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(71) **Applicants**: **TEMPLE UNIVERSITY-OF THE COMMONWEALTH SYSTEM OF HIGHER EDUCATION** [US/US]; Broad & Montgomery Avenue, Philadelphia, PA 19122 (US). **THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA** [US/US]; 3160 Chestnut Street, Philadelphia, PA 19104 (US).

(72) **Inventors**: **ANDRADE, Rodrigo, B.**; 916 Clover Hill Road, Wynnewood, PA 19096 (US). **GLASSFORD, Ian, M.**; 3000 Valley Forge Circle, Apt. 1353, Valley Forge, PA 19406 (US). **COOPERMAN, Barry, S.**; 2200 Arch Street, Unit 1208, Philadelphia, PA 19103 (US).

(74) **Agents**: **ORBIN, Alia, M.** et al.; Riverside Law, LLP, Glenhardie Corporate Center, 1285 Drummers Lane, Suite 202, Wayne, PA 19087 (US).

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(54) **Title**: METHODS OF IDENTIFYING NOVEL ANTIBIOTICS USING IN SITU CLICK CHEMISTRY

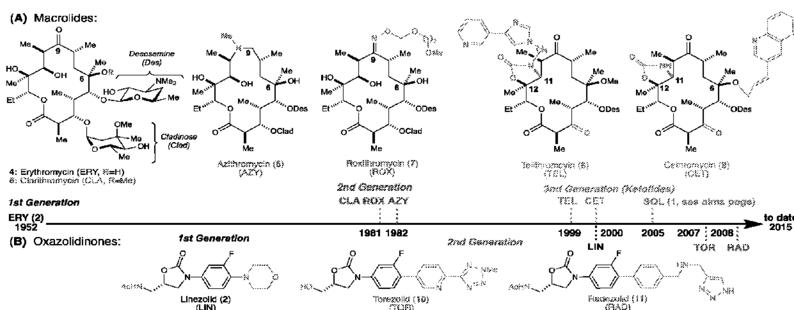


Figure 1

(57) **Abstract**: The present invention includes a method of *in situ* click chemistry for identifying novel compounds useful for treating bacterial infections. The present invention further includes compositions of compounds identified by the method of the invention. The present invention further includes a novel method treating, preventing an infection disease or disorder associated with microbial infection using a compound identified by the method of the invention.

WO 2016/210239 A1

TITLE OF THE INVENTION

Methods of Identifying Novel Antibiotics using *In Situ* click chemistry

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims priority from U.S. Provisional Application Serial Nos. 62/184,007, filed on June 24, 2015, and 62/257,411, filed on November 19, 2015, all of which applications are incorporated by reference herein in their entireties.

10 STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with government support under grant number AI080968 awarded by the National Institutes of Health. The government has certain rights in the invention.

15

BACKGROUND OF THE INVENTION

The discovery and development of antibiotics is a true success story in medicine, resulting in the saving of countless lives in the past century (Walsh, 2003, Nat Rev Microbiol 1:65-70). Major surgeries, including organ transplantation, would 20 not be viable without these indispensable drugs. Bacterial resistance is a formidable 21st century global public health threat. If left unaddressed, there is a risk of moving toward a "post-antibiotic era." While resistance is a natural consequence of antibiotic use, the rate at which pathogenic bacteria have evaded multiple classes of drugs (including those of last resort) has markedly outpaced the introduction of new ones. 25 New antibiotics are desperately needed to fill this void (Fox, 2006, Nat Biotechnol 24:1521-8; Wright et al., 2014, Angew Chem Int Ed 53:8840-69).

Each year approximately two million Americans develop hospital-acquired infections that result in 99,000 deaths, primarily due to antibacterial-resistant pathogens. The cost to the US health care system has been estimated to be between 30 \$21 and \$34 billion (Bush et al., 2011, Nat Rev Microbiol 9:894-6). Community-acquired pneumonia (CAP) is the leading cause of death from infectious disease in the United States and the sixth most common cause of death (File, 2004, J Med 117:39-50). *Streptococcus pneumoniae* is the most common cause of CAP, followed by other bacteria such as *Haemophilus influenzae*, *Mycoplasma pneumoniae* and *Legionella*

species. Macrolides, which have successfully been deployed to treat CAP, are now ineffective against many *S. pneumoniae* strains containing erythromycin-resistant methylase (*erm*) genes.

Over half of all known antibiotics, including macrolides, target the
5 bacterial ribosome (Tenson and Makin, 2006 Mol Microbiol 59:1664-77). Macrolide antibiotics reversibly bind in the peptidyl transferase center located in the 50S subunit, thus inhibiting protein synthesis by blocking the passage of nascent oligopeptides (Spahn and Prescott, 1996, J Mol Med 74:423-39; Yan et al., 2005, Antimicrob. Agents Chemother. 49:3367-3372). The availability of high-resolution
10 crystal structures of major antibiotics bound to bacterial ribosomes such as *D. radiodurans* (2001), *H. marismortuii* (2002), and most recently *E. coli* (2010) have both corroborated earlier biochemical mode-of-action studies and enabled structure-based drug design to be pursued in earnest (Schlunzen et al., 2001, Nature 413:814-21; Hansen et al., 2002, Molecular Cell, 10:117-28; Dunkle et al., 2010, Proc Natl
15 Acad Sci U.S.A. 107:17152-7). Of these three organisms, the *E. coli* structure is most relevant to pathogenic bacteria (Dunkle et al., 2010, Proc Natl Acad Sci U.S.A. 107:17152-7; Mankin, 2008, Curr Opin Microbiol 11:414-21).

Macrolides are one of the safest and most effective drug classes in medicine; however, resistance has compromised efficacy. To date, three generations
20 have been developed with only the lattermost targeting bacterial resistance (Fox, 2006, Nat Biotechnol. 24:1521-8; Wright et al., 2014, Agnew Chem. Int. Ed. 53:8840-69). Single next-generation macrolides will not keep pace with the inevitable onset of resistance.

There is a critical need for antibiotics that address antibiotic
25 resistance. Developing novel antibiotics is severely challenged by the short lag time between the availability of a new antibiotic and its associated clinical resistance. Bacteria reproduce rapidly and readily share genetic information thereby allowing for the widespread transfer of resistance mechanisms between bacterial species. Hence, an expedited process for discovering highly potent antibiotics is
30 required.

Fragment-based drug design (FBDD) is based on the idea that weakly-bound fragments that complex with the drug target may be linked to create a new drug. The use of fragments in drug design has been shown to yield a higher percentage of hits in high throughput screens (HTS) compared to traditional

compound screens (Hajduk and Greer, 2007, Nat. Rev. Drug. Discov. 6:211-9; Hann et al., 2001, J. Chem. Inf. Model 41:856-864; Schuffenhaur et al., 2005, curr. Top Med. Chem. 5:751-762) meaning fragments are better able to predict activity than standard chemical libraries.

5 There is a critical need in the art for multiple future-generation antibiotics to tackle both current and future resistance mechanisms.

BRIEF SUMMARY OF THE INVENTION

The present invention includes a method of identifying a compound. In
10 one embodiment, the method includes the steps of reacting a first fragment and a second fragment using a click chemistry reaction, wherein the click chemistry reaction is catalyzed by a biological target to form a compound, and identifying the compound. In another embodiment, the method includes the steps of obtaining a first library of fragments and a second library of fragments, selecting the first fragment
15 from the first library of fragments, wherein the first fragment exhibits affinity for the biological target, selecting a plurality of fragments from the second library of fragments, wherein at least one fragment of the plurality of fragments exhibits affinity for the biological target, contacting the first fragment and the plurality of fragments with the biological target, forming a complex comprising the biological target, the
20 first fragment, and the second fragment from the at least one fragment of the plurality of fragments, reacting the first fragment and the second fragment using the click chemistry reaction to form the compound, wherein the click chemistry reaction is catalyzed by a biological target to form the compound, isolating the compound; and identifying the compound. In another embodiment, the first library of fragments
25 comprises an at least one fragment, wherein the at least one fragment comprises at least one azide. In another embodiment, the second library of fragments comprises an at least one fragment, wherein the at least one fragment comprises at least one alkyne. In another embodiment, the biological target is selected from the group consisting of a biomolecule, a bacterial cell and a bacterial cell lysate. In another embodiment, the
30 biomolecule is a bacterial organelle. In another embodiment, the bacterial organelle is at least a part of a ribosome. In another embodiment, the at least a part of a ribosome is selected from the group consisting of an *E. coli* ribosome, a *S. aureus* ribosome, a *S. pneumonia* ribosome, a *H. influenzae* ribosome, a *M. catarrhalis* ribosome, a *S. pyogenes* ribosome, a *N. gonorrhoeae* ribosome, and a *L. pneumophila* ribosome. In

another embodiment, at least a part of a ribosome is a drug resistant ribosome. In another embodiment, the drug resistant ribosome is selected from the group consisting of a *E. coli* DK A2058G ribosome, *E. coli* SQ171 A2058G ribosome, *S. aureus* UCN 14 ribosome, *S. aureus* UCN 17 ribosome, *S. aureus* UCN18 ribosome, *S. aureus* ATCC 33591 ribosome, *S. pneumoniae* 2196 ribosome, *S. pneumoniae* 655 ribosome, *S. pneumoniae* 1369 ribosome, *S. pneumoniae* 319 ribosome, and *S. pneumoniae* 2634 ribosome. In another embodiment, the at least a part of a ribosome is selected from the group consisting of a 70S ribosomal subunit and the 50S ribosomal subunit. In another embodiment, the at least one compound further comprises separating the compound from the biological target by a method selected from the group consisting of size exclusion chromatography, chomolith chromatography, and monolith chromatography. In another embodiment, identifying the compound further comprises subjecting the compound to Q-TOF LC-MS.

The present invention also includes a compound identified by a method of the invention or a pharmaceutically acceptable salt or prodrug thereof. In one embodiment, the compound is an antibiotic. In another embodiment, the antibiotic is selected from the group consisting of a macrolide, an oxazolidinone, an aminoglycoside, and a peptide antibiotic.

The present invention also includes a pharmaceutical composition comprising one or more compounds identified by amethod of the invention and a pharmaceutically acceptable carrier.

The present invention also includes a method of treating or preventing an infection, disease or disorder associated with a microbial infection in a subject in need thereof. In one embodiment, the method includes the step of administering a therapeutically effective amount of a composition comprising at least one compound identified by a method of the invention to the subject. In one embodiment, the microbial infection is associated with a pathogen from the group consisting of: *Streptococcus pneumonia*, *Haemophilus influenzae*, and *Mycoplasma pneumoniae* and *Legionella*

BRIEF DESCRIPTION OF THE DRAWINGS

The following detailed description of preferred embodiments of the invention will be better understood when read in conjunction with the appended drawings. For the purpose of illustrating the invention, there are shown in the

drawings embodiments which are presently preferred. It should be understood, however, that the invention is not limited to the precise arrangements and instrumentalities of the embodiments shown in the drawings.

Figure 1, comprising Figures 1A and 1B, depicts the discovery timeline of macrolide and oxazolidinone antibiotics. Figure 1A depicts discovery timeline of macrolides based on flagship erythromycin (ERY, **2**) with changes in red. Figure 1B depicts discovery timeline of oxazolidinone based on flagship linezolid (LIN, **85**) with changes in red.

Figure 2 depicts the chemical structure of Solithromycin (SOL, **1**) Linezolid (LIN, **85**) with sites targeted for *in situ* click circled in blue and regions targeted for chemical modification in red.

Figure 3 depicts the novel ribosome-templated azide/alkyne *in situ* click synthesis of SOL (**1**).

Figure 4 depicts the structures of TEL (**6**) and desmethyl analogs **10-13**.

Figure 5 depicts the ribosome-templated *in situ* click strategy for antibiotic synthesis. Sequential and proximal binding of azide- and alkyne-bearing fragments (e.g., **8** and **9**, respectively) leads to irreversible *anti* (1,4)- and/or *syn* (1,5)-triazole formation by co-localization. The order in which fragments bind the target is dependent on target affinity.

Figure 6 depicts the novel application of Hofmann elimination to CLA (**3**) to access natural **16** and unnatural **17** desosamine analogs.

Figure 7 depicts the results of *in situ* click experiments with *E. coli* 70S ribosomes, 50S subunit, 70S with inhibitor azithromycin (AZY, 25 mM) and negative controls (30S subunit, BSA, and buffer only). Mass counts (normalized) correspond to the combined *anti*-**1** (solithromycin) and *syn*-**1** regioisomer ions.

Figure 8 depicts a concise multigram synthesis of N11-tethered macrolide azide **8** and bis-azide **21** from CLA (**3**).

