Letter

# Inhibition of Nonessential Bacterial Targets: Discovery of a Novel Serine *O*-Acetyltransferase Inhibitor

Joana Magalhães, Nina Franko, Samanta Raboni, Giannamaria Annunziato, Päivi Tammela, Agostino Bruno, Stefano Bettati, Andrea Mozzarelli, Marco Pieroni,* Barbara Campanini, and Gabriele Costantino
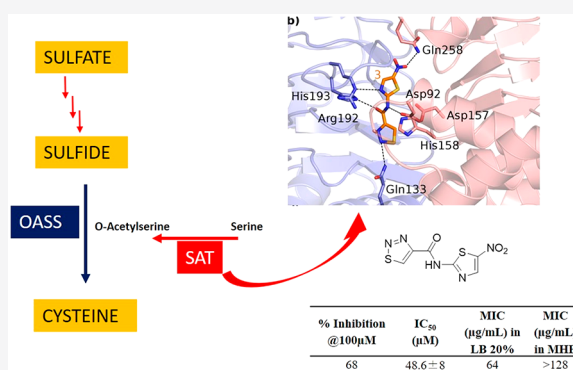
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**ABSTRACT:** In ϒ-proteobacteria and Actinomycetales, cysteine biosynthetic enzymes are indispensable during persistence and become dispensable during growth or acute infection. The biosynthetic machinery required to convert inorganic sulfur into 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 the analysis of the overall physicochemical properties allowed the selection of 73 compounds that were purchased and evaluated on the recombinant enzyme. Six compounds displaying an $IC_{50}$ <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.



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

Antimicrobial resistance (AMR) is one of the most threatening insults to public health worldwide. Features related to this upset are worrying, in terms of both loss of human lives and costs for hospitalization.[1] Experts estimate that 10 million people will die every year due to AMR 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, and protozoa) to nullify the therapeutic effects of antimicrobials, and it can affect anyone, at any age, in any country. In general, the 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 nonessential targets, such as those related to bacterial virulence 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 the 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 Actinomyce-tales (*Mycobacterium tuberculosis*)[10,13] because it has been shown that suppression or reduction of the cysteine biosyn-thesis led to decreased bacterial fitness and infectivity.[14,15] In
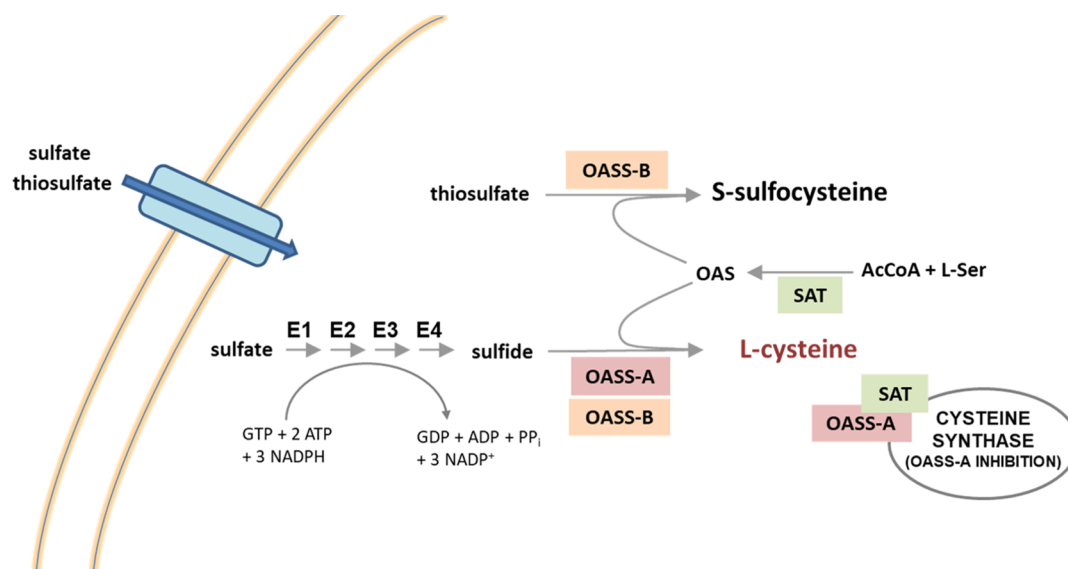
**Figure 1.** Reductive sulfate assimilation pathway in *E. coli* and *Salmonella*. OASS and SAT are highlighted. The enzymes that catalyze sulfate reduction are: E1, ATP sulfurylase; E2, APS kinase; E3, PAPS sulfotransferase; and E4, NADPH sulfite reductase.
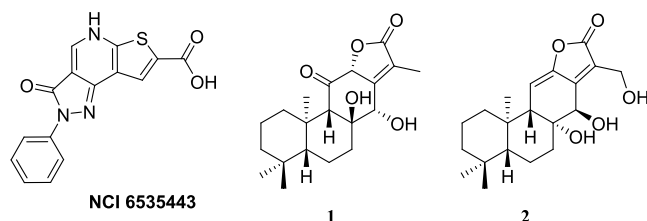


