



Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

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



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ARTICLE INFO

Article history:

Received 22 January 2014

Accepted 27 January 2014

Available online 7 February 2014

Keywords:

Group A Streptococcus
Virulence inhibitor
Streptokinase
Antibiotic
Phenotypic screening
Target identification
Tag-free photoprobes
Photo-crosslinking
Click chemistry

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 step-wise, ‘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.

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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

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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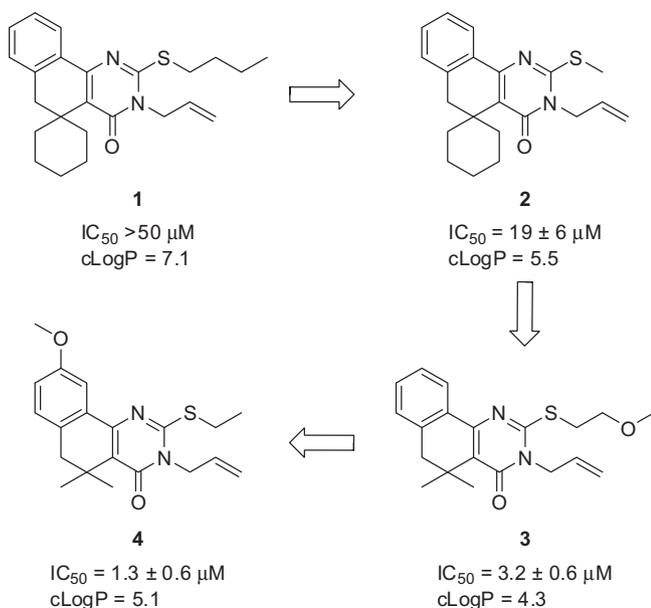
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<http://dx.doi.org/10.1016/j.bmcl.2014.01.079>

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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) studies⁵ 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,



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

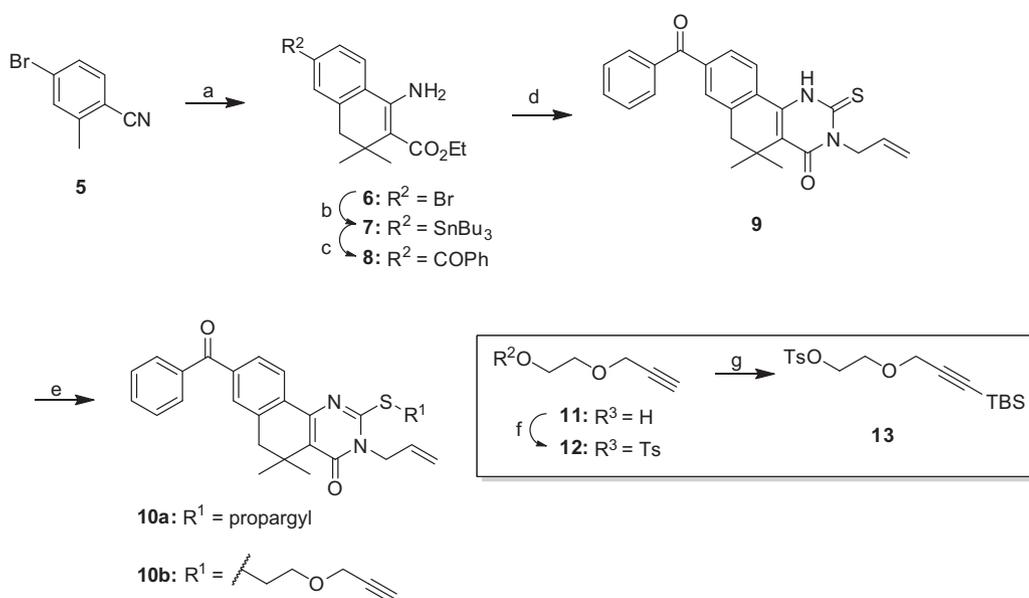
taken together with the lack of antibiotic leads discovered via bacterial target-based screening,⁸ suggest phenotypic screening may be a potentially more fruitful tool for identifying novel antibacterial agents. However, phenotypic screening does not explicitly identify the molecular target of individual 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