Figure 9 depicts the synthesis of (*E*)- and (*Z*)-C9-tethered azides **23-26** and bis-azides **91-92**.

Figure 10 depicts the synthesis of O6-tethered azides **29-30** and bis-azides **93-94**.

Figure 11 depicts the novel Des-modified analogs **16-17** from secondary amines **31-39**.

Figure 12 depicts the synthesis of N3'-functionalized Des analogs for *in situ* click from epoxide **15**.

Figure 13 depicts the analog prioritization from 15-alkyne *in situ* click experiment with azide **8** based on mass count (MC) % increase. *Kd*, % translation
5 inhibition, and MIC data shown.

Figure 14 depicts the results of *in situ* click with azide **8**, **9**, and 70S *E. coli* pikR2 ribosomes (dimethylated at A2058).

Figure 15, comprising Figures 15A and 15B depicts the overview of *in situ* click competition experiments. Figure 15A depicts mono-azide in situ click
10 competition experiments to access novel analogs **57-60** functionalized at four sites. Figure 15B depicts bis-azide in situ click competition experiments to access novel analogs **95-96.**

Figure 16 depicts the results of MIC evaluation of SOL, **43-56** against WT, resistant *E. coli*, *S. aureus*, *S. pneumoniae*.

15 Figure 17, comprising Figure 17A and Figure 17B, depicts the structures of solithromycin (**1**) and telithromycin (**6**). Figure 17A depicts the structures of solithromycin (**1**), precursors **8** and **9**, Figure 17B depicts the structure of telithromycin (**6**).

Figure 18, comprising Figure 18A and Figure 18B, depict the
20 structures derived from *in situ* click experiments. Figure 18 A depicts alkyne fragments in the training set. Figure 18B depicts the regioisomeric *anti* (1,4)- and *syn* (1,5)-triazoles derived from *in situ* click experiments (R = Fragment).

Figure 19 depicts the results of *in situ* click experiment with azide **8** and alkynes **3**, **5**, **15**, **16**, and **10**. Mix represents inseparable *anti*- and *syn*-isomers.
25 Normalized mass count percent increases are calculated from the ratio of the ribosome-templated reaction to the background reaction. Results are an average of two experiments.

Figure 20 depicts the results of *in situ* click experiment with azide **8** and alkynes **14**, **11**, 7, **12**, and **16**. Mix represents inseparable *anti*- and *syn*-isomers.
30 Normalized mass count percent increases are calculated from the ratio of the ribosome-templated reaction to the background reaction. Results are an average of two experiments.

Figure 21 depicts the results of *in situ* click experiment with azide **8** and alkynes **3**, **5-18**. Mix represents an inseparable mixture of *anti*- and *syn*-isomers.

Mass count percent increases are calculated from the ratio of the ribosome-templated reaction to the background reaction. Results are an average of five experiments.

Figure 22 depicts an overview of ribosome-templated bis-azide in situ click to efficiently target new binding sites.

5 Figure 23, comprising Figures 23A through 23D depicts synthesis of oxazolidinone cores. Figure 23A depicts the synthesis of **99-101**. Figure 23B depicts the synthesis of **104**. Figure 23C depicts the synthesis of **105-107**. Figure 23D depicts the synthesis of **108-110**.

Figure 24 depicts an overview of mono- and bis-azide ISCC for 10 preparation of oxazolidinone analogs.

Figure 25 depicts the K_d fits in triplicate of **1**, **8,** and **71-84**.

Figure 26 depicts the results of experimental examples showing cell-free translation inhibition of **1**, **8,** and **71-84**.

Figure 27 depicts the chemical structures of intermediates **A**-**G**.

15 Figure 28 depicts the chemical structures of **1, 8,** and **71-76**.

Figure 29 depicts the chemical structures of **77-84**.

Figure 30 depicts an exemplary method for *in cellulo* and *ex cellulo* click chemistry.

Figure 31 depicts exemplary methods of bacterial antibiotic targets.

20 Figure 32 depicts exemplary fragments possessing known RNA-recognition motifs.

Figure 33 depicts a cross section of the 50S ribosome showing PTC, exit tunnel and proximal antibiotic binding sites.

Figure 34 depicts the *in cellulo* variant of ISCC combined with MIC 25 bioassay in a 96-well plate format.

Figure 35 depicts results from an *in cellulo* experiment in MIC format using resistant S. aureus UCN14 strain (A2058T). ND=not determined.

Figure 36 depicts the structures of aminoglycoside inhibitor plazomicin and peptide antibiogic negamycin sites targeted for *in situ* click circled in blue and 30 regions targeted for chemical modification in red.

DETAILED DESCRIPTION

The present invention relates to the unexpected discovery that cellular organelles can serve as templates for *in situ* click chemistry for the discovery of novel

compounds useful as antibiotics. In one aspect, the invention provides methods that allow for rapid screening of possible antibiotics while eliminating the time consuming and costly synthesis and purification of undesired molecules. The present invention also provides compounds identified using the methods of the invention, and compositions comprising same. The present invention also provides methods for treating a microbial infection using compounds of the invention.

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

As used herein, each of the following terms has the meaning associated with it in this section.

The articles "a" and "an" are used herein to refer to one or to more than one (*i.e.*, to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

"About" as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of ±20% or ±10%, more preferably ±5%, even more preferably ±1%, and still more preferably ±0.1% from the specified value, as such variations are appropriate to perform the disclosed methods.

The terms "patient," "subject," or "individual" are used interchangeably herein, and refer to any animal, or cells thereof whether *in vitro* or *in situ*, amenable to the methods described herein. In a non-limiting embodiment, the patient, subject or individual is a human.

As used herein, the term "composition" or "pharmaceutical composition" refers to a mixture of at least one compound useful within the invention with a pharmaceutically acceptable carrier. The pharmaceutical composition facilitates administration of the compound to a patient or subject. Multiple techniques of administering a compound exist in the art including, but not limited to, intravenous, oral, aerosol, parenteral, ophthalmic, pulmonary and topical administration.

A "therapeutic" treatment is a treatment administered to a subject who exhibits signs of pathology, for the purpose of diminishing or eliminating those signs.

As used herein, the term "treatment" or "treating" is defined as the application or administration of a therapeutic agent, i.e., a compound of the invention (alone or in combination with another pharmaceutical agent), to a patient, or application or administration of a therapeutic agent to an isolated tissue or cell line from a patient (e.g., for diagnosis or ex vivo applications), who has a condition contemplated herein, a symptom of a condition contemplated herein or the potential to develop a condition contemplated herein, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve or affect a condition contemplated herein, the symptoms of a condition contemplated herein or the potential to develop a condition contemplated herein. Such treatments may be specifically tailored or modified, based on knowledge obtained from the field of pharmacogenomics.

As used herein, a "solvate" of a molecule refers to a complex between the molecule and a finite number of solvent molecules. In one embodiment, the solvate is a solid isolated from solution by precipitation or crystallization. In another embodiment, the solvate is a hydrate.

By the term "specifically bind" or "specifically binds," as used herein, is meant that a first molecule preferentially binds to a second molecule (e.g., a particular receptor or enzyme), but does not necessarily bind only to that second molecule.

The terms "inhibit" and "antagonize", as used herein, mean to reduce a molecule, a reaction, an interaction, a gene, an mRNA, and/or a protein's expression, stability, function or activity by a measurable amount or to prevent entirely. Inhibitors are compounds that, e.g., bind to, partially or totally block stimulation, decrease, prevent, delay activation, inactivate, desensitize, or down regulate a protein, a gene, and an mRNA stability, expression, function and activity, e.g., antagonists.

As used herein, the terms "effective amount," "pharmaceutically effective amount" and "therapeutically effective amount" refer to a nontoxic but sufficient amount of an agent to provide the desired biological result. That result may be reduction and/or alleviation of the signs, symptoms, or causes of a disease, or any other desired alteration of a biological system. An appropriate therapeutic amount in any individual case may be determined by one of ordinary skill in the art using routine experimentation.

As used herein, the term “pharmaceutically acceptable” refers to a material, such as a carrier or diluent, which does not abrogate the biological activity or properties of the compound, and is relatively non-toxic, i.e., the material may be administered to an individual without causing undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained.

As used herein, the language “pharmaceutically acceptable salt” refers to a salt of the administered compounds prepared from pharmaceutically acceptable non-toxic acids, including inorganic acids, organic acids, solvates, hydrates, or clathrates thereof. Examples of such inorganic acids are hydrochloric, hydrobromic, hydroiodic, nitric, sulfuric, phosphoric, acetic, hexafluorophosphoric, citric, gluconic, benzoic, propionic, butyric, sulfosalicylic, maleic, lauric, malic, fumaric, succinic, tartaric, amsonic, pamoic, p-tolunenesulfonic, and mesylic. Appropriate organic acids may be selected, for example, from aliphatic, aromatic, carboxylic and sulfonic classes of organic acids, examples of which are formic, acetic, propionic, succinic, camphorsulfonic, citric, fumaric, gluconic, isethionic, lactic, malic, mucic, tartaric, para-toluenesulfonic, glycolic, glucuronic, maleic, furoic, glutamic, benzoic, anthranilic, salicylic, phenylacetic, mandelic, embonic (pamoic), methanesulfonic, ethanesulfonic, pantothenic, benzenesulfonic (besylate), stearic, sulfanilic, alginic, galacturonic, and the like. Furthermore, pharmaceutically acceptable salts include, by way of non-limiting example, alkaline earth metal salts (e.g., calcium or magnesium), alkali metal salts (e.g., sodium-dependent or potassium), and ammonium salts.

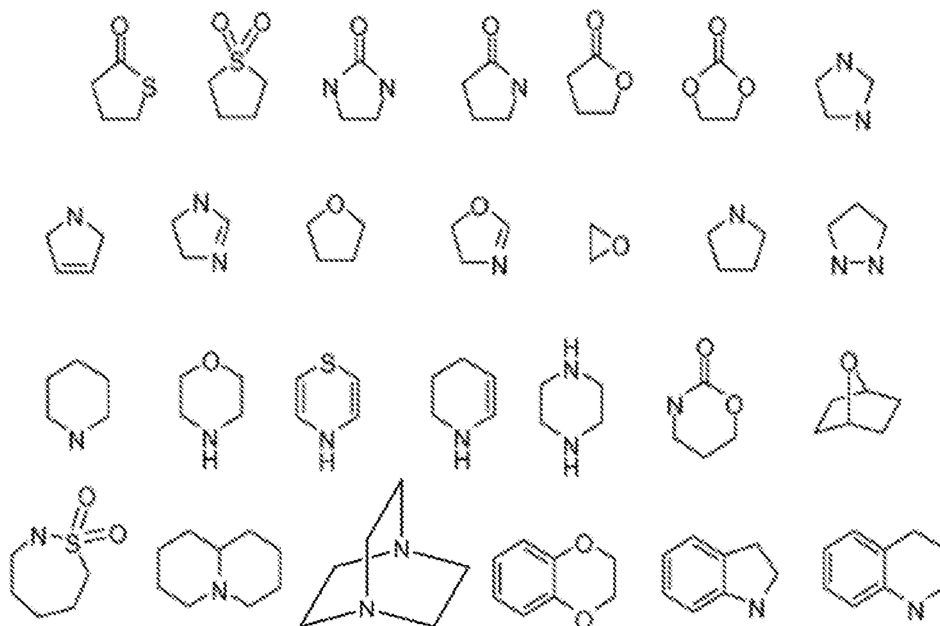
As used herein, the term “pharmaceutically acceptable carrier” means a pharmaceutically acceptable material, composition or carrier, such as a liquid or solid filler, stabilizer, dispersing agent, suspending agent, diluent, excipient, thickening agent, solvent or encapsulating material, involved in carrying or transporting a compound useful within the invention within or to the patient such that it may perform its intended function. Typically, such constructs are carried or transported from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be “acceptable” in the sense of being compatible with the other ingredients of the formulation, including the compound useful within the invention, and not injurious to the patient. Some examples of materials that may serve as pharmaceutically acceptable carriers include: sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives,

such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients, such as cocoa butter and suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; surface active agents; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; phosphate buffer solutions; and other non-toxic compatible substances employed in pharmaceutical formulations. As used herein, "pharmaceutically acceptable carrier" also includes any and all coatings, antibacterial and antifungal agents, and absorption delaying agents, and the like that are compatible with the activity of the compound useful within the invention, and are physiologically acceptable to the patient. Supplementary active compounds may also be incorporated into the compositions. The "pharmaceutically acceptable carrier" may further include a pharmaceutically acceptable salt of the compound useful within the invention. Other additional ingredients that may be included in the pharmaceutical compositions used in the practice of the invention are known in the art and described, for example in Remington's Pharmaceutical Sciences (Genaro, Ed., Mack Publishing Co., 1985, Easton, PA), which is incorporated herein by reference.