**Figure 2.** Chemical structures of some SAT inhibitors reported in the literature.[26,28]
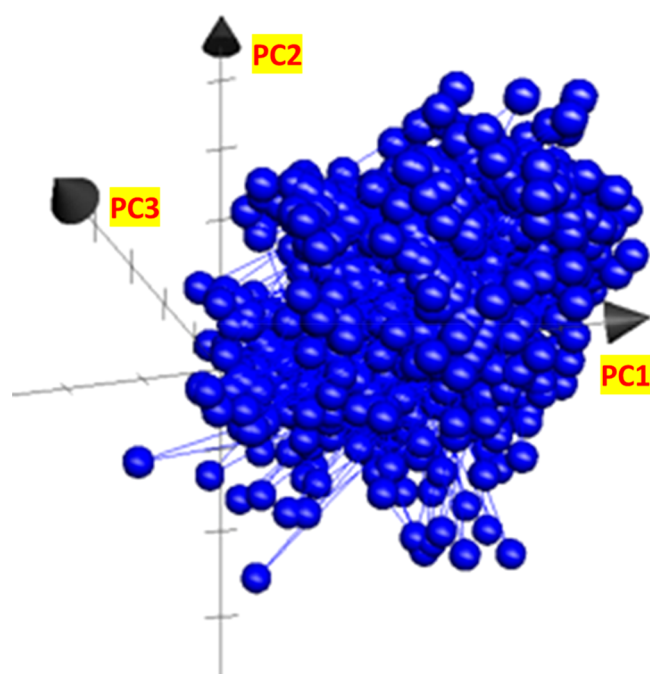


**Figure 3.** Graphical representation of the principal components of the 1354 compounds. PC1 (shape, size, and polarizability) is represented on the *x* axis, PC2 (aromaticity and conjugation) is on the *y* axis, and PC3 (lipophilicity, polarity, and H-bond capacity) is represented on the *z* axis.

both vegetative and swarm cell populations, the 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 sulfur, bacteria and plants possess highly conserved enzymes that enable its biosynthesis through the reductive sulfate assimilation pathway (Figure 1).[10,16]

Sulfate and thiosulfate, the most abundant forms of extracellular sulfur, enter the cell through specific transporters. Sulfate 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 the activation of sulfate by ATP with the formation of adenosine 5′-phosphosulfate (APS), 3′-phosphoadenosine-5′-phosphosulfate (PAPS) is synthesized. Afterward, 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 catalyzes a *β*-replacement reaction on *O*-acetylserine (OAS), synthesized by serine acetyltransferase (SAT).[19] It was demonstrated that the inhibition of OASS was able to revert the bacterial resistance to fluoroquinolones and tetracycline.[14,15] This 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 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 a thorough investigation of the structure−activity relationships for these OASS inhibitors,[21−24] we have deemed it to be of interest to investigate the effects of SAT inhibition, which is the upstream enzyme of the cysteine synthase complex.[25−28] It is notable that the inhibition of SAT is expected to exert partially distinct effects with respect to those brought about by OASS inhibition. Indeed, the inhibition of OASS is expected to induce the