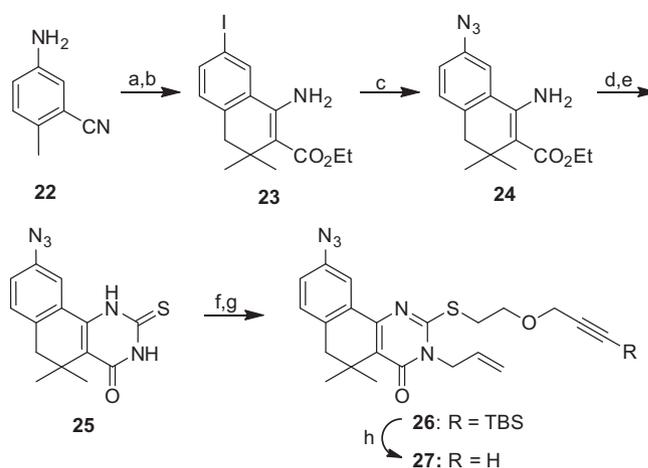
Scheme 2. Synthesis of benzophenone-functionalized tag-free probes **10a–b**. Reagents and conditions: (a) LDA, 1 h; then ethyl 3,3-dimethyl acrylate, ZnI₂, diglyme, –78 °C to rt, 2 h, 50%; (b) (Bu₃Sn)₂, Pd(PPh₃)₄, toluene, reflux, 16 h, 37%; (c) 1 atm CO, PdCl₂(PPh₃)₂, iodobenzene, DMF, 60 °C, 4 h, 58%; (d) allyl isothiocyanate, AcOH, EtOH, 70 °C, 16 h, 27%; (e) propargyl bromide or 12, Cs₂CO₃, 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%.

to synthesize a diverse series of compounds with one of three photolabile groups (benzophenone, diazirine, aryl azide) at different points on the scaffold to maximize the probability of identifying the target.

The chemistry to install benzophenone, diazirine, and aryl azide functionality onto the core scaffold of this class of compounds required three distinct synthetic routes. Synthesis of the benzophenone-based probes (**10a–b**, Scheme 2) began with the LDA-promoted Michael addition of 4-bromo-2-methylbenzonitrile (**5**) to ethyl 3,3-dimethyl acrylate and subsequent ZnI₂-mediated cyclization to afford β-aminoester **6**.²⁵ Stannylation via halogen-metal exchange²⁶ followed by carbonylative Stille coupling²⁷ successfully delivered benzophenone **8**. Acid-catalyzed addition of the amine to allyl isothiocyanate and subsequent cyclization generated 2-thioxopyrimidinone **9** that underwent S-alkylation with propargyl bromide or **12** (generated via tosylation of commercially available propynol ethoxylate **11**) under mildly basic conditions to generate probes **10a** and **10b**.

Diazirine probes **19a–d** were synthesized via an analogous route beginning from aryl methyl ether **14** (Scheme 3). Demethylation and protection of the resulting phenol as a silyl ether resulted in **15**, which could be employed in the synthesis of β-aminoester **16** in a manner similar to the one used to generate **6** in Scheme 3. In the course of our investigation, we found that cyclization with allyl isothiocyanate in the presence of cesium carbonate installed the desired 2-thioxopyrimidinone ring system while simultaneously cleaving the silyl ether protecting group, affording common intermediate **17**. Successive chemoselective alkylations at sulfur and oxygen were exploited to install the diazirine and alkyne linker moieties in four different arrangements, yielding probes **19a–d**. Diazirine-containing alkylating agent **21** was synthesized from 4-hydroxy-2-butanone (**20**) via diaziridination with 7N methanolic ammonia and hydroxylamine *O*-sulfonic acid,²⁸ followed by oxidation and tosylation.²⁹

