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Development of tag-free photoprobes for studies aimed at identifying the target of novel Group A Streptococcus antivirulence agents

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ABSTRACT

We previously reported the identification and development of novel inhibitors of streptokinase (SK) expression by Group A Streptococcus (GAS), originating from a high throughput cell-based phenotypic screen. Although phenotypic screening is well-suited to identifying compounds that exert desired biological effects in potentially novel ways, it requires follow-up experiments to determine the macromolecular target(s) of active compounds. We therefore designed and synthesized several classes of chemical probes for target identification studies, guided by previously established structure–activity relationships. The probes were designed to first irreversibly photolabel target proteins in the intact bacteria, followed by cell lysis and click ligation with fluorescent tags to allow for visualization on SDS–PAGE gels. This stepwise, 'tag-free' approach allows for a significant reduction in molecular weight and polar surface area compared to full-length fluorescent or biotinylated probes, potentially enhancing membrane permeability and the maintenance of activity. Of the seven probes produced, the three most biologically active were employed in preliminary target identification trials. Despite the potent activity of these probes, specific labeling events were not conclusively observed due to a considerable degree of nonspecific protein binding. Nevertheless, the successful synthesis of potent biologically active probe molecules will serve as a starting point for initiating more sensitive methods of probe-based target identification.

The growing threat of bacterial resistance to antibiotic treatment necessitates the development of new antibacterial agents with novel mechanisms of action.[1] Bacterial pathogens express genes known as virulence factors that do not contribute to the growth and maintenance of the cell, but are critical for infection in the host. As a result, virulence-attenuating therapeutics have emerged as a potential mechanism for managing bacterial infection without selecting for mutants that are resistant to treatment.[2] Our group has applied this paradigm toward the development of a new class of anti-virulence antibiotics that suppress the expression of streptokinase (SK), a bacterial activator of human plasmin, that plays a direct role in enhancing Group A Streptococcus (GAS) virulence.[3] The lead compound of this series (**1**, Scheme 1) was identified via a cell-based high-throughput screen (HTS) for compounds that reduce *ska* gene transcription without inhibiting bacterial growth.[4] Optimization of the lead compound through structure-activity relationship (SAR) studies[5] led to a number of incremental improvements in activity and lipophilicity (**2** and **3**), eventually resulting in the discovery of potent analog **4** with a 35-fold greater potency and 2-log reduction in *c* Log*P* compared to lead 1.

Phenotype-based HTS strategies, like the one used to identify 1, return hit compounds with physicochemical properties sufficient for activity in whole cells and do not rely on *a priori* knowledge of the affected biological pathway, making them useful for discovering compounds with novel mechanisms of action.[6,7] These advantages,

Abbreviations: SK, streptokinase; GAS, Group A Streptococcus; HTS, high-throughput screen; SAR, structure–activity relationship; TPSA, topological polar surface area; *c* Log*P*, logarithm of the calculated octanol–water partition coefficient; LDA, lithium diisopropylamide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; SILAC, stable isotope labeling by amino acids in cell culture; THY, Todd–Hewitt media with 0.2% yeast extract; ESI-MS, electrospray ionization mass spectrometry.

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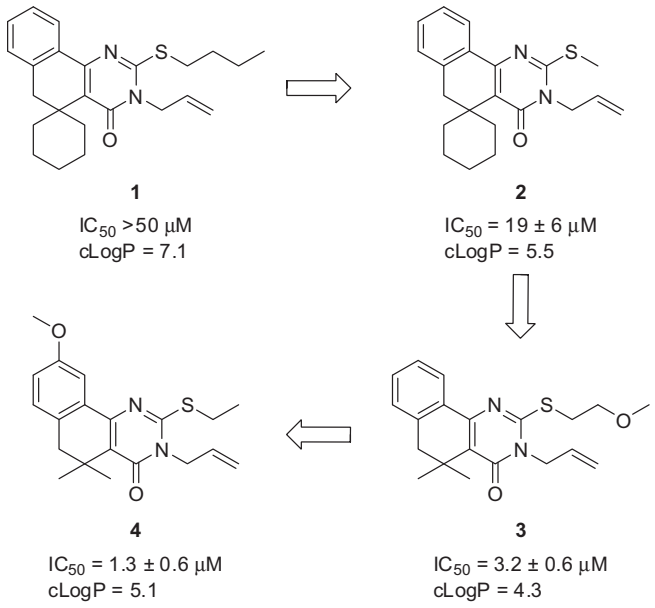