**Scheme 1. Indirect Assay of SAT Activity[a]**
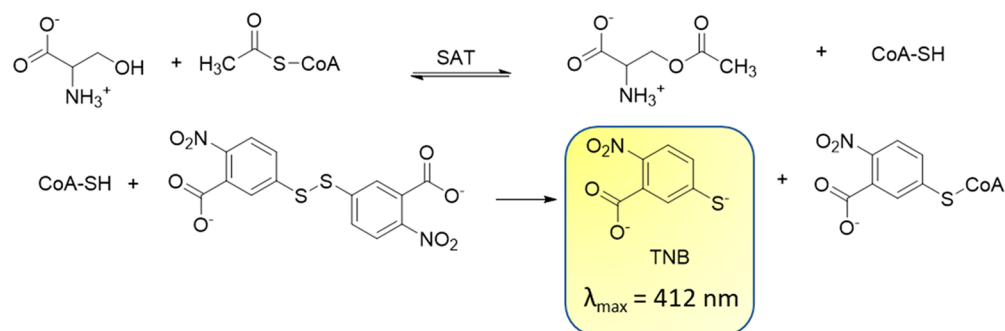


[a]CoA released by SAT activity spontaneously reacted with DTNB with the release of one molecule of TNB for each molecule of CoA produced.
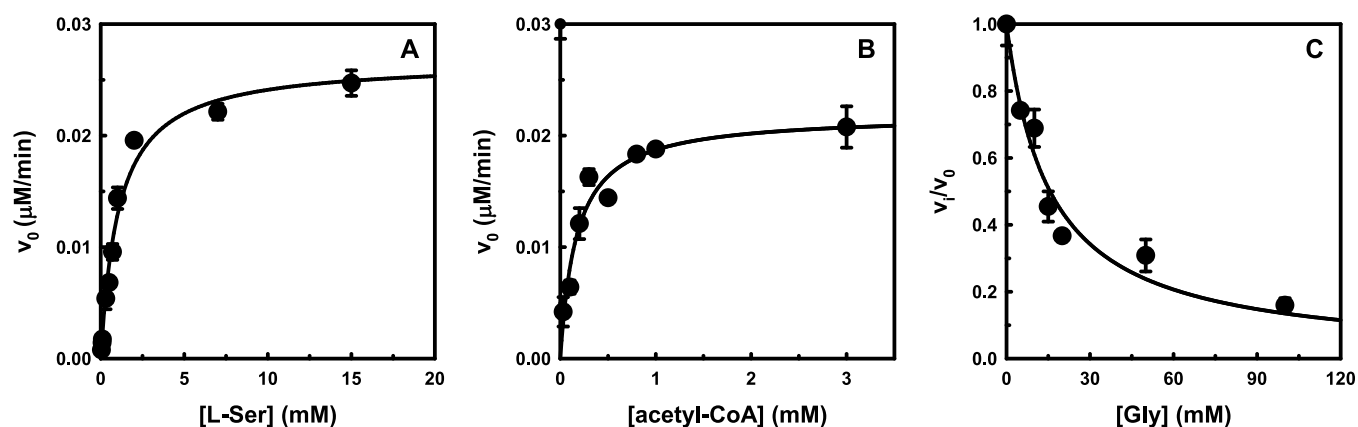


**Figure 4.** Validation of the indirect assay for the screening of potential SAT inhibitors. (A) Initial reaction rate as a function of L-Ser concentration. The line through data points represents the fitting to the Michaelis−Menten equation with $k_{cat}$ = 3813 ± 169 min$^{-1}$, $K_m$ = 1.07 ± 0.15 mM, and $k_{cat}/K_m$ = 3563 min$^{-1}$ mM$^{-1}$. (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, and $k_{cat}/K_m$ = 18 400 min$^{-1}$ mM$^{-1}$. (C) Relative activity as a function of glycine concentration. The line through data points represents the fitting to a hyperbolic equation with IC$_{50}$ = 15 ± 1 mM.
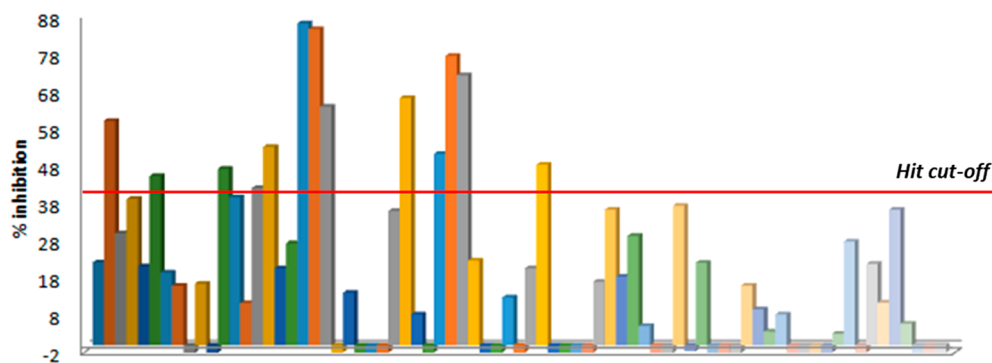