As used herein, the term "heterocycloalkyl" or "heterocyclyl" refers to a heteroalicyclic group containing one to four ring heteroatoms each selected from O, S and N. In one embodiment, each heterocycloalkyl group has from 4 to 10 atoms in its ring system, with the proviso that the ring of said group does not contain two adjacent O or S atoms. In another embodiment, the heterocycloalkyl group is fused with an aromatic ring. In one embodiment, the nitrogen and sulfur heteroatoms may be optionally oxidized, and the nitrogen atom may be optionally quaternized. The heterocyclic system may be attached, unless otherwise stated, at any heteroatom or carbon atom that affords a stable structure. A heterocycle may be aromatic or non-aromatic in nature. In one embodiment, the heterocycle is a heteroaryl.

An example of a 3-membered heterocycloalkyl group includes, and is not limited to, aziridine. Examples of 4-membered heterocycloalkyl groups include, and are not limited to, azetidine and a beta lactam. Examples of 5-membered heterocycloalkyl groups include, and are not limited to, pyrrolidine, oxazolidine and thiazolidinedione. Examples of 6-membered heterocycloalkyl groups include, and are

not limited to, piperidine, morpholine and piperazine. Other non-limiting examples of heterocycloalkyl groups are:

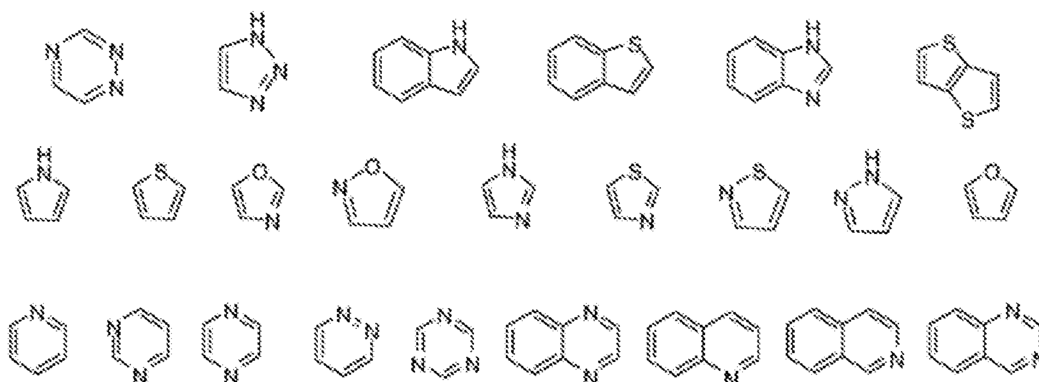


Examples of non-aromatic heterocycles include monocyclic groups such as aziridine, oxirane, thiirane, azetidine, oxetane, thietane, pyrrolidine, pyrroline, pyrazolidine, imidazoline, dioxolane, sulfolane, 2,3-dihydrofuran, 2,5-dihydrofuran, tetrahydrofuran, thiophane, piperidine, 1,2,3,6-tetrahydropyridine, 1,4-dihydropyridine, piperazine, morpholine, thiomorpholine, pyran, 2,3-dihydropyran, tetrahydropyran, 1,4-dioxane, 1,3-dioxane, homopiperazine, homopiperidine, 1,3-dioxepane, 4,7-dihydro-1,3-dioxepin, and hexamethyleneoxide.

As used herein, the term “aromatic” refers to a carbocycle or heterocycle with one or more polyunsaturated rings and having aromatic character, i.e. having $(4n + 2)$ delocalized π (pi) electrons, where n is an integer.

As used herein, the term “aryl” or “arene,” employed alone or in combination with other terms, means, unless otherwise stated, a carbocyclic aromatic system containing one or more rings (typically one, two or three rings), wherein such rings may be attached together in a pendent manner, such as a biphenyl, or may be fused, such as naphthalene. Examples of aryl groups include phenyl, anthracyl, and naphthyl. Preferred examples are phenyl and naphthyl, most preferred is phenyl.

As used herein, the term “heteroaryl” or “heteroaromatic” refers to a heterocycle having aromatic character. A polycyclic heteroaryl may include one or more rings that are partially saturated. Examples include the following moieties:



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Examples of heteroaryl groups also include pyridyl, pyrazinyl, pyrimidinyl (particularly 2- and 4-pyrimidinyl), pyridazinyl, thienyl, furyl, pyrrolyl (particularly 2-pyrrolyl), imidazolyl, thiazolyl, oxazolyl, pyrazolyl (particularly 3- and 5-pyrazolyl), isothiazolyl, 1,2,3-triazolyl, 1,2,4-triazolyl, 1,3,4-triazolyl, tetrazolyl, 1,2,3-thiadiazolyl, 1,2,3-oxadiazolyl, 1,3,4-thiadiazolyl and 1,3,4-oxadiazolyl.

Examples of polycyclic heterocycles and heteroaryls include indolyl (particularly 3-, 4-, 5-, 6- and 7-indolyl), indolinyl, quinolyl, tetrahydroquinolyl, isoquinolyl (particularly 1- and 5-isoquinolyl), 1,2,3,4-tetrahydroisoquinolyl, cinnolinyl, quinoxalinyl (particularly 2- and 5-quinoxalinyl), quinazolinyl, phthalazinyl, 1,8-naphthyridinyl, 1,4-benzodioxanyl, coumarin, dihydrocoumarin, 1,5-naphthyridinyl, benzofuryl (particularly 3-, 4-, 5-, 6- and 7-benzofuryl), 2,3-dihydrobenzofuryl, 1,2-benzisoxazolyl, benzothienyl (particularly 3-, 4-, 5-, 6-, and 7-benzothienyl), benzoxazolyl, benzothiazolyl (particularly 2-benzothiazolyl and 5-benzothiazolyl), purinyl, benzimidazolyl (particularly 2-benzimidazolyl), benzotriazolyl, thioxanthinyl, carbazolyl, carbolinyl, acridinyl, pyrrolizidinyl, and quinolizidinyl.

A "biological target" or "biomolecule" can be any biological molecule involved in biological pathways associated with any of various diseases and conditions, including, those associated with a bacterial infection. The biological target may also be referred to as the "target biomacromolecule" or the "biomacromolecule." The biological target can be a receptor, such as enzyme receptors, ligand-gated ion channels, G-protein-coupled receptors, and transcription factors. The biologically target is preferably a bacterial target. The bacterial target may be a protein or protein complex, such as enzymes, membrane transport proteins, hormones, and antibodies. In

one particularly preferred embodiment, the protein biological target is a bacterial ribosome.

As used herein, the terms "bioconjugation" and "conjugation," unless otherwise stated, refers to the chemical derivatization of a macromolecule with another
5 molecular entity. The molecular entity can be any molecule and can include a small molecule or another macromolecule. Examples of molecular entities include, but are not limited to, compounds of the invention, other macromolecules, polymers or resins, such as polyethylene glycol (PEG) or polystyrene, non-immunogenic high molecular weight compounds, fluorescent, chemiluminescent radioisotope and bioluminescent
10 marker compounds, antibodies, biotin, diagnostic detector molecules, such as a maleimide derivatized fluorescein, coumarin, a metal chelator or any other modifying group. The terms bioconjugation and conjugation are used interchangeably throughout the Specification.

An "effective amount" of a delivery vehicle is that amount sufficient to
15 effectively bind or deliver a compound.

As used herein, the term "potency" refers to the dose needed to produce half the maximal response (ED_{50}).

As used herein, the term "efficacy" refers to the maximal effect (E_{max}) achieved within an assay.

20 Ranges: throughout this disclosure, various aspects of the invention can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges
25 as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range.

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Description

The present invention relates to the unexpected discovery that ribosomes can act as templates to synthesize biologically active antibiotics by *in situ*

click chemistry. Thus, the present invention provides a method for identifying novel antibiotics.

In one aspect, the methods of the invention include the use of *in situ* click chemistry for the identification of compounds that bind to and target a specific cellular organelle. The *in situ* click chemistry approach permits the irreversible target-guided synthesis of high affinity ligands from fragments. In one embodiment, the methods of the invention include the use of the [3+2] cycloaddition reaction to conjugate two molecules that are each independently bound to the template. The conjugation reaction serves to produce a single compound with a high degree of binding affinity for the template, and thus improved antimicrobial activity. In some embodiments, the method of the invention identifies a compound by reacting a first fragment and a second fragment through a click chemistry reaction to form a compound, wherein a biological target catalyzes the click chemistry reaction, and identifying the compound.

In one embodiment, a method of identifying a compound is provided. The method comprises obtaining a obtaining a first library of fragments and a second library of fragments; selecting the first fragment from the first library of fragments, wherein the first fragment exhibits affinity for the biological target; selecting a plurality of fragments from the second library of fragments, wherein at least one fragment of the plurality of fragments exhibits affinity for the biological target; contacting the first fragment and the plurality of fragments with the biological target; forming a complex comprising the biological target, the first fragment, and the second fragment from the at least one fragment; reacting the first fragment and the second fragment through the click chemistry reaction to form the compound, wherein the biological target catalyzes the click chemistry reaction; isolating the compound; and identifying the compound.

In one embodiment, the first library of fragments comprises an at least one fragment, wherein the at least one fragment comprises at least one azide. In another embodiment, the second library of fragments comprises an at least one fragment, wherein the at least one fragment comprises at least one.

In one embodiment, the biological target includes, but is not limited to, a biomolecule, a bacterial cell and a bacterial cell lysate. In some embodiments the biomolecule is a bacterial organelle. In one embodiment the bacterial organelle is at

least a part of a ribosome. In certain emodiments, the at least one part of a ribosme is a 70S ribosomal subunit or a 50S ribosomal subunit.

In some embodiments, the method of identifying a compound is carried out *in cellulo* by incubating the first fragment and the library of second fragments with cells comprising the biological target, wherein a first fragment and a second fragment bind the biological target in the cell and wherein a click chemistry reaction is catalyzed by the biological target in the cell to form a compond. One of the main benefits of *in cellulo* click chemistry is that the biological target does not have to be chosen a priori; moreover, a compound formed by click chemistry is truly effective under the conditions of cellular use. Accordingly, in one embodiment, the biological target is *in cellulo*. In another embodiment, the biological target is in a bacterial cell. In yet another embodiment, the biological target is a bacterial cell or bacterial cell lysate.

In one embodiment the bacterial strain includes, but is not limited to, *E. coli, S. aureus, S. pneumonia*, *H. influenzae*, *M. catarrhalis*, *S. pyogenes*, *N. gonorrhoeae*, and *L. pneumophila*. In some embodiments the bacterial strain is a drug resistant strain. In certain embodiments the drug resistant bacterial strain includes but is not limited to *E. coli* DK A2058G, *E. coli* SQ171 A2058G, *S. aureus* UCN 14, *S. aureus* UCN 17, *S. aureus* UCN18, *S. aureus* ATCC 33591, *S. pneumoniae* 2196, *S. pneumoniae* 655, *S. pneumoniae* 1369, *S. pneumoniae* 319, and *S. pneumoniae* 2634.

In certain embodiments, the method further comprises separating the compound from the biological target. In some aspects the compound is separated from the biological target using chomolith chromatography or monolith chromatography. In other aspects, the compound is separated from the biological target using reverse phase HPLC, or size exclusion chromatography.

In some embodiments, the method further comprises subjecting the compound to Q-TOF LC-MS.

In one aspect, the present invention provides a compound identified using the methods of the invention, or a pharmaceutically acceptable salt or prodrug thereof. In one embodiment, the compound is an antibiotic. In certain embodiments the antibiotic includes, but is not limited to, a macrolide and an oxazolidinone.

In another aspect, the invention provides a pharmaceutical composition comprising one or more compounds identified by the methods of the invention and a

pharmaceutically acceptable carrier. In one embodiment, the pharmaceutical compound further comprises a known antibiotic.

The present invention further includes a method of treating or preventing an infection, disease or disorder associated with microbial infection in a subject in need thereof wherein the method comprises administering a therapeutically effective amount of a composition comprising at least one compound of the invention. In another embodiment the infection, disease, or disorder is associated with *Streptococcus pneumonia, Haemophilus influenzae,* and *Mycoplasma pneumoniae* or *Legionella.*

Screening

The present invention relates to the discovery of new methods for the synthesis and identification of compounds with antimicrobial activity. In one embodiment, the molecules bind a bacterial ribosome. It is contemplated herein that a single compound may not be ideal for a wide variety of bacterial infections. Accordingly, the present invention includes an *in situ* click chemistry method of identifying a compound useful as an antibiotic. In one aspect of the invention, the bacterial ribosome assembles its own inhibitor when incubated with small molecule fragments bearing terminal alkynes and azides. These fragments may undergo a cycloaddition reaction to form triazoles with a greater affinity for the ribosome.