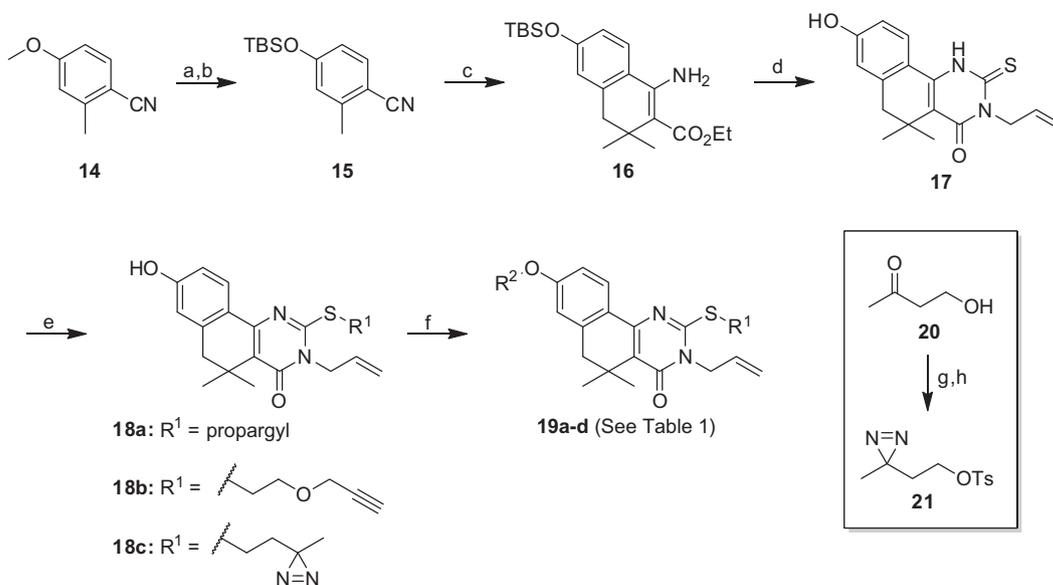
Synthesis of the aryl azide probe **27** began from commercially available 2-cyano-3-methylaniline **22** (Scheme 4). Masking the aniline functionality of **22** as a mono- or bis-Boc carbamate, diphenyl imine, or azide proved incompatible with the LDA/ZnI₂ cyclization conditions. Finally, it was found that modified Sandmeyer



Scheme 4. Synthesis of aryl azide-functionalized tag-free probe **27**. Reagents and conditions: (a) NaNO₂, HCl, then KI, H₂O, 0 °C; (b) LDA, then ethyl 3,3-dimethyl acrylate, ZnI₂, diglyme, −78 °C to rt, 3 h, 16% over 2 steps; (c) NaN₃, CuI, *N,N*-dimethyl ethylene diamine, Na-ascorbate, DMSO/H₂O (5:1), rt, 3 h, 85%; (d) benzoyl isothiocyanate, EtOH, 70 °C, 3 h; (e) KOH, EtOH/H₂O, 2:1, 70 °C, 3 h, 54% over 2 steps; (f) **13** (Scheme 2), NaHCO₃, 70 °C, DMF, 16 h; (g) NaOMe, allyl bromide, EtOH, 70 °C, 3 h, 42% over 2 steps; (h) TBAF, THF, 0 °C, 1 h, 65%.

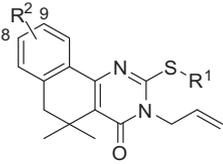
conditions to transform the aniline to the aryl iodide³⁰ permitted the formation of β-aminoester **23** in sufficient yields. Conversion to the corresponding aryl azide **24** via Cu(I)-mediated S_NAr proceeded in excellent yields.³¹ Stepwise generation of the unsubstituted 2-thioxopyrimidinone with benzoyl isothiocyanate and potassium hydroxide afforded **25**,³² which was then selectively S-alkylated with TBS-protected alkyne-containing unit **13**. Alkyne protection was necessary to minimize cross-reactivity during the subsequent *N*-allylation under basic conditions to furnish **26**. Finally, fluoride ion-promoted removal of the silyl group yielded the desired aryl azide probe **27**.

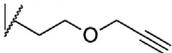
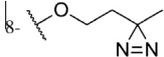
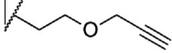
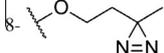
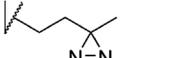
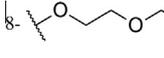
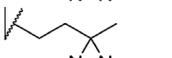
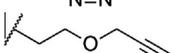
All completed tag-free affinity probes were assessed for their inhibitory effects on streptokinase expression using our previously described assay.⁴ Briefly, GAS culture supernatant was mixed with