**Figure 5.** Determination of the inhibitory potential of the 73 purchased compounds against *St*SAT at time point 180s.

accumulation of OAS, which is converted to *N*-acetylserine (NAS), a natural inducer of the biosynthetic operon,[29] and cysteine depletion. Conversely, the inhibition of SAT is expected to deplete the cell not only of cysteine but also of the natural inducer. SAT catalyzes 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 the exploitation of SAT inhibition holds promise as an antibacterial adjuvant strategy, only a limited amount of literature about potential inhibitors is reported. In one case, after a virtual screening (VS) based on the *E. coli* SAT crystal structure, a compound with an IC$_{SAT50}$ of 72 $\mu$M (Figure 2), was identified.[30] However, in the following studies, the compound was found to inhibit *E. histolytica* proliferation at

**Table 1. StSAT 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.

a concentration of 0.61 μM, ruling out the possibility that the 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 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 and 25 μg/mL). Inspired by these encouraging preliminary data, we herein report the VS of a library of compounds to find potential inhibitors of SAT. As the most relevant result, we were able to identify a hit compound showing weak antibacterial properties, which is consistent with the inhibition of SAT.

**Results and Discussion.** *Virtual Screening.* A crucial step of a VS is the selection of the chemical library. In general, to cross the bacterial membrane, antibiotics must possess a series of peculiar physicochemical characteristics, such as molecular weight (MW), LogD, and polar surface area (PSA), which are poorly represented in commercial chemical libraries.[31−33] Therefore, compound structures were retrieved from the ChemDiv anti-infective (8523), antibacterial (5460), 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 Lipinski'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 the Experimental Procedures in the Supporting Information). The VS identified a pool of 1409 compounds, which was reduced to 1391 hits after visual inspection. The analysis of commercial antibacterial products with activity toward Gram-negative pathogens suggests an average ClogD of −2.8 and a PSA of 165 Å as preferred characteristics.[32] Therefore, 37 molecules with PSA <200 and ClogD <2 were initially cherry-picked to undergo activity assays.