Any fragments that can be combined by *in situ* click chemistry to create compounds are useful in the present invention, as would be understood by one skilled in the art. In one embodiment, the method comprises the steps of providing a biological target as a templating enzyme and at least two fragments for the biomolecule to assemble, and identifying compounds formed by Q-TOF LC-MS. In another embodiment, the biological target is at least a part of a ribosome.

In situ click chemistry

In situ click chemistry offers an attractive new approach to molecular probe discovery, since it is not dependent on the screening of final compounds, laboriously prepared through traditional means, but rather allows the enzyme to select and combine building blocks that fit into its binding site to assemble its own inhibitor molecules. For example, with just 200 building blocks (100 mono-azides and 100 mono-acetylenes), one can quickly scan through 20,000 possible combinations

(100x100x2; the factor `2` accounts for possible syn- or anti-triazole formation) without actually having to make these compounds. This number becomes even larger, with the same number of building blocks, if one includes di- or tri-azides or -acetylenes, thereby providing the enzyme with greater flexibility to choose the appropriate building block and functional group at the same time. The screening method is as simple as determining whether or not the product has been formed in a given test mixture by LC/MS. A compound that is formed by the enzyme is likely to be a good and selective binder, due to the multivalent nature of the interaction.

In one embodiment, the *in situ* click chemistry is catalyzed by a biological target. In some embodiments, the biological target is an organelle of a cell. In one embodiment, the cell is a bacterial cell. In some aspects, the biological target is a bacterial ribosome or a part of a bacterial ribosome. In another embodiment, the biological target is a bacterial cell lysate. In another embodiment, the biological target is a bacterial cell wall. In yet another embodiment the biological target is a bacterial cell. In another embodiment, the biological target is a part of a bacterial cell. In another embodiment the part of a bacterial cell includes, but is not limited to 30S ribosomal subunit, 50S ribosomal subunit, DNA gyrase, DNA topoisomerase, dihydropteroate synthetase, MurA-MurF, MurZ, penicillin-binding protein, transpeptidase, cell membrane, isoprenyl pyrophosphate, RNA polymerase, enolpyruvyl transferase, and fatty acid precursors.

In one embodiment, the method of the invention includes contacting a first fragment and a plurality of second fragments with the biological target, the first fragment and at least one of the pluarility of second fragments each having an affinity for the biological target so that the the first fragment and one of the plurality of second fragments form a complex with the biological target.

In one embodiment, contacting the biological target comprises contacting the first fragment with the biological target and then contacting the pluarility of second fragments with the biological target and the first fragment.

In another embodiment, contacting the biological target comprises contacting the pluarility of second fragments with the biological target and then contacting the first fragment with the biological target and pluarility of second fragments.

In yet another embodiment contacting the biological target comprises combining the first fragment and the pluarility of second fragments to form a mixture and then contacting the mixture with the biological target.

In one embodiment, the method of the invention includes forming a complex comprising the biological target, the first fragment, and the second fragment from the at least one fragment of the plurality of fragments. In one embodiment both the first fragment and the second fragment of the complex have an affinity for the biological target.

In one embodiment, wherein the biological target is a ribosome, the first fragment and the second fragment individually bind the ribosome. The ribosome catalyzes a cycloaddition reaction to form a single compound comprising the first fragment and the second fragment.

In another embodiment, wherein the biological target is a bacterial cell, the first fragment and the second fragment individually bind a component of the bacterial cell. The bacterial cell can be of any phylum, class, family, genus or species of bacterial, including but not limited to, *E. coli*, *S. aureus*, *S. pneumonia*, *H. influenzae*, *M. catarrhalis*, a *S. pyogenes*, *N. gonorrhoeae*, and *L. pneumophila*. In one aspect, the first fragment bind to any part of the bacteria, including but not limited to a cell wall, a ribosome, a flagelluim, a plasma membrane, a pilus, a nucleoid, 30S ribosomal subunit, 50S ribosomal subunit, DNA gyrase, DNA topoisomerase, dihydropteroate synthetase, MurA-MurF, MurZ, penicillin-binding protein, transpeptidase, cell membrane, isoprenyl pyrophosphate, RNA polymerase, enolpyruvyl transferase, and a fatty acid precursor.

In another embodiment, wherein the biological target is a bacterial cell lysate, the first fragment and the second fragment individually bind a component contained within the bacterial cell lysate. The bacterial cell lysate can be derived from any phylum, class, family, genus or species of bacterial, including but no limited to, *E. coli*, *S. aureus*, *S. pneumonia*, *H. influenzae* ribosome, *M. catarrhalis*, a *S. pyogenes* ribosome, *N. gonorrhoeae*, and *L. pneumophila*. In one aspect, the first fragment bind to any part of the bacteria, including but not limited to a cell wall, a ribosome, a flagelluim, a plasma membrane, a pilus, a nucleoid, 30S ribosomal subunit, 50S ribosomal subunit, DNA gyrase, DNA topoisomerase, dihydropteroate synthetase, MurA-MurF, MurZ, penicillin-binding protein, transpeptidase, cell

membrane, isoprenyl pyrophosphate, RNA polymerase, enolpyruvyl transferase, and a fatty acid precursor.

In some embodiments, the step contating a first fragment and a plurality of second fragments are with the biological target further comprises adding a reaction buffer. In one embodiment, the buffer comprises Tris-HCl (pH 7.5), $MgCl_2$, NH_4Cl, and β-mercaptoethanol. In another aspect, the biological target has a concentration of about 1 μM to about 20 μM. In another aspect, the first fragment and the second fragment each have concentrations of about 0.5 to about 20 μM. In another embodiment the first fragment and the second fragment each have concentrations of about 0.5 to about 20 mM

In another embodiment, the step forming a complex comprising the biological target, the first fragment, and the second fragment from the at least one fragment of the plurality of fragments further comprises incubating the complex at a suitable temperature, as would be understood by one of ordinary skill in the art. In one embodiment, the temperature ranges from about 20°C to about 27°C. In another embodiment, the temperature ranges from about 20°C to about 37°C.

In yet another embodiment, the step forming a complex comprising the biological target, the first fragment, and the second fragment from the at least one fragment of the plurality of fragments further comprises incubating the complex for a suitable period of time, as would be understood by one of ordinary skill in the art. In one embodiment, the time ranges from of about 24 hours to about 48 hours. In one embodiment, the plurality of fragments comprises from about 2 fragments to about 15 fragments. In another embodiment, the plurality of fragments comprises about 15 fragments.

In some embodiments, the first fragment and the second fragment contain at least one functional group capable of participating in a click reaction. In one embodiment, the functional group is an alkyne. In another embodiment, the functional group is an azide. In some embodiments, the first fragment is comprised of two or more functional groups. In one embodiment, the first fragment may contain 2, 3, 4, or even more functional groups. In other embodiments, the second fragment is comprised of two or more functional groups. In one embodiment, the second fragment may contain 2, 3, 4, or even more functional groups. In one embodiment, the two or more functional groups are identical. In another embodiment, the two or more

functional groups are a combination of different functional groups. In another embodiment, both the first fragment and the second fragment are each comprised of more than one functional group.

In one embodiment, the first fragment comprises an alkyne functional group. In another embodiment, the first fragment comprises 2, 3, or more alkyne groups. In one embodiment, the first fragment comprises an azide functional group. In another embodiment, the first fragment comprises 2, 3, or more azide groups.

In one embodiment, the second fragment comprises an alkyne functional group. In another embodiment, the second fragment comprises 2, 3, or more alkyne groups. In one embodiment, the second fragment comprises an azide functional group. In another embodiment, the second fragment comprises 2, 3, or more azide groups.

In some embodiments, the fragment comprises at least a part of a known compound or antibiotic. In some aspects the known antibiotic includes, but is not limited to, a macrolide or oxazolidinone or those described in Wilson (2009, Crit Rev Biochem Mol Biol 44:363-433), which is incorporated by reference herein in its entirety. Thus, in one aspect, the invention provides a method for identifying new derivatives of known inhibitors. In a non-limiting example, a compound of a known class of antibiotics can be modified to include a functional group which can participate in an *in situ* click chemistry reaction using the methods of the invention. New derivatives identified through click chemistry have an advantage over the known compound by being more potent or active against drug resistant bacterial strains.

In some embodiments, the fragment comprises compounds possessing RNA-recognition elements. In one embodiment, the fragment comprises a 5-membered arene. In another embodiment, the fragment comprises a 6-membered arene. In another embodiment, the fragment comprises a 5-membered heterocycle. In yet another embodiment, the fragment comprises a 6-membered heterocycle. In another embodiment, the fragment comprises an aliphatic amine. In one embodiment, the fragment comprises an alicyclic amine. In one embodiment, the fragment comprises a melamine. In one embodiment, the fragment comprises a pyrene. In one embodiment, the fragment comprises a BenzoC. In one embodiment, the fragment comprises an quinazolinone. In one embodiment, the fragment comprises hydantoin. In one embodiment, the fragment comprises a nucleobase or a derivative thereof. In

one embodiment, the nucleobase includes, but is not limited to adenine, guanine, thymine, cytosine, uracil, 2-amino adenine, and dihydrouracil.

Fragment Identification

5 In one embodiment, the method of the invention comprises obtaining a first library of fragments and a second library of fragments; selecting the first fragment from the first library of fragments, wherein the first fragment exhibits affinity for the biological target; and selecting a plurality of fragments from the second library of fragments, wherein at least one fragment of the plurality of 10 fragments exhibits affinity for the biological target. Thus, the invention includes method of selecting fragments.

In another aspect, the methods of the invention may be used for the development of novel macrolide antibiotics, include pursuing congeners based on the desosamine sugar, which engages in hydrogen bonding and electrostatic interactions 15 with neighboring RNA bases, or building upon interactions of the ARM with the ribosome by adding a second ARM. Preliminary tests of macrolide analogs bearing multiple ARM moieties indicate more favorable properties compared to ligands bearing a single ARM. Such analogs may exploit the ribosome-templated *in situ* click chemistry approach discussed previously using multiple types of precursors, thereby 20 expanding the number of potential congeners possible in a single *in situ* reaction.

As described herein, the compounds identified by *in situ* click Chemistry can be easily modified to create a library of compounds, as would be understood by one skilled in the art. In one embodiment, compounds are modified using single-step free energy perturbation (SSFEP) analysis.

25 In one embodiment, the screening method involves identifying a plurality of fragments that exhibit affinity for the binding site of the target enzyme. A functional group capable of participating in a click chemistry reaction, such as an azide or alkynyl group, is also attached to the molecule, optionally via a linker. Individual members of the plurality of molecules are then mixed with the biological 30 target and individual members of a plurality or library of compounds that may exhibit affinity for a substrate binding site of the enzyme. The members of the substrate-binding library have been chemically modified to include at least one click chemistry functional group compatible with the functional group of the library of cofactor-binding molecules. Thus, any pair of compounds, one from each library that exhibits

affinity for the binding sites of the enzyme will covalently bond via the click chemistry functional groups *in situ*. The screening process can utilize conventional screening equipment known in the art such as multi-well microtiter plates.

Compound isolation and identification

5 The compound may be isolated using any method known in the art, such as size exclusion chromatograph (SEC), HPLC, 2 dimensional (2D) thin layer chromatography (TLC), preparatory TLC, crystallization, silica gel chromatography, and any other standard separation technology. In one embodiment, a compound is isolated from the biological target using HPLC. In another embodiment, a compound 10 is isolated from the biological target using SEC. In another embodiment, a compound is isolated from the biological target using ultra centrifugation. SEC is particularly advantageous because it provides complete separation of the biological target from the compound. Furthermore, when a mixture of compounds is present, SEC provides some additional separation between the compounds of the mixture.

15 After isolating the compound from the biological target, the compound may be identified using any method known in the art. A mass spectrometer may be used for sequential, automated data analysis of the screening process. Exemplary spectrometer equipment that can be used include the Agilent MSD 1100 SL system, linear ion trap systems (ThermoFinnigan LTQ), quadrupole ion trap (LCQ), or a 20 quadrupole time-of-flight (QTOF from Waters or Applied Biosystems). Each of these analyzers have very effective HPLC interfaces for LC-MS experiments. In another embodiment, the compound is identified using nuclear magnetic resonance spectroscopy (NMR).