Scheme 3. Synthesis of diazirine-functionalized tag-free probes **19a–d**. Reagents and conditions: (a) BBr₃, DCM, 0 °C–rt, 16 h; (b) TBS-Cl, imidazole, DCM, 0 °C–rt, 16 h, 96% over 2 steps; (c) LDA, then ethyl 3,3-dimethyl acrylate, ZnI₂, diglyme, −78 °C to rt, 3 h, 63%; (d) allyl isothiocyanate, Cs₂CO₃, EtOH, 70 °C, 16 h, 57%; (e) **12**, **21**, or propargyl bromide, NaHCO₃, 70 °C, DMF, 3–6 h, 45–71%; (f) **12**, **21**, or propargyl bromide, Cs₂CO₃, 70 °C, DMF, 3 h, 16–64%; (g) 7 N NH₃:MeOH, hydroxylamine-*O*-sulfonic acid, 0 °C–rt, 16 h; then I₂, 0 °C, 30 min; (h) Ts-Cl, pyridine, 0–4 °C, 24 h, 15% over 3 steps.

Table 1
Activity data for tag-free photoprobes **10**, **19**, and **27**

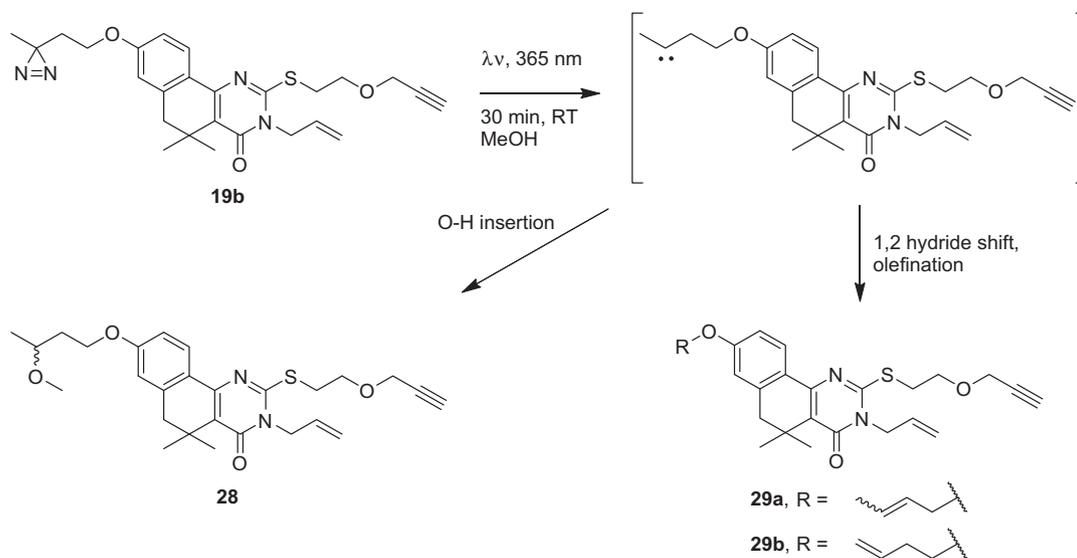


Compd	R ¹	R ²	T/C (5 μM) ^a	Growth T/C (50 μM) ^b	IC ₅₀ ^c (μM)
10a	Propargyl	8-Bz	0.77 ± 0.34	1.09 ± 0.04	nd
10b		8-Bz	0.51 ± 0.07	0.94 ± 0.18	3.8 ± 0.6
19a	Propargyl		1.07 ± 0.16	0.96 ± 0.09	nd
19b			0.49 ± 0.34	1.07 ± 0.02	0.33 ± 0.70
19c			0.71 ± 0.40	1.01 ± 0.09	nd
19d		8-O-Propargyl	0.89 ± 0.16	1.02 ± 0.01	nd
27		9-N ₃	0.30 ± 0.08	0.98 ± 0.04	0.19 ± 0.26
4	Et	9-OMe	0.10 ± 0.06	0.73 ± 0.07	1.3 ± 0.6

^a Amount of SK activity in GAS culture measured via colorimetric assay (A_{405}) after incubation with 5 μM test compound for 24 h divided by SK activity measured in GAS culture 24 h after treatment with DMSO control, +/- standard deviation.

^b Growth inhibition as measured by OD₆₀₀ of GAS culture 24 h after treatment with 50 μM test compound divided by OD₆₀₀ of control GAS culture treated with DMSO control after 24 h, +/- standard deviation.