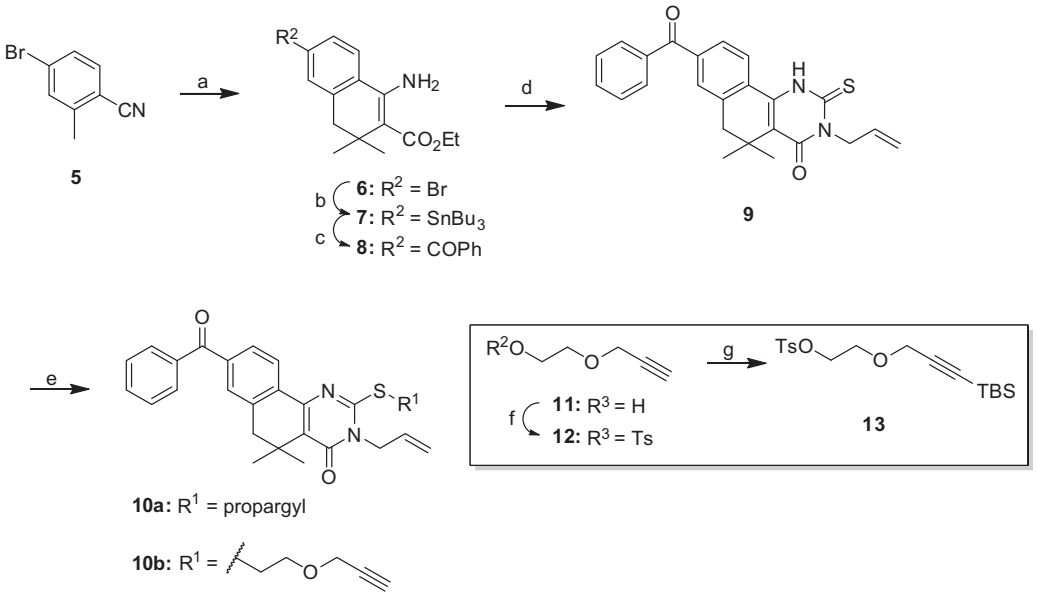
Scheme 1. Selected compounds from the SAR effort leading to potent analogs of screening hit **1**.

taken together with the lack of antibiotic leads discovered via bacterial target-based screening,[8] suggest phenotypic screening may be a potentially more fruitful tool for identifying novel antibacterial agents. However, phenotypic screening does not explicitly identify the molecular target of individual hit compounds, and therefore they must be established through subsequent studies. In the context of our project, identifying the target of this compound series would be important for (a) helping to establish a biochemical assay with which to improve the potency and specificity of the series, and (b) elucidating potentially novel virulence control pathways.

The potent activity of our compounds against SK expression, combined with RNA microarray data indicating the down-regulation of other important GAS virulence factors,[4] suggests that their macromolecular target(s) are involved in the upstream regulation of GAS virulence mechanisms. Several proteins governing GAS virulence have been studied in detail,[9] including Mga,[10] Rgg,[11,12] and CovR/CovS,[13] but the genomic sequencing of several clinically relevant GAS serotypes has revealed multiple well-conserved virulence control elements that remain uncharacterized.[14] Thus, identifying the target of this compound series has the potential to ascertain novel control mechanisms and further elucidate the complex nature of GAS virulence.

The use of chemical probes is a proven strategy for successfully establishing the protein targets of small molecules.[15–18] We chose to pursue a tandem photolabeling-bioorthogonal conjugation strategy that has become widespread since the development of click chemistry.[19–21] In this approach, a small-molecule analog of a potent compound possessing a photoreactive group and a terminal alkyne is covalently crosslinked to target proteins in the intact cellular milieu with UV light. After cell lysis, an azide-modified fluorescent or biotin-derived moiety can then be appended to the alkyne-functionalized protein(s) via copper(I)-mediated click chemistry, resulting in target proteins with covalently attached tags for visualization or selective purification. The lower molecular weight and topological polar surface area (TPSA) of these 'tag-free'[20] compounds compared to traditional biotinylated probes increases their likelihood of being cell-permeable, allowing them to be used in whole-cell systems rather than lysates. Cell-permeable affinity probes are advantageous in that their biological activity can be confirmed in phenotypic assays before beginning target identification studies. The probes also have access to all proteins in their native cellular conformations.

We envisioned the design of several tag-free photoprobes based on structural insights gained from our SAR studies on this scaffold.[5] Although the maintenance of a high level of potency was a primary concern, the nature and positioning of the UV-active and terminal alkyne groups were equally important to us as they are crucial for ensuring a compatible orientation for labeling.[22,23] A number of different photolabile groups have been successfully employed in the literature, varying in their stability, reactivity, and preference for carbon–carbon or carbon–heteroatom bond formation.[24] The unknown nature of the binding site ultimately determines the structural features necessary for a functional probe, so we set out



Scheme 2. Synthesis of benzophenone-functionalized tag-free probes **10a**–**b**. Reagents and conditions: (a) LDA, 1 h; then ethyl 3,3-dimethyl acrylate, ZnI_2, diglyme, −78 °C to rt, 2 h, 50%; (b) $(Bu_3Sn)_2$, $Pd(PPh_3)_4$, toluene, reflux, 16 h, 37%; (c) 1 atm CO, $PdCl_2(PPh_3)_2$, iodobenzene, DMF, 60 °C, 4 h, 58%; (d) allyl isothiocyanate, AcOH, EtOH, 70 °C, 16 h, 27%; (e) propargyl bromide or 12, Cs_2CO_3, 2-butanone, 70 °C, 16 h, 69–70%; (f) Ts–Cl, pyridine, 0–4 °C, 24 h, 72%; (g) *n*BuLi, −78 °C, THF, 2 h, then TBS-OTf, −78 °C to rt, 1 h, 63%.