Once the compounds are identified, they may then be synthesized 25 using any synthetic method known by those of ordinary skill in the art. After the compounds have been synthesized, they can be evaluated for desired efficacy and potency, including, but not limited to: minimum inhibitory concentration (MIC) assays against wild-type and resistant strains of microorganisms such as *E. coli*, *S. pneumoniae*, *H. influenzae*, and *S. aureus*, K_d, protein synthesis inhibition, maximum 30 tolerated dose in mice, efficacy of each agent in a systemic lethal infection model in mice, protective dose for 50% of animals (PD50), and *in vivo* efficacy in a mouse model of bacterial pneumonia.

The desired efficacy and potency properties can vary based on the specific application. Accordingly, a compound with properties useful for a specific

anti-bacterial application can be selected based on the bioimaging properties deemed most important for that specific application. For example, in one embodiment, a compound useful as an anti-bacterial can be selected based primarily on the compound's MIC in a drug resistant bacterial strain. However, the compound can be chosen based on any selection criteria, as would be understood by a person skilled in the art, and the evaluation and selection of a compound as anti-bacterial is not limited to any specific property listed herein. In one embodiement, a compound inhibiting bacteria selected from the group consisting of *Streptococcus pneumonia, Haemophilus influenzae,* and *Mycoplasma pneumoniae* and *Legionella* is selected. In another embodiment a compound inhibiting the drug resistant strain *E. coli* DKpkk3535 or *E. coli* DK 2058G is selected. In yet another embodiment, compounds which inhibit mutant 70S E. coli ribosomes dimethylated at position A2058 are selected.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures, embodiments, claims, and examples described herein. Such equivalents were considered to be within the scope of this invention and covered by the claims appended hereto. For example, it should be understood, that modifications in reaction conditions, including but not limited to reaction times, reaction size/volume, and experimental reagents, such as solvents, catalysts, pressures, atmospheric conditions, e.g., nitrogen atmosphere, and reducing/oxidizing agents, with art-recognized alternatives and using no more than routine experimentation, are within the scope of the present application.

Compositions

The compositions of the present invention are generally compounds derived from *in situ* click chemistry. The compounds include compounds identified using the methods described herein. In one embodiment, the compounds of the present invention target bacteria. In another embodiment, the compounds of the present invention are antibiotics. In one embodiment, the compounds of the present invention target the ribosome. In yet another embodiment the compounds of the present invention include, but are not limited to, macrolides, ketolides, PTF inhibitors, azalides, streptogramins, enacyloxins, thiopeptides, kasugamycin, kirromysins,

pactamycins, EF-Tu inhibitors, pleuromutilins, edeines, EF2 inhibitors, translocation inhibitors, oxazolidinones aminoglycosides, tetracyclines, chloramphenicols, clindamycins, ansamycins, carbacephems, carbapenems, cephalosporins, lincosamides, lipopeptides, monobactams, nitrofurans, penicillins, quinolones,
5 fluoroquinolones, sulfonamides, and peptide antibiotics.

The compounds of the present invention may be synthesized using techniques well-known in the art of organic synthesis. The starting materials and intermediates required for the synthesis may be obtained from commercial sources or synthesized according to methods known to those skilled in the art.

10 The compounds of the invention may possess one or more stereocenters, and each stereocenter may exist independently in either the *R* or *S* configuration. In one embodiment, compounds described herein are present in optically active or racemic forms. It is to be understood that the compounds described herein encompass racemic, optically-active, regioisomeric and stereoisomeric forms,
15 or combinations thereof that possess the therapeutically useful properties described herein. Preparation of optically active forms is achieved in any suitable manner, including by way of non-limiting example, by resolution of the racemic form with recrystallization techniques, synthesis from optically-active starting materials, chiral synthesis, or chromatographic separation using a chiral stationary phase. In one
20 embodiment, a mixture of one or more isomer is utilized as the therapeutic compound described herein. In another embodiment, compounds described herein contain one or more chiral centers. These compounds are prepared by any means, including stereoselective synthesis, enantioselective synthesis and/or separation of a mixture of enantiomers and/ or diastereomers. Resolution of compounds and isomers thereof is
25 achieved by any means including, by way of non-limiting example, chemical processes, enzymatic processes, fractional crystallization, distillation, and chromatography.

The methods and formulations described herein include the use of *N*-oxides (if appropriate), crystalline forms (also known as polymorphs), solvates,
30 amorphous phases, and/or pharmaceutically acceptable salts of compounds having the structure of any compound of the invention, as well as metabolites and active metabolites of these compounds having the same type of activity. Solvates include water, ether (e.g., tetrahydrofuran, methyl tert-butyl ether) or alcohol (e.g., ethanol)

solvates, acetates and the like. In one embodiment, the compounds described herein exist in solvated forms with pharmaceutically acceptable solvents such as water, and ethanol. In another embodiment, the compounds described herein exist in unsolvated form.

5 In one embodiment, the compounds of the invention may exist as tautomers. All tautomers are included within the scope of the compounds presented herein.

In one embodiment, compounds described herein are prepared as prodrugs. A "prodrug" refers to an agent that is converted into the parent drug *in vivo*. 10 In one embodiment, upon *in vivo* administration, a prodrug is chemically converted to the biologically, pharmaceutically or therapeutically active form of the compound. In another embodiment, a prodrug is enzymatically metabolized by one or more steps or processes to the biologically, pharmaceutically or therapeutically active form of the compound.

15 In one embodiment, sites on, for example, the aromatic ring portion of compounds of the invention are susceptible to various metabolic reactions. Incorporation of appropriate substituents on the aromatic ring structures may reduce, minimize or eliminate this metabolic pathway. In one embodiment, the appropriate substituent to decrease or eliminate the susceptibility of the aromatic ring to metabolic 20 reactions is, by way of example only, a deuterium, a halogen, or an alkyl group.

Compounds described herein also include isotopically-labeled compounds wherein one or more atoms is replaced by an atom having the same atomic number, but an atomic mass or mass number different from the atomic mass or mass number usually found in nature. Examples of isotopes suitable for inclusion in 25 the compounds described herein include and are not limited to ^{2}H, ^{3}H, ^{11}C, ^{13}C, ^{14}C, ^{36}Cl, ^{18}F, ^{123}I, ^{125}I, ^{13}N, ^{15}N, ^{15}O, ^{17}O, ^{18}O, ^{32}P, and ^{35}S. In one embodiment, isotopically-labeled compounds are useful in drug and/or substrate tissue distribution studies. In another embodiment, substitution with heavier isotopes such as deuterium affords greater metabolic stability (for example, increased in vivo half-life or reduced 30 dosage requirements). In yet another embodiment, substitution with positron emitting isotopes, such as ^{11}C, ^{18}F, ^{15}O and ^{13}N, is useful in Positron Emission Topography (PET) studies for examining substrate receptor occupancy. Isotopically-labeled compounds are prepared by any suitable method or by processes using an appropriate isotopically-labeled reagent in place of the non-labeled reagent otherwise employed.

In one embodiment, the compounds described herein are labeled by other means, including, but not limited to, the use of chromophores or fluorescent moieties, bioluminescent labels, or chemiluminescent labels.

The compounds described herein, and other related compounds having different substituents are synthesized using techniques and materials described herein and as described, for example, in Fieser & Fieser's Reagents for Organic Synthesis, Volumes 1-17 (John Wiley and Sons, 1991); Rodd's Chemistry of Carbon Compounds, Volumes 1-5 and Supplementals (Elsevier Science Publishers, 1989); Organic Reactions, Volumes 1-40 (John Wiley and Sons, 1991), Larock's Comprehensive Organic Transformations (VCH Publishers Inc., 1989), March, Advanced Organic Chemistry 4th Ed., (Wiley 1992); Carey & Sundberg, Advanced Organic Chemistry 4th Ed., Vols. A and B (Plenum 2000, 2001), and Green & Wuts, Protective Groups in Organic Synthesis 3rd Ed., (Wiley 1999) (all of which are incorporated by reference for such disclosure). General methods for the preparation of compound as described herein are modified by the use of appropriate reagents and conditions, for the introduction of the various moieties found in the formula as provided herein.

Compounds described herein are synthesized using any suitable procedures starting from compounds that are available from commercial sources, or are prepared using procedures described herein.

Methods of the Invention

The invention includes a method of treating or preventing an infection, disease or disorder in a subject in need thereof. The method comprises administering to the subject an effective amount of a therapeutic composition comprising a compound of the invention. In one embodiment, the subject is further administered an additional thereaputic. In another embodiment, the method further comprises inhibiting a bacterial ribosome.

In one embodiment, the infection disease or disorder includes but is not limited to infections caused by *Streptococcus pneumonia, Haemophilus influenzae,* and *Mycoplasma pneumoniae and Legionella.*

In one embodiment, the subject is a mammal. In another embodiment, the mammal is a human.

Combination Therapies

The compounds of the present invention may be useful in combination with one or more additional compounds. These additional compounds may comprise compounds of the present invention or therapeutic agents known to treat, prevent, or reduce the symptoms or effects of a disease in a subject. In one embodiment, the disease is a bacterial infection. In another embodiment, the bacterial infection is a Gram positive bacterial infection.

In some embodiments, the invention includes antibacterial agents, such as those described in Wilson (2009, Crit Rev Biochem Mol Biol 44:363-433), which is incorporated by reference herein in its entirety. Non-limiting examples of antibacterial agents contemplated within the invention include, but are not limited to, lipopeptide, fluoroquinolone, ketolide, cephalosporin, amikacin, gentamicin, kanamycin, neomycin, netilmicin, paromomycin, streptomycin, tobramycin, cefacetrile, cefadroxil, cefalexin, cefaloglycin, cefalonium, cefaloridine, cefalotin, cefapirin, cefatrizine, cefazaflur, cefazedone, cefazolin, cefradine, cefroxadine, ceftezole, cefaclor, cefamandole, cefmetazole, cefonicid, cefotetan, cefoxitin, cefprozil, cefuroxime, cefuzonam, cefcapene, cefdaloxime, cefdinir, cefditoren, cefetamet, cefixime, cefmenoxime, cefodizime, cefotaxime, cefpimizole, cefpodoxime, cefteram, ceftibuten, ceftiofur, ceftiolene, ceftizoxime, ceftriaxone, cefoperazone, ceftazidime, cefclidine, cefepime cefluprenam, cefoselis, cefozopran, cefpirome, cefquinome, cefaclomezine, cefaloram, cefaparole, cefcanel, cefedrolor, cefempidone, cefetrizole, cefivitril, cefmatilen, cefmepidium, cefovecin, cefoxazole, cefrotil, cefsumide, ceftaroline, ceftioxide, cefuracetime, imipenem, primaxin, doripenem, meropenem, ertapenem, flumequine, nalidixic acid, oxolinic acid, piromidic acid pipemidic acid, rosoxacin, ciprofloxacin, enoxacin, lomefloxacin, nadifloxacin, norfloxacin, ofloxacin, pefloxacin, rufloxacin, balofloxacin, gatifloxacin, grepafloxacin, levofloxacin, moxifloxacin, pazufloxacin, sparfloxacin, temafloxacin, tosufloxacin, clinafloxacin, gemifloxacin, sitafloxacin, trovafloxacin, prulifloxacin, azithromycin, erythromycin, clarithromycin, dirithromycin, roxithromycin, telithromycin, amoxicillin, ampicillin, bacampicillin, carbenicillin, cloxacillin, dicloxacillin, flucloxacillin, mezlocillin, nafcillin, oxacillin, penicillin g, penicillin v, piperacillin, pivampicillin, pivmecillinam, ticarcillin, sulfamethizole, sulfamethoxazole, sulfisoxazole, trimethoprim-sulfamethoxazole, demeclocycline, doxycycline, minocycline, oxytetracycline, tetracycline, linezolid, clindamycin,

metronidazole, vancomycin, vancocin, mycobutin, rifampin, nitrofurantoin, and chloramphenicol.

A synergistic effect may be calculated, for example, using suitable methods such as, for example, the Sigmoid-E_{max} equation (Holford & Scheiner, 1981, Clin. Pharmacokinet. 6:429-453), the equation of Loewe additivity (Loewe & Muischnek, 1926, Arch. Exp. Pathol Pharmacol. 114:313-326) and the median-effect equation (Chou & Talalay, 1984, Adv. Enzyme Regul. 22:27-55). Each equation referred to above may be applied to experimental data to generate a corresponding graph to aid in assessing the effects of the drug combination. The corresponding graphs associated with the equations referred to above are the concentration-effect curve, isobologram curve and combination index curve, respectively.