^c Concentration at which 50% maximal inhibitory effect against SK expression was achieved +/- standard deviation. Each data point is derived from the statistical treatment of at least 3 replicates. nd = not determined.



Scheme 5. UV-induced reactivity of probe compound **19b** in methanol.

human plasma, and the resulting plasmin activity was assayed by turnover of a plasmin-specific chromogenic substrate by monitoring absorbance at 405 nm. Activity data are reported in [Table 1](#) as the ratio of plasmin activity in test cultures divided by plasmin activity in a control culture treated with DMSO alone. Since retaining high potency is important for target identification, 50% streptokinase expression inhibition at 5 μM was used as the

cutoff value for further evaluating probe compounds. Compounds meeting this threshold were more completely characterized with full IC₅₀ determinations. Results are summarized in [Table 1](#).

Benzophenone **10b**, with its *S*-propargyloxyethyl click ligation sidechain, was found to be more potent than the corresponding *S*-propargyl analog **10a**. This trend was also evident in diazirine probes **19a** and **19b**. Disappointingly, reversing the positioning of

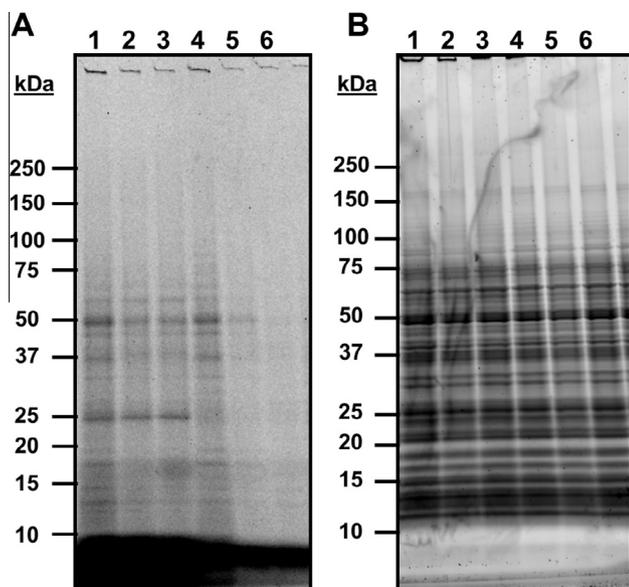


Figure 1. Crosslinking trials using probe **10b**. GAS cultures were grown in the presence of **10b** with or without competitor **3**, then subjected to UV crosslinking conditions. The cells were lysed and a fluorescent Alexa Fluor 488 moiety was appended to probe-protein adducts via click chemistry, then proteins were isolated and separated via SDS-PAGE. (A) Fluorescent visualization of proteins from crosslinking and competition experiments with **10b** on PAGE gel. (B) Total protein stain of gel with Sypro Ruby stain. Lane 1: 100 μ M **10b**; Lane 2: 100 μ M **10b** + 500 μ M **3**; Lane 3: 100 μ M **10b** + 1 mM **3**; Lane 4: 10 μ M **10b**; Lane 5: 1 μ M **10b**; Lane 6: 100 μ M **10b**, no UV crosslinking.

the alkyne and diazirine in probes **19c** and **19d** resulted in complete loss of activity. Azide probe **27**, on the other hand, possessed potency slightly greater than lead compound **4**, the most potent compound derived from our prior SAR studies. Fortuitously, we were able to identify a sufficiently active probe containing each of the selected photolabile groups (**10b**, **19b**, and **27**) for use in target identification studies.

Representative probe **19b** was subjected to crosslinking conditions in methanol to assess whether the core scaffold would exhibit undesired photo-induced reactivity. Exposure of a methanolic

solution of **19b** to 365 nm UV light led to conversion of the parent compound into 2 new isolable compounds within 30 min (Scheme 5). NMR and ESI-MS analysis of the purified products identified the major product as **28**, consistent with carbene insertion into the O–H bond of methanol (43% yield). The most prominent minor product consisted of alkene regioisomers that resulted from the two possible 1,2 hydride shifts (**29**, 27% combined yield) common for carbenes adjacent to saturated carbons.³³ A previously reported study involving methanol capture by aliphatic diazirines observed analogous insertion and rearrangement products in similar ratios.³⁴ Importantly, this study established that the remainder of the template is stable to degradation by UV irradiation.