The analysis of the remaining 1354 compounds was carried out using ChemGPS software[34,35] to evaluate the scaffold similarity and purchase scaffolds as diverse as possible. Principal component (PC) analysis was carried out, and in Figure 3, the representation of the 1354 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 StSAT Inhibition.* Whereas the *St*SAT 3D structure has not yet been solved, our group has a long-standing expertise in the characterization of cysteine biosynthetic enzymes from *Salmonella*, as previously reported.[11,17,23] Because our ultimate goal is to develop molecules that are effective in blocking cysteine biosynthesis in *Salmonella*, we chose to test the compounds identified in the
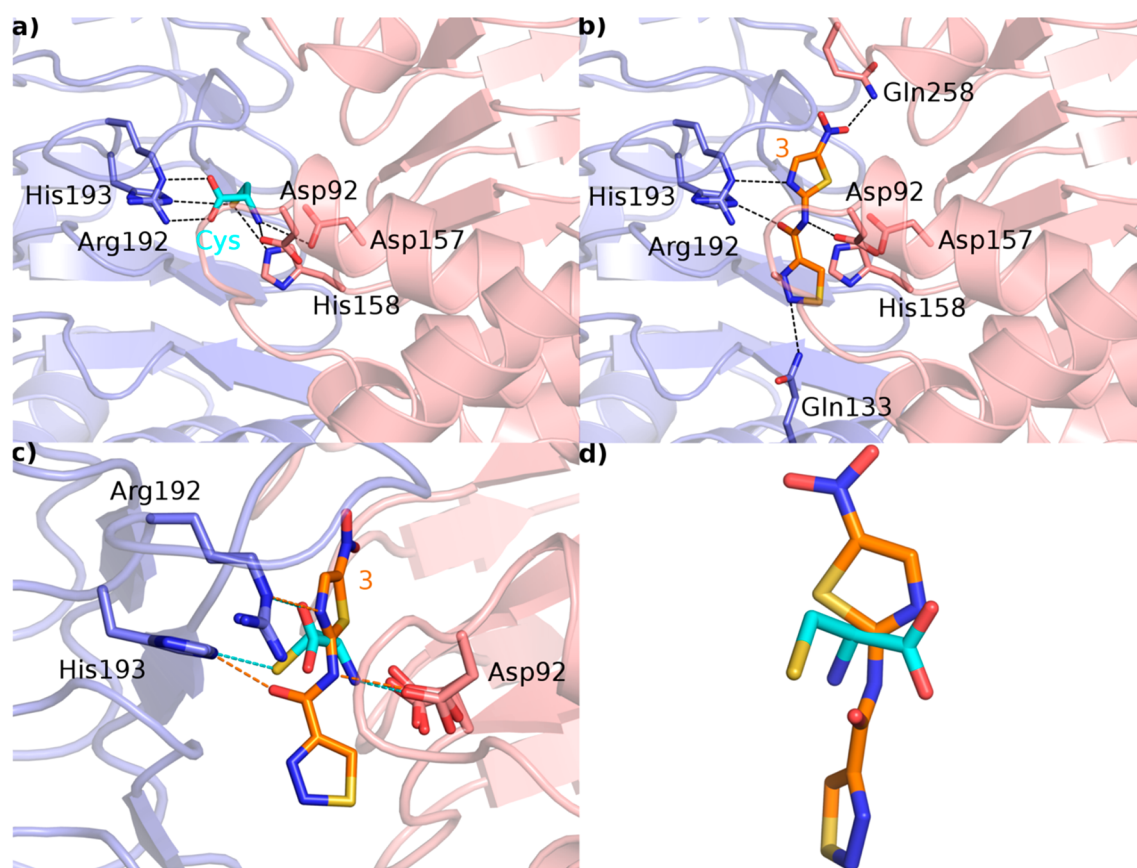
**Figure 6.** (a) X-ray crystal structure of *Ec*SAT in complex with Cys (PDB code: 1T3D). The two monomeric chains of SAT are depicted as blue and pink transparent cartoons, the amino acid side chains are depicted as sticks and are color-coded depending on the chain they belong to, the feedback inhibitor Cys is depicted as cyan sticks, and relevant H bonds are depicted as dashed black lines. (b) Binding mode of **3** (orange sticks) into the SAT active site. The color code is the same as that reported in panel a. (c) Superposition of the SAT-Cys and SAT-**3** complexes. Highlighted with dashed lines are H bonds conserved in both complexes (cyan dashed lines for Cys; orange dashed lines for **3**). (d) Superposition of **3** (orange sticks) and Cys (cyan sticks).

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 catalyzes the transfer of the acetyl group of acetyl-CoA to serine, with the release of CoASH. SAT activity can be determined by measuring the decrease in absorbance at 232 nm due to the hydrolysis of the thioester bond of acetyl-CoA.[36,37] However, this methodology is not suitable for screening of compound libraries because most organic molecules display high absorbance at 232 nm.

