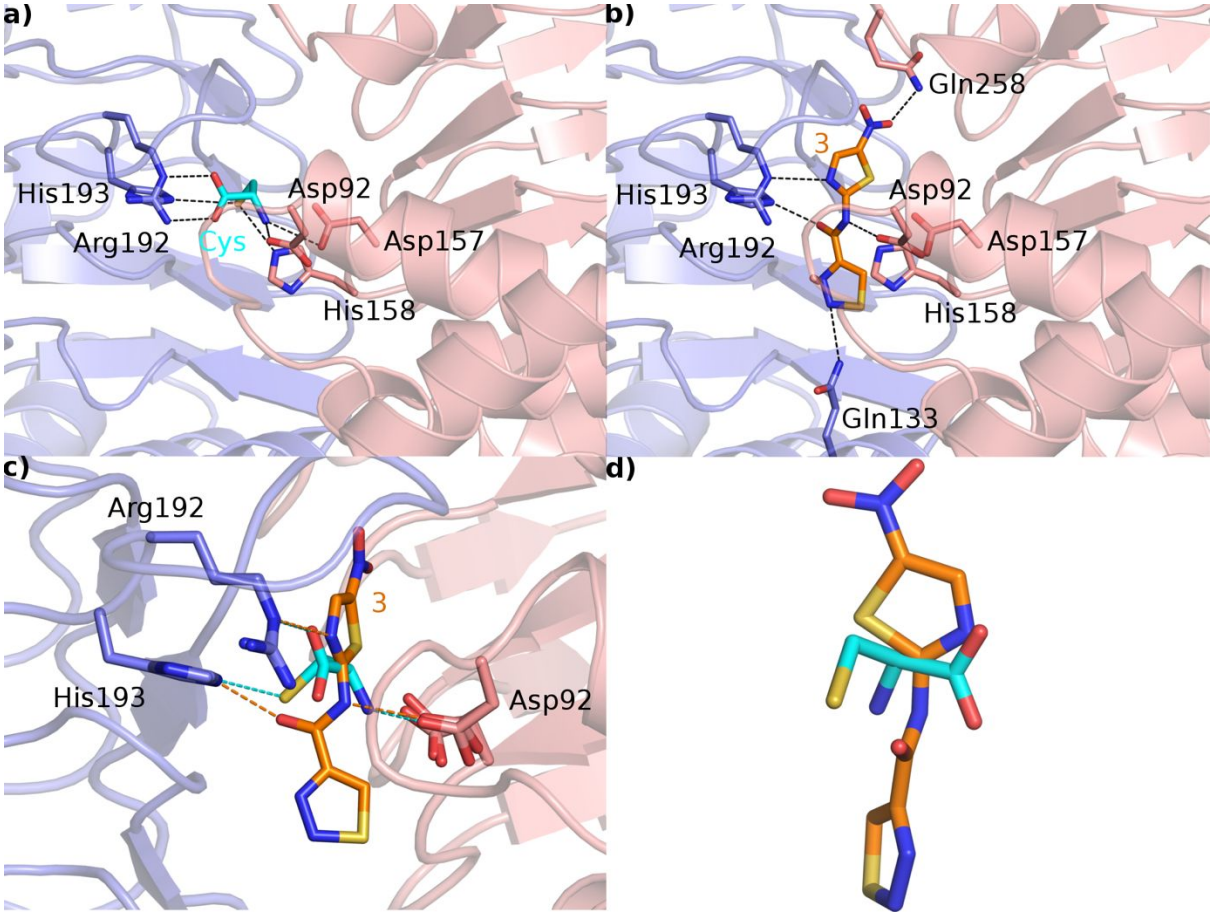
the Michaelis-Menten equation with $k_{cat}$ = 3128 ± 186 $min^{-1}$, $K_m$ = 0.17 ± 0.04 mM, $k_{cat}/K_m$ = 18400 $min^{-1}$ $mM^{-1}$. Panel C: relative activity as function of glycine concentration. The line through data points represents the fitting to a hyperbolic equation with $IC_{50}$ = 15 ± 1 mM.