Having confirmed the photostability of our probe scaffold, we began photo-crosslinking³⁵ and fluorescent tagging assays. Briefly, GAS cultures were incubated in the presence of probe compounds for 30 min, then exposed to long-wave UV radiation for 5 (**19b**, **27**) or 10 (**10b**) min to promote crosslinking. The cells were lysed and pelleted, and the resulting lysates were subjected to click chemistry conditions with Alexa Fluor 488 azide. Proteins were then precipitated via methanol/chloroform/water solution, separated via SDS-PAGE gel, and fluorescently visualized. Total protein levels were subsequently visualized by staining with Sypro Ruby (see Supplemental data for more detailed protocol).

Benzophenone probe **10b** appeared to crosslink to a distinct subpopulation of proteins when separated on a polyacrylamide gel and fluorescently imaged (Fig. 1). Tagging was found to be dose-dependent, evidenced by lower fluorescent signal in protein bands from samples tagged with lower probe concentrations. An identically treated control omitting the UV crosslinking step (Lane 6) did not fluorescently tag any proteins. Staining the gel to visualize total protein content with Sypro Ruby stain confirmed an abundance of protein in the sample, indicating that only a small subset of proteins were crosslinked to **10b** and tagged with Alexa Fluor 488. Although the intensity of several protein bands appears to have been diminished by the introduction of soluble competitor **3**, these findings could not be reliably reproduced in biotin pull-down experiments (*data not shown*). The presence of several other protein bands on the gel that did not lose intensity in the presence of **3** implies some degree of nonspecific protein binding by **10b**.

Crosslinking experiments undertaken with diazirine **19b** and azide **27** (Figs. 2 and 3) showed similar trends to **10b**. In each case

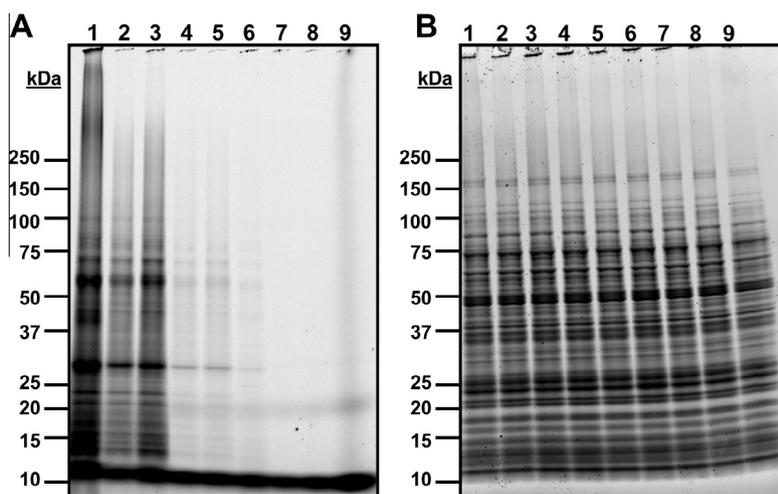


Figure 2. Crosslinking trials using diazirine probe **19b**. GAS cultures were grown in the presence of **19b** with or without competitor **3**, then subjected to UV crosslinking conditions. The cells were lysed and a fluorescent Alexa Fluor 488 moiety was appended to probe-protein adducts via click chemistry, then proteins were isolated and separated via SDS-PAGE. (A) Fluorescent visualization of proteins from crosslinking and competition experiments with **19b** on PAGE gel. (B) Total protein stain of gel with Sypro Ruby stain. Lane 1: 100 μ M **19b**; Lane 2: 10 μ M **19b**; Lane 3: 10 μ M **19b** + 100 μ M **3**; Lane 4: 1 μ M **19b**; Lane 5: 1 μ M **19b** + 100 μ M **3**; Lane 6: 100 μ M **19b**, no UV crosslinking; Lane 7: 10 μ M **19b**, no UV crosslinking; Lane 8: 1 μ M **19b**, no UV crosslinking; Lane 9: no probe, no UV crosslinking.