Administration/Dosage/Formulations

The regimen of administration may affect what constitutes an effective amount. The therapeutic formulations may be administered to the subject either prior to or after the onset of an infection, disease or disorder. Further, several divided dosages, as well as staggered dosages may be administered daily or sequentially, or the dose may be continuously infused, or may be a bolus injection. Further, the dosages of the therapeutic formulations may be proportionally increased or decreased as indicated by the exigencies of the therapeutic or prophylactic situation.

Administration of the compositions of the present invention to a patient, preferably a mammal, more preferably a human, may be carried out using known procedures, at dosages and for periods of time effective to treat infections, diseases or disorders in the patient. An effective amount of the therapeutic compound necessary to achieve a therapeutic effect may vary according to factors such as the state of the disease or disorder in the patient; the age, sex, and weight of the patient; and the ability of the therapeutic compound to treat infections, diseases or disorders in the patient. Dosage regimens may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. A non-limiting example of an effective dose range for a therapeutic compound of the invention is from about 1 and 5,000 mg/kg of body weight/per day. One of ordinary skill in the art would be able to study the relevant factors and make

the determination regarding the effective amount of the therapeutic compound without undue experimentation.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of this invention may be varied so as to obtain an amount of the active ingredient that is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.

In particular, the selected dosage level depends upon a variety of factors including the activity of the particular compound employed, the time of administration, the rate of excretion of the compound, the duration of the treatment, other drugs, compounds or materials used in combination with the compound, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well, known in the medical arts.

A medical doctor, e.g., physician or veterinarian, having ordinary skill in the art may readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

In particular embodiments, it is especially advantageous to formulate the compound in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the patients to be treated; each unit containing a predetermined quantity of therapeutic compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical vehicle. The dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the therapeutic compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding/formulating such a therapeutic compound for the treatment of infections in a patient.

In one embodiment, the compositions of the invention are formulated using one or more pharmaceutically acceptable excipients or carriers. In one embodiment, the pharmaceutical compositions of the invention comprise a therapeutically effective amount of a compound of the invention and a pharmaceutically acceptable carrier.

The carrier may be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity may be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms may be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it is preferable to include isotonic agents, for example, sugars, sodium chloride, or polyalcohols such as mannitol and sorbitol, in the composition. Prolonged absorption of the injectable compositions may be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate or gelatin. In one embodiment, the pharmaceutically acceptable carrier is not DMSO alone.

In one embodiment, the compositions of the invention are administered to the patient in dosages that range from one to five times per day or more. In another embodiment, the compositions of the invention are administered to the patient in range of dosages that include, but are not limited to, once every day, every two, days, every three days to once a week, and once every two weeks. It is readily apparent to one skilled in the art that the frequency of administration of the various combination compositions of the invention varies from individual to individual depending on many factors including, but not limited to, age, disease or disorder to be treated, gender, overall health, and other factors. Thus, the invention should not be construed to be limited to any particular dosage regime and the precise dosage and composition to be administered to any patient is determined by the attending physical taking all other factors about the patient into account.

Compounds of the invention for administration may be in the range of from about 1 μg to about 10,000 mg, about 20 μg to about 9,500 mg, about 40 μg to about 9,000 mg, about 75 μg to about 8,500 mg, about 150 μg to about 7,500 mg, about 200 μg to about 7,000 mg, about 3050 μg to about 6,000 mg, about 500 μg to about 5,000 mg, about 750 μg to about 4,000 mg, about 1 mg to about 3,000 mg, about 10 mg to about 2,500 mg, about 20 mg to about 2,000 mg, about 25 mg to about 1,500 mg, about 30 mg to about 1,000 mg, about 40 mg to about 900 mg, about 50 mg

to about 800 mg, about 60 mg to about 750 mg, about 70 mg to about 600 mg, about 80 mg to about 500 mg, and any and all whole or partial increments therebetween.

In some embodiments, the dose of a compound of the invention is from about 1 mg and about 2,500 mg. In some embodiments, a dose of a compound of the invention used in compositions described herein is less than about 10,000 mg, or less than about 8,000 mg, or less than about 6,000 mg, or less than about 5,000 mg, or less than about 3,000 mg, or less than about 2,000 mg, or less than about 1,000 mg, or less than about 500 mg, or less than about 200 mg, or less than about 50 mg. Similarly, in some embodiments, a dose of a second compound as described herein is less than about 1,000 mg, or less than about 800 mg, or less than about 600 mg, or less than about 500 mg, or less than about 400 mg, or less than about 300 mg, or less than about 200 mg, or less than about 100 mg, or less than about 50 mg, or less than about 40 mg, or less than about 30 mg, or less than about 25 mg, or less than about 20 mg, or less than about 15 mg, or less than about 10 mg, or less than about 5 mg, or less than about 2 mg, or less than about 1 mg, or less than about 0.5 mg, and any and all whole or partial increments thereof.

In one embodiment, the present invention is directed to a packaged pharmaceutical composition comprising a container holding a therapeutically effective amount of a compound of the invention; and instructions for using the compound to treat, prevent, or reduce one or more symptoms of infections, diseases or disorders in a patient.

Formulations may be employed in admixtures with conventional excipients, i.e., pharmaceutically acceptable organic or inorganic carrier substances suitable for oral, parenteral, nasal, intravenous, subcutaneous, enteral, or any other suitable mode of administration, known to the art. The pharmaceutical preparations may be sterilized and if desired mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure buffers, coloring, flavoring and/or aromatic substances and the like. They may also be combined where desired with other active agents, e.g., other analgesic agents.

Routes of administration of any of the compositions of the invention include oral, nasal, rectal, intravaginal, parenteral, buccal, sublingual or topical. The compounds for use in the invention may be formulated for administration by any suitable route, such as for oral or parenteral, for example, transdermal, transmucosal

(*e.g.*, sublingual, lingual, (trans)buccal, (trans)urethral, vaginal (*e.g.*, trans- and perivaginally), (intra)nasal and (trans)rectal), intravesical, intrapulmonary, intraduodenal, intragastrical, intrathecal, subcutaneous, intramuscular, intradermal, intra-arterial, intravenous, intrabronchial, inhalation, and topical administration.

5 Suitable compositions and dosage forms include, for example, tablets, capsules, caplets, pills, gel caps, troches, dispersions, suspensions, solutions, syrups, granules, beads, transdermal patches, gels, powders, pellets, magmas, lozenges, creams, pastes, plasters, lotions, discs, suppositories, liquid sprays for nasal or oral administration, dry powder or aerosolized formulations for inhalation, compositions 10 and formulations for intravesical administration and the like. It should be understood that the formulations and compositions that would be useful in the present invention are not limited to the particular formulations and compositions that are described herein.

15 *Oral Administration*

For oral application, particularly suitable are tablets, dragees, liquids, drops, suppositories, or capsules, caplets and gelcaps. The compositions intended for oral use may be prepared according to any method known in the art and such compositions may contain one or more agents selected from the group consisting of 20 inert, non-toxic pharmaceutically excipients that are suitable for the manufacture of tablets. Such excipients include, for example an inert diluent such as lactose; granulating and disintegrating agents such as cornstarch; binding agents such as starch; and lubricating agents such as magnesium stearate. The tablets may be uncoated or they may be coated by known techniques for elegance or to delay the 25 release of the active ingredients. Formulations for oral use may also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert diluent.

For oral administration, the compounds of the invention may be in the form of tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., polyvinylpyrrolidone, 30 hydroxypropylcellulose or hydroxypropylmethylcellulose); fillers (e.g., cornstarch, lactose, microcrystalline cellulose or calcium phosphate); lubricants (e.g., magnesium stearate, talc, or silica); disintegrates (e.g., sodium starch glycollate); or wetting agents (e.g., sodium lauryl sulphate). If desired, the tablets may be coated using suitable methods and coating materials such as OPADRY™ film coating systems

available from Colorcon, West Point, Pa. (e.g., OPADRY™ OY Type, OYC Type, Organic Enteric OY-P Type, Aqueous Enteric OY-A Type, OY-PM Type and OPADRY™ White, 32K18400). Liquid preparation for oral administration may be in the form of solutions, syrups or suspensions. The liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, methyl cellulose or hydrogenated edible fats); emulsifying agent (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters or ethyl alcohol); and preservatives (e.g., methyl or propyl p-hydroxy benzoates or sorbic acid).

Granulating techniques are well known in the pharmaceutical art for modifying starting powders or other particulate materials of an active ingredient. The powders are typically mixed with a binder material into larger permanent free-flowing agglomerates or granules referred to as a "granulation." For example, solvent-using "wet" granulation processes are generally characterized in that the powders are combined with a binder material and moistened with water or an organic solvent under conditions resulting in the formation of a wet granulated mass from which the solvent must then be evaporated.

Melt granulation generally consists in the use of materials that are solid or semi-solid at room temperature (i.e. having a relatively low softening or melting point range) to promote granulation of powdered or other materials, essentially in the absence of added water or other liquid solvents. The low melting solids, when heated to a temperature in the melting point range, liquefy to act as a binder or granulating medium. The liquefied solid spreads itself over the surface of powdered materials with which it is contacted, and on cooling, forms a solid granulated mass in which the initial materials are bound together. The resulting melt granulation may then be provided to a tablet press or be encapsulated for preparing the oral dosage form. Melt granulation improves the dissolution rate and bioavailability of an active (i.e. drug) by forming a solid dispersion or solid solution.

U.S. Patent No. 5,169,645 discloses directly compressible wax-containing granules having improved flow properties. The granules are obtained when waxes are admixed in the melt with certain flow improving additives, followed by cooling and granulation of the admixture. In certain embodiments, only the wax itself melts in the melt combination of the wax(es) and additives(s), and in other cases both the wax(es) and the additives(s) melt.

The present invention also includes a multi-layer tablet comprising a layer providing for the delayed release of one or more compounds of the invention, and a further layer providing for the immediate release of a medication for treatment of G-protein receptor-related diseases or disorders. Using a wax/pH-sensitive polymer mix, a gastric insoluble composition may be obtained in which the active ingredient is entrapped, ensuring its delayed release.

Parenteral Administration

For parenteral administration, the compounds of the invention may be formulated for injection or infusion, for example, intravenous, intramuscular or subcutaneous injection or infusion, or for administration in a bolus dose and/or continuous infusion. Suspensions, solutions or emulsions in an oily or aqueous vehicle, optionally containing other formulatory agents such as suspending, stabilizing and/or dispersing agents may be used.

Additional Administration Forms

Additional dosage forms of this invention include dosage forms as described in U.S. Patents Nos. 6,340,475; 6,488,962; 6,451,808; 5,972,389; 5,582,837; and 5,007,790. Additional dosage forms of this invention also include dosage forms as described in U.S. Patent Applications Nos. 20030147952; 20030104062; 20030104053; 20030044466; 20030039688; and 20020051820. Additional dosage forms of this invention also include dosage forms as described in PCT Applications Nos. WO 03/35041; WO 03/35040; WO 03/35029; WO 03/35177; WO 03/35039; WO 02/96404; WO 02/32416; WO 01/97783; WO 01/56544; WO 01/32217; WO 98/55107; WO 98/11879; WO 97/47285; WO 93/18755; and WO 90/11757.

Controlled Release Formulations and Drug Delivery Systems

In one embodiment, the formulations of the present invention may be, but are not limited to, short-term, rapid-offset, as well as controlled, for example, sustained release, delayed release and pulsatile release formulations.

The term sustained release is used in its conventional sense to refer to a drug formulation that provides for gradual release of a drug over an extended period of time, and that may, although not necessarily, result in substantially constant blood

levels of a drug over an extended time period. The period of time may be as long as a month or more and should be a release which is longer that the same amount of agent administered in bolus form.

For sustained release, the compounds may be formulated with a suitable polymer or hydrophobic material which provides sustained release properties to the compounds. As such, the compounds for use the method of the invention may be administered in the form of microparticles, for example, by injection or in the form of wafers or discs by implantation.

In one embodiment of the invention, the compounds of the invention are administered to a patient, alone or in combination with another pharmaceutical agent, using a sustained release formulation.

The term delayed release is used herein in its conventional sense to refer to a drug formulation that provides for an initial release of the drug after some delay following drug administration and that mat, although not necessarily, includes a delay of from about 10 minutes up to about 12 hours.

The term pulsatile release is used herein in its conventional sense to refer to a drug formulation that provides release of the drug in such a way as to produce pulsed plasma profiles of the drug after drug administration.

The term immediate release is used in its conventional sense to refer to a drug formulation that provides for release of the drug immediately after drug administration.

As used herein, short-term refers to any period of time up to and including about 8 hours, about 7 hours, about 6 hours, about 5 hours, about 4 hours, about 3 hours, about 2 hours, about 1 hour, about 40 minutes, about 20 minutes, or about 10 minutes and any or all whole or partial increments thereof after drug administration after drug administration.