Because the transfer of the acetyl group of acetyl-CoA to serine occurs with the 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 from 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 the literature determined using the direct assay.[39] In particular, the IC$_{50}$ for glycine is in very good agreement with the $K_i$ of $13 \pm 4$

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 $\mu$M (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 27 s intervals because the total number of compounds allowed such measurements. Nevertheless, the 180 s time point was selected as the optimal endpoint measurement for calculation of the % inhibition because it provided a higher robustness of the parameters Z′, SW, and S/B. Thirteen compounds, displaying a percentage of inhibition ≥40% in the primary screen, were retested at 100 $\mu$M, and, after the confirmatory testing, dose−response experiments were performed for six compounds displaying a percentage of inhibition >50% at 100 $\mu$M concentration. These six compounds (Table 1) displayed micromolar IC$_{50}$ values toward *St*SAT, with compound **7** being the most potent inhibitor with an IC$_{50}$ of 14 $\mu$M (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 move forward, we decided to investigate the in-cell antimicrobial activity of compounds **3**−**8**.

*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 a 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 Müller-Hinton broth (MHB) and in minimal media (LB 20%) with a 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 $\mu$g/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 $\mu$g/mL). Results were confirmed upon the resynthesis 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 the *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; and (iii) a 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).

In summary, 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, the 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 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 a 96-well plate format, yielding six compounds displaying an IC$_{50}$ <100 $\mu$M 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 toward Gram-negative *E. coli* in the cell-based assay.

Although we are 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 (pan assay interference compounds), 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 aggre-gators according to the software "Aggregator Advisor" (http://advisor.bkslab.org/).

## ASSOCIATED CONTENT

### Supporting Information

## AUTHOR INFORMATION

### Corresponding Author
**Marco Pieroni** − *P4T group, University of Parma, 43124 Parma, Italy; Centro Interdipartimentale "Biopharmanet-tec", Università degli Studi di Parma, 43124 Parma, Italy;* orcid.org/0000-0001-9190-3712; Email: marco.pieroni@unipr.it

### Authors
**Joana Magalhães** − *P4T group, University of Parma, 43124 Parma, Italy*

**Nina Franko** − *Laboratory of Biochemistry and Molecular Biology, Department of Food and Drug, University of Parma, 43124 Parma, Italy*

**Samanta Raboni** − *Laboratory of Biochemistry and Molecular Biology, Department of Food and Drug, University of Parma, 43124 Parma, Italy; Institute of Biophysics, CNR, 56124 Pisa, Italy*

**Giannamaria Annunziato** − *P4T group, University of Parma, 43124 Parma, Italy*

**Päivi Tammela** − *Drug Research Program, Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, Helsinki FI-00014, Finland;* orcid.org/0000-0003-4697-8066

**Agostino Bruno** − *P4T group, University of Parma, 43124 Parma, Italy;* orcid.org/0000-0002-2731-980X

**Stefano Bettati** − *Department of Medicine and Surgery, University of Parma, 43125 Parma, Italy; National Institute of Biostructures and Biosystems, 00136 Rome, Italy; Institute of Biophysics, CNR, 56124 Pisa, Italy;* orcid.org/0000-0001-6787-0594

**Andrea Mozzarelli** − *Laboratory of Biochemistry and Molecular Biology, Department of Food and Drug, University of Parma, 43124 Parma, Italy; National Institute of Biostructures and Biosystems, 00136 Rome, Italy; Institute of Biophysics, CNR, 56124 Pisa, Italy;* orcid.org/0000-0003-3762-0062

**Barbara Campanini** − *Laboratory of Biochemistry and Molecular Biology, Department of Food and Drug, University of Parma, 43124 Parma, Italy*

**Gabriele Costantino** − *P4T group and Centro Interdipartimentale Misure (CIM) 'G. Casnati', University of Parma, 43124 Parma, Italy; Centro Interdipartimentale "Biopharmanet-tec", Università degli Studi di Parma, 43124 Parma, Italy*

Complete contact information is available at:
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### Author Contributions

J.M.: synthesis, virtual screening, set up of the *St*SAT assay, and preliminary microbiological investigation; N.F.: data

### ■ ABBREVIATIONS

AMR, antimicrobial resistance; APS, adenosine 5′-phosphosulfate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MHB, Müller-Hinton broth; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant *S. aureus*; NAS, *N*-acetylserine; OASS, *O*-acetylserine sulfhydrylase; PAINS, pan assay interference compounds; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PSA, polar surface area; SAT, serine acetyltransferase; TNB, 5-thio-2-nitrobenzoic acid; VS, virtual screening.

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