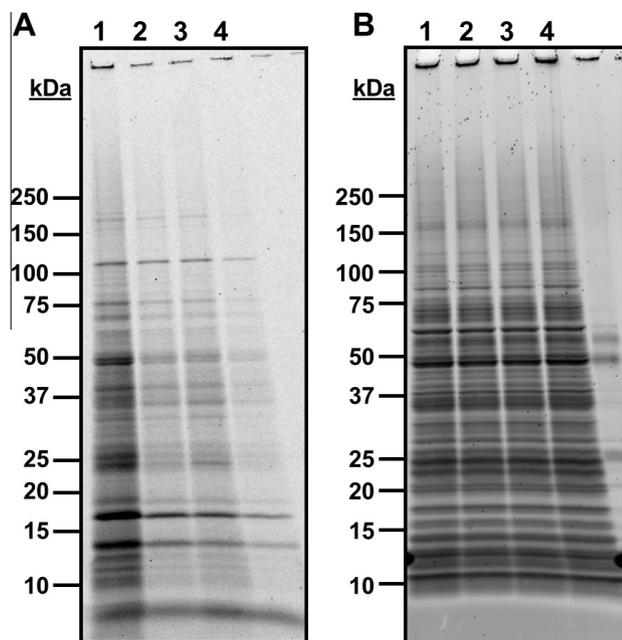


Figure 3. Crosslinking trials using azide probe **27**. GAS cultures were grown in the presence of **27** with or without competitor **3**, then subjected to UV crosslinking conditions. The cells were lysed and a fluorescent Alexa Fluor 488 moiety was appended to probe–protein adducts via click chemistry, then proteins were isolated and separated via SDS–PAGE. (A) Fluorescent visualization of proteins from crosslinking and competition experiments with **27** on PAGE gel. (B) Total protein stain of gel with Sypro Ruby stain. Lane 1: 100 μM **27**; Lane 2: 10 μM **27**; Lane 3: 10 μM **27** + 100 μM **3**; Lane 4: 1 μM **27**.

the intensity of fluorescently-tagged proteins was dependent on both the concentration of the probe compound and the exposure to UV light. Qualitative comparison of the fluorescent and total protein stains suggests that the fluorescent intensity was generally proportional to protein abundance, with a few exceptions. Bands at approximately 30 K and 60 K (probe **19b**, Fig. 2) and 18 K and 110 K (probe **27**, Fig. 3), for example, appear to be disproportionately labeled compared to their relative abundance, but the lack of competition-induced signal reduction suggests these are due to nonspecific interactions as well.

In summary, we report the synthesis of 7 tag-free photoaffinity probe compounds designed to determine the target of a novel class of Group A Streptococcal virulence inhibitors. Three probes, each with a different photolabile moiety, were found to be sufficiently active for use in target identification studies. After confirming adequate photoactivation of representative probe **19b** without destruction of the overall template, we performed preliminary target identification studies. Crosslinking in whole cells, followed by cell lysis and covalent attachment of a fluorescent tag, led to relatively widespread fluorescent labeling that appears consistent with nonspecific labeling of a subset of total GAS proteins.

While biotin/fluorescent tagging and SDS–PAGE separation have been used successfully in the past for target identification, the nature of affinity chromatography and PAGE gel visualization usually limits the effectiveness of this method to probes that potently interact with high-abundance proteins.³⁶ The possibility of the target being a bacterial transcription factor, among the least abundant proteins in the cellular milieu,³⁷ along with the high propensity for nonspecific binding exhibited by these compounds, suggests that visual inspection of SDS–PAGE gels may not be sufficiently sensitive to identify our target. Future studies will employ proteomic techniques that offer a far greater level of resolution, particularly stable isotope labeling by amino acids in cell culture

(SILAC)³⁶ and GAS genome phage display library screening.³⁸ These techniques solve the issue of low abundance by vastly expanding the detection limit for specific interactions and expressing the entire proteome at equal levels, respectively. The development of more potent probes is also underway. Using probes with greater potency should enhance labeling efficiency and allow for the use of lower probe concentrations, reducing the likelihood of widespread nonspecific labeling.

Acknowledgments

This work was supported by a University of Michigan Life Sciences Institute Innovation Partnership grant to S.D.L., and an NIH Pharmacological Sciences Training Program fellowship (Grant T32 GM007767) to B.D.Y.

Supplementary data

Supplementary data (methods for activity determination, target identification, and solvent capture studies, as well as detailed synthetic procedures and characterization data for all synthesized compounds) associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmcl.2014.01.079>.

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