**Figure 5**



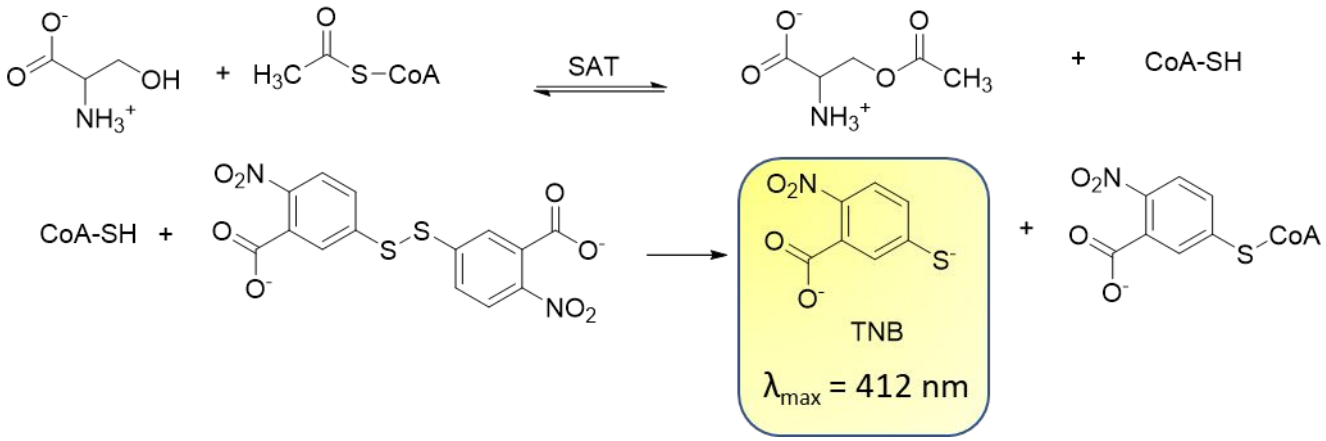

Determination of the inhibitory potential of the 73 purchased compounds against *St*SAT at time point 180s.

**Figure 6**



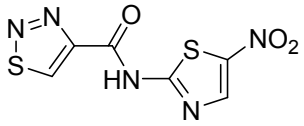
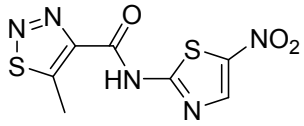
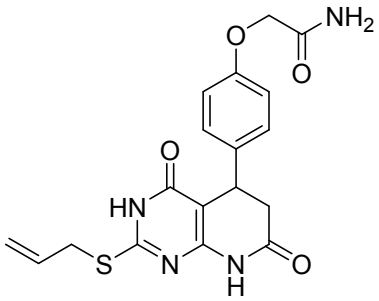
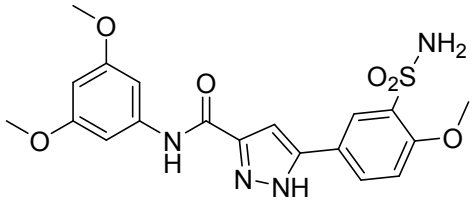
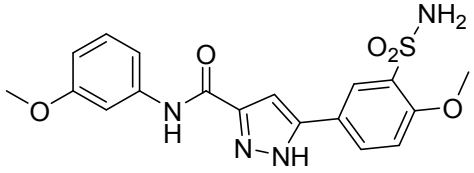
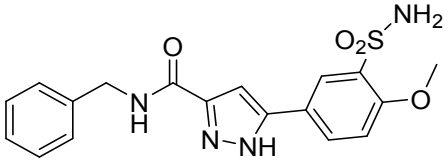

**a)** X-ray crystal structure of *Ec*SAT in complex with Cys (PDB code: 1T3D). The two monomeric chains of SAT are depicted as blue and pink transparent cartoon, amino acid side chains are depicted as sticks and color coded depending on the chain they belong to, the feedback inhibitor Cys is depicted in cyan sticks, while relevant H-bonds are depicted as dashed black lines; **b)** Binding mode of **3** (orange sticks) into SAT active site, the color code is the same reported in **a)**; **c)** Superposition of the SAT-Cys and SAT-**3** complexes, highlighted with dashed line are H-bonds conserved in both complexes (cyan dash lines for Cys, orange ones for **3**); **d)** Superposition of **3** (orange sticks) and Cys (cyan sticks).

**Scheme 1**




Indirect assay of SAT activity. CoA released by SAT activity spontaneously react with DTNB with the release of one molecule of TNB for each molecule of CoA produced.

**Table 1** – *St*SAT inhibition, $IC_{50StSAT}$, and MIC values against *E. coli* of the hits derived from Virtual Screening.

| Cmpd | Structure | % Inhibition @100μM | $IC_{50}$ (μM) | MIC (μg/mL) in LB 20% | MIC (μg/mL) in MHB |
|---|---|---|---|---|---|
| **3** |  | 68 | 48.6±8.43 | 64 | >128 |
| **4** |  | 72 | 47.8±5.35 | >128 | >128 |
| **5** |  | 57 | 84.1±5.39 | >128 | >128 |
| **6** |  | 52 | False positive[a] | >128 | >128 |
| **7** |  | 75 | 13.6±2.28 | >128 | >128 |
| **8** |  | 79 | 52.8±2.38 | >128 | >128 |

[a]Changing the dose between 200 and 50 μM always caused the same inhibition. At higher concentration, the compound was not soluble.