As used herein, rapid-offset refers to any period of time up to and including about 8 hours, about 7 hours, about 6 hours, about 5 hours, about 4 hours, about 3 hours, about 2 hours, about 1 hour, about 40 minutes, about 20 minutes, or about 10 minutes, and any and all whole or partial increments thereof after drug administration.

Dosing

The therapeutically effective amount or dose of a compound of the present invention depends on the age, sex and weight of the patient, the current medical condition of the patient and the progression of infection in the patient being treated. The skilled artisan is able to determine appropriate dosages depending on these and other factors.

A suitable dose of a compound of the present invention may be in the range of from about 0.01 mg to about 5,000 mg per day, such as from about 0.1 mg to about 1,000 mg, for example, from about 1 mg to about 500 mg, such as about 5 mg to about 250 mg per day. The dose may be administered in a single dosage or in multiple dosages, for example from 1 to 4 or more times per day. When multiple dosages are used, the amount of each dosage may be the same or different. For example, a dose of 1 mg per day may be administered as two 0.5 mg doses, with about a 12-hour interval between doses.

It is understood that the amount of compound dosed per day may be administered, in non-limiting examples, every day, every other day, every 2 days, every 3 days, every 4 days, or every 5 days. For example, with every other day administration, a 5 mg per day dose may be initiated on Monday with a first subsequent 5 mg per day dose administered on Wednesday, a second subsequent 5 mg per day dose administered on Friday, and so on.

In the case wherein the patient's status does improve, upon the doctor's discretion the administration of the inhibitor of the invention is optionally given continuously; alternatively, the dose of drug being administered is temporarily reduced or temporarily suspended for a certain length of time (i.e., a “drug holiday”). The length of the drug holiday optionally varies between 2 days and 1 year, including by way of example only, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 10 days, 12 days, 15 days, 20 days, 28 days, 35 days, 50 days, 70 days, 100 days, 120 days, 150 days, 180 days, 200 days, 250 days, 280 days, 300 days, 320 days, 350 days, or 365 days. The dose reduction during a drug holiday includes from 10%-100%, including, by way of example only, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%.

Once improvement of the patient's conditions has occurred, a maintenance dose is administered if necessary. Subsequently, the dosage or the frequency of administration, or both, is reduced, as a function of the viral load, to a

level at which the improved disease is retained. In one embodiment, patients require intermittent treatment on a long-term basis upon any recurrence of symptoms and/or infection.

The compounds for use in the method of the invention may be formulated in unit dosage form. The term "unit dosage form" refers to physically discrete units suitable as unitary dosage for patients undergoing treatment, with each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect, optionally in association with a suitable pharmaceutical carrier. The unit dosage form may be for a single daily dose or one of multiple daily doses (e.g., about 1 to 4 or more times per day). When multiple daily doses are used, the unit dosage form may be the same or different for each dose.

Toxicity and therapeutic efficacy of such therapeutic regimens are optionally determined in cell cultures or experimental animals, including, but not limited to, the determination of the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between the toxic and therapeutic effects is the therapeutic index, which is expressed as the ratio between LD_{50} and ED_{50}. The data obtained from cell culture assays and animal studies are optionally used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with minimal toxicity. The dosage optionally varies within this range depending upon the dosage form employed and the route of administration utilized.

Those skilled in the art recognizes, or is able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures, embodiments, claims, and examples described herein. Such equivalents were considered to be within the scope of this invention and covered by the claims appended hereto. For example, it should be understood, that modifications in reaction conditions, including but not limited to reaction times, reaction size/volume, and experimental reagents, such as solvents, catalysts, pressures, atmospheric conditions, e.g., nitrogen atmosphere, and reducing/oxidizing agents, with art-recognized alternatives and using no more than routine experimentation, are within the scope of the present application.

It is to be understood that wherever values and ranges are provided herein, all values and ranges encompassed by these values and ranges, are meant to be

encompassed within the scope of the present invention. Moreover, all values that fall within these ranges, as well as the upper or lower limits of a range of values, are also contemplated by the present application.

The following examples further illustrate aspects of the present invention. However, they are in no way a limitation of the teachings or disclosure of the present invention as set forth herein.

EXPERIMENTAL EXAMPLES

The invention is further described in detail by reference to the following experimental examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Thus, the invention should in no way be construed as being limited to the following examples, but rather, should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

Example 1: Methods of identifying novel antibiotics using *in situ* click methods

Since its isolation from the soil bacterium *Saccharopolyspora erythraea* in 1952, the flagship macrolide antibiotic erythromycin (**2**) has enjoyed success in the clinic for the safe and effective treatment of upper and lower respiratory, skin and soft-tissue disorders (Figure 1) (Maguire et al., 2008, RNA 14:188-95; Iacoviello and Zinner, 2002, Macrolide Antibiotics 15-24). Poor pharmacokinetic properties associated with **2** led to the development of 2nd-generation macrolides clarithromycin (**3**, Taisho/Abbott), azithromycin (**4**, Pliva/Pfizer), and roxithromycin (**5**, Roussel-Uclaff) (Adachi and Morimoto, 2002, Macrolide Antibiotics 53-72; Bright et al., 1988, J Antibiot 41:1029-47; Gasc et al., 1991, J Antibiot 44:313-30). A major breakthrough came in 1988 when Abbott introduced the C11-12 oxazolidinone motif bearing an *N*-butylarene to preclude O12-C9 ketalization and participate in π–π interactions with the ribosome to address resistance to **2-5** (Baker et al., 1988, J Org Chem 53:2340-5). Aventis used this strategy to discover telithromycin (**6**) in 1999, and Abbott placed the side chain at O6 to yield cethromycin (**7**) in 2000 (Bryskier, 2002, Macrolide Antibiotics 97-140; Ma et al., 2001, J Med Chem 44:4137-56).

In 2005, solithromycin (**1**, SOL, Figure 2) was discovered by employing Cu(I)-catalyzed combinatorial click chemistry at Optimer Pharmaceuticals.

SOL is currently in Phase III clinical trials and is the best-in-class ketolide developed to date. Using isolated 70S *E. coli* ribosomes, it is unambiguously shown that this validated bacterial drug target is capable of templating the formation of **1** from macrolide-tethered azide **8** and 3-ethynylaniline (**9**) precursors (Figure 3). The significance of this finding is that *in situ* click chemistry with isolated bacterial ribosomes, including those of pathogenic bacteria (e.g., MRSA, *S. pneumoniae, S. aureus*), will greatly accelerate the pace of antibiotic discovery by allowing the target to template and assemble the best inhibitor by sampling various partners in a manner similar to fragment-based drug design (Maguire et al., 2008, RNA 14:188-195; Farmer and Reitz, 2008, Practice of Medicinal Chemistry 3rd Ed 228-43). Another strength of this method lies in the capacity for the ribosome to assemble either the 1,4- (e.g., **1**) or 1,5-regioisomer, thus doubling the exploration of chemical space. This is not the case in metal-mediated processes, which greatly favor one regioisomer (Mamidyala and Finn, 2010, Chem Soc Rev 39:1252-61).

The data presented herein is innovative as it describes the development of novel *in situ* click methodology using wild-type and resistant bacterial ribosomes to prioritize the synthesis and accelerate the delivery of potent, selective macrolide antibiotics and provides novel synthetic routes to unexplored desosamine analogs at the C3'-amino substituent, which plays a role in bioactivity and resistance.

Four desmethyl (i.e., replacing CH_3 with H) analogs of telithromycin (**6**, TEL) (Figure 4), designed, synthesized, and evaluated, including 4,8,10-tridesmethyl TEL (**10**), 4,10-didesmethyl TEL (**11**), 4,8-didesmethyl TEL (**12**), and 4-desmethyl TEL (**13**), in addition to 4,8,10-tridesmethyl cethromycin (Velvadapu et al., 2011, J Org Chem 76:7516-27; Velvadapu et al., 2011, ACS Med Chem Lett 2:68-72; Velvadapu et al., 2012, ACS Med Chem Lett 3:211-15; Wagh et al., 2012, ACS Med Chem Lett 3:1013-18; Glassford et al., 2014, ACS Med Chem Lett 5:1021-6; Wagh et al., 2013, ACS Med Chem Lett 4:1114-8). In this approach it was hypothesized that replacing the 4-methyl group with hydrogen would avoid a steric clash with the mutant A2058G ribosome (Tu et al., 2005, Cell 121:257-70). While 4-desmethyl TEL (**13**) was equipotent with wild-type ribosomes, it was four-fold less potent against the A2058G mutant and thus not supportive of our desmethyl hypothesis. Removing the methyl groups on the macrolactone ring decreased potency. Although not wishing to be bound by any particular theory, these results suggest that structure-based drug design requires targeting both wild-type and mutant targets and

underscores the importance of the proposed *in situ* click approach wherein the mutant ribosome selects its own inhibitor.

Ribosome-templated in situ click.

5 The ribosome is a complex molecular machine composed by weight of 2/3 RNA and 1/3 protein whose singular purpose is to synthesize proteins by decoding mRNA in concert with aminoacylated tRNAs (Tenson and Mankin, 2006, Mol Microbiol 59:1664-77; Spahn and Prescott, 1996, J Mol Med 74:423-39). The bacterial ribosome, whose molecular weight is 2.5M Daltons, represents the largest 10 macromolecule to template the azide-alkyne Huisgen [3+2] cycloaddition reaction (i.e., *in situ* click reaction). This novel method is summarized in Figure 5 wherein the tighter binder docks first to the ribosome (i.e., macrolide azide, **A**) followed by a second fragment (i.e., alkyne, **B**). The resultant proximity of the reactive groups markedly lowers the activation energy barrier leading to irreversible triazole 15 formation (red line). Thermodynamically, the ribosome is paying the entropic penalty of bringing the two reactants together. Accordingly, the result of this experiment is a mixture of triazole products wherein the major product(s) formed are also the best inhibitors. Less labor-intensive variants of the *in situ* click obviate the need to isolate the ribosomes by directly using intact bacterial cells or cell lysates.

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Exploring new macrolide chemical space.

Many innovations have occurred since the discovery of erythromycin (**2**) in 1952 (Wright et al., 2014, Chem Int Ed 53:8840-8869). All involved exploring novel chemical space to address physicochemical properties and/or resistance. The 25 Hofmann elimination reaction when applied to clarithromycin (**3**) allows gram-scale access to 2,3-epoxide **15** in 75% yield (Figure 6). Heating **15** with a secondary amine and 10 mol% $Sc(OTf)_3$ gave a mixture of 3'-amino analog **16** and novel 2'-amino analog **17**. Side chains are installed on these novel analogs and are employed in *in situ* click experiments.

30 New side chain analogs and tethers that improve on those in TEL, CET, and SOL are designed. Next, fragment synthesis (i.e., macrolide azide, alkyne), validation/prioritization via *in situ* click chemistry, and synthesis of novel desosamine-modified analogs are carried out. Finally potency is determined and MOA is established. Successful ligands have significant changes in their chemical

structures as compared to SOL (**1**) while maintaining or surpassing its efficacy, including activity against a range of wild-type and resistant bacterial strains. The inclusion of chemical variability as a criterion will achieve our goal of developing novel macrolide antibiotics that will preempt future ribosomal modifications
5 associated with unforeseen mechanisms of resistance. Finally, the best candidates are tested in extensive MIC assays against a broader panel of resistant strains with the most promising 3-5 compounds being subjected to IND-enabling studies.

Synthesis of designed analogs

10 *E. coli* 70S ribosomes and 50S subunits reproducibly template the [3+2] cycloaddition reaction between macrolide azide **8** and 3-ethynylaniline (**9**) to prepare SOL (**1**, Figure 3, Figure 7), thus demonstrating that bacterial ribosomes are capable of effecting *in situ* click chemistry (Sharpless and Manetsch, 2006, Expert Opin Drug Discov 1:525-38). Since erythromycin (**2**) and congeners bind *E. coli*
15 ribosomes with nM K_d values, the macrolide-tethered azide precursor **8** of SOL would be readily anchored in the 50S subunit (Figure 5); addition of excess 3-ethynylaniline (**9**), which has a markedly lower affinity for the ribosome, would sample various binding regions including those corresponding to SOL's side chain (Yan et al., 2005, Antimicrob Agents Chemother 49:3367-72). Accordingly, it was hypothesized that
20 the ribosome would make favorable interactions with the transition state leading to **1** and pay the entropic penalty of bringing **8** and **9** together, ultimately resulting in irreversible formation of triazole **1** (Sharpless and Manetsch, 2006, Expert Opin Drug Discov 1:525-38; Mocharla et al., 2004, Agnew Chem Int Ed 44:116-20). After varying concentrations of ribosome, azide, and alkyne in buffer, 5 μM ribosome, 5
25 μM azide and 5 mM alkyne at room temperature for 24-48 h resulted in the formation of **1** and its 1,5-regioisomer (~2:1 ratio) in 8- to 16-fold greater amounts than in the absence of 70S or 50S ribosomal subunits (e.g., buffer, BSA, or 30S subunit). An Agilent 6520B Q-TOF LC-MS instrument was used and extracted ion chromatograms were analyzed to locate and quantify the masses of interest (Figure 7, normalized to
30 highest value). Retention times of both triazole products were confirmed by independent synthesis via thermal cycloaddition, and SOL (**1**) was prepared by Cu(I)-catalysis. Several lines of evidence strongly support the *in situ* click process: (1) in the absence of 70S or 50S ribosomal subunits (i.e., only buffer), there was 16-fold less product formation showing only mass counts due to the thermal cycloaddition

background reaction; (2) the 30S subunit, which does not have a macrolide binding site, also displayed an ion profile similar to background; (3) in the presence of ribosomal inhibitor azithromycin (AZY, 25 μM) that competes for the binding site, 70S ribosomes were unable to template the process; (4) replacing ribosomes with
5 bovine serum albumin (BSA), a standard negative control used to rule out non-competitive binding, resulted in ion counts similar to those of the background cycloaddition; and finally, (5) the ratio of regioisomers in all negative controls (i.e., 30S, BSA, and buffer reactions) and the inhibition experiment was 1:1 whereas in the presence of 70S ribosome and 50S subunits, the product ratio was 2:1 favoring **1**,
10 which is a hallmark of selectivity (Sharpless and Manetsch, 2006, Expert Opin Drug Discov 1:525-38). Percent translation inhibition data on solithromycin (**1**) and a library of other analogs using established assays were obtained. Using ribosome-templated *in situ* click process, general mass counts correlated with target inhibition, allowing classification of inhibitors into good, medium, and poor binders.
15

Synthesis of macrolide-tethered azides at N11, C9, and O6.

The application of *in situ* click chemistry toward the discovery of novel macrolide antibiotics first requires the synthesis of suitable azide and aryl alkyne reactants (Figure 8). Procurement of alkyne partners identified by modeling, as
20 discussed above, is through commercial vendors or chemical synthesis employing established alkynylation reactions of commercial aryl halide, triflate or aldehyde precursors (e.g., Sonogashira coupling, Corey-Fuchs, Seyferth-Gilbert homologations, etc.) (Sonogashira, 2002, J Organomet Chem 653:46-9; Corey and Fuchs, 1972, Tetrahedron Lett 13:3769-72; Gilbert and Weerasooriya, 1982, J Org Chem 47:1837-
25 45; Seyferth et al., 1971 J Org Chem 36:1379-86). Three logical, validated positions to tether the side chains, were targeted, specifically N11, C9, and O6. Of the three sites, the first (N11) has been the most utilized (e.g., TEL, SOL) (Xu et al., 2012, Antibiotic Discovery and Development, pp 181-228).

Figure 8 above shows the 9-step, gram-scale synthesis of macrolide-
30 tethered azide **8** at N11 used in the *in situ* click synthesis of **1** from commercial clarithromycin (**3**). By reacting **18** with the appropriate amino alcohol (in red), the linker type/length can be varied informed by molecular modeling. Two-step azide installation, cladinose removal, oxidation, installation of the 2-fluoro group with *t*-

BuOK and *N*-fluorobenzenesulfonimide (NFSI), and finally removal of the 2'-OAc (Des) are all established operations that are employed.

The synthesis of C9-tethered macrolide azides **23-26** focuses on the oxime functionality (Figure 9). Roxithromycin (**5**), a 2nd generation macrolide derived from **2**, features an (*E*)-oxime ether at C9 (Gasc et al., 1991, J Antibiot 44:313-330). Roussel-Uclaff showed that the (*Z*)-oximes of **21** and **22** is prepared from the (*E*)-oxime by base-mediated isomerization; moreover, nucleophiles react site-selectively at the C9 keto over C3 (Beebe et al., 2004, Bioorg Med Chem Lett 14:2417-21). Based on results from molecular modeling, the ω-azido hydroxylamine possessing the optimal tether length (in red) is reacted with **21-22** to access (*E*)-oxime ether **23-24**. If modeling suggests the (Z)-isomer is superior, the C9 oxime is isomerized (*E*→*Z*) then alkylated with the appropriate electrophile (in red) to prepare **25-26**. In both cases, methanolysis as a final step removes the acetate protecting group at the 2' hydroxyl of Des.

The synthesis of O6-tethered macrolide azides **29-30** starts from readily available cethromycin (**7**) precursor **27** on mulitgram scale (Figure 10) (Cao et al., 2013, J Chem Res, 37:107-9). Results from molecular modeling will determine the optimal linker length. Accordingly, one of two approaches is taken to procure the requisite azide. Butyl and greater azides ($n \geq 4$) employ olefin cross-metathesis of **27** or **28** and the appropriate ω-alkenyl azide and phosphine-free Hoveyda-Grubbs 2nd generation catalyst (HG-II) (Garber et al., 2000, J Am Chem Soc 122:8168-79). Subsequent and precedented chemoselective reduction of the olefin in the presence of the azide by diimide reduction with Myers' *o*-nitrobenzenesulfonyl hydrazide (NBSH) and Et_3N affords **29** and **30** after fluorination and methanolysis (Myers et al., 1997, J Org Chem 62:7507; Haukaas and O'Doherty, 2002, Org Lett 4:1771-4). The propyl series (n=3) employs hydroboration of the terminal olefin and two-step conversion of the alcohol to the azide. To access the ethyl (n=2) series, the allyl group is subjected to ozonolysis and reduced to the alcohol (Ma et al., 2001, J Med Chem 44:4137-4156). Mesylation of the alcohol, substitution with NaN_3, and steps shown in Figure 8 procures the material.

Synthesis of desosamine-modified analogs

A concise synthesis of D-desosamine (Des), in addition to Des analogs at the C3' position has been reported (Velvadapu and Andrade, 2008, Carbohydr Res

343:145-50). Recently, Hofmann reaction was employed to execute the same 2,3-epoxide ring-opening method without removing Des and reglycosylating (Figure 11). This markedly reduces the steps, time, and cost involved in preparing novel Des-modified analogs. Significantly, this route enables the first synthesis of *N,N'*-
5 disubstituted Des analogs from epoxide **15** (Figure 6), which is utilized to prepare novel analogs of SOL (**1**) and access analogs for *in situ* click. Previous modifications of Des at the 3'-amino position have been limited to monosubstitution via sequential dealkylation/*N*-alkylation (Ying and Tang, 2010, Curr Top Med Chem 10:1441-69). To date, a small library of clarithromycin (**3**) analogs **16-17** has been made by
10 $Sc(OTf)_3$-catalyzed ring-opening of **15** with secondary amines **31-39** in good to excellent yields and varying ratios of isomeric C3':C2' products (Figure 11). The C3' amino group can now be fully explored and novel macrolide analogs can be accessed.

Synthesis of Des-modified analogs.

15 3'-modified Des donors are prepared by (1) regioselective ring-opening of **15** with the appropriate secondary amines as in Figure 11 to access novel macrolides. Other Lewis acids, solvents, additives, and heating regimes are screened such that we can predictably prepare the desired regioisomer. The design and synthesis of >100 unique analogs are subjected to *in situ* click and evaluated.

20

Synthesis of macrolide-tethered azides at N3'.

Access to N3'-functionalized azides for *in situ* click chemistry follows the approach described above and is detailed in Figure 12. Epoxide **15** is reacted with the appropriate secondary amine prepared using standard synthetic techniques to
25 furnish **40**. After acetylation of the newly formed 2' hydroxyl of Des, the tactics outlined in Figure 8 transform **40** into either N3'-functionalized azides **41-42**.

Optimize in situ click with wild-type and resistant E. coli ribosomes

The ribosome-templated *in situ* click method with azide **8** has been
30 reproducibly extended from a single alkyne (Figure 7), 5-alkyne, and most recently a 15-alkyne competition experiment (Figure 13). The utility of the method is its ability to reproducibly prioritize analogs as determined by MIC against *E. coli* and *S. pneumoniae*. The method's selectivity is particularly pronounced in the more clinically relevant latter strain. In addition, protein synthesis inhibition data consistent

with the MIC results was obtained. K_d values are measured for these analogs using the well-established competition binding experiments with [^{14}C]erythromycin (Petropoulos et al., 2009, J Mol Biol 385:1179-92).

For the 15-membered competition experiment, both ribosome and azide **8** concentrations were increased from 5 μM to 10 μM, and 0.5 mM of each of the 15 alkynes were used for a total alkyne concentration of 7.5 mM. A negative control containing no ribosomes was also set up and incubated in the same way to provide data for the background reaction. Assays were incubated at room temperature for 24 h before injecting onto an Agilent 6520B Q-TOF LC-MS using a C8 reverse-phase column. Extracted ion chromatograms $(M+H)^+$ were generated for all expected triazole products, and peaks were integrated to obtain mass counts (MC). Values in Figure 13 are reported as MC percent increase of product formed in the presence of ribosomes relative to the respective background reaction in the absence of ribosomes (i.e., |MC|/background MC x 100). It is important to compare these values relative to the background reaction since background rates can differ markedly from analog to analog. In competition *in situ* click experiments, Sharpless reported background reaction rates were similar amongst analogs tested; thus, calculating MC difference and normalizing to the best performer (highest MC) was sufficient for rank-ordering compounds (Manetsch et al., 2004, J Am Chem Soc 126: 12809-18; Lewis et al., 2002, Agnew Chem Int Ed 41:1053-7; Krasiński et al., 2005, J Am Chem Soc 127:6686-92; Grimster et al., 2012, J Am Chem Soc 134:6732-40; Mocharla et al., 2004, Agnew Chem Int Ed 44:116-20). This approach was used in the proof-of-concept binary experiments (Figure 7).

Co-crystal structures of bacterial ribosomes (e.g., *E. coli, H. marismortui, D. radiodurans*) with ketolides (TEL, SOL, CET) have shown that although the ribosomes of these different species are highly conserved in their binding of ketolides, side chain orientations of bound ketolides vary greatly (Dunkle et al., 2010, Proc Natl Acad Sci U.S.A.107:17152-17157). There are currently no co-crystal structures of resistant ribosomes; however, results from GCMC/MD simulation allows for rationalization on how ribosomal modification (*N,N'*-dimethyl A2058) or mutation (A2058G) negatively impacts the binding of ketolide antibiotics.

The impact of ribosomal changes on drug efficacy is not always proportional amongst compounds (i.e., the binding/activity of a set of compounds does not retain its rank order when tested against a resistant bacterium or ribosome). It

is possible for a compound with low activity against wild-type bacteria to be a top-performing compound against resistant bacteria. For this very reason, resistant ribosomes were targeted for *in situ* click assays. Mutant 70S *E. coli* ribosomes dimethylated at position A2058 were isolated and using cell free protein synthesis inhibition SOL (**1**) was shown to have an IC_{50} of 30 μM, which is thirty-fold greater than wild-type 70S ribosomes. Thus, successful binary *in situ* click experiment has been designed with these ribosomes to make SOL (**1**) and its 1,5 isomer (Figure 14). The concentration of azide **8** was increased to 50 μM whereas other parameters remained the same as the wild- type binary experiment (see Figure 7).

The results, like the wild-type binary experiment in Figure 3, showed an increase of both SOL (**1**) and its 1,5 isomer over the background (i.e., no ribosomes). Remarkably, the ratio of **1** to its 1,5 isomer switched, suggesting the latter is more active against *E. coli* (pikR2) and possibly other *erm*-modified resistant bacteria. With recent advances in the isolation of pathogenic bacterial ribosomes, the *in situ* click method may allow for custom design of resistance-specific antibiotics

Using in situ click in the preparation of novel analogs

Reaction conditions and data analysis for the *in situ* click method are continually optimized, which is a complex, dynamic system containing mixtures of azide **8**, excess alkynes, as well as macromolecules (i.e., RNA and proteins) that make up the ribosome. As discussed, differences in background azide/alkyne cycloaddition rates can affect the comparison of mass counts (MC) in the assay, so that it is not straightforward to use observed MC as quantitative indicators of the amount of triazole products formed. Individual triazole formation in the presence of ribosomes must be analyzed relative to their respective background reaction before comparing to other formed alkynes.

The current protocol consists of incubating a mixture of 10 μM azide **8**, 15 different alkynes (0.5 mM each), and 10 μM *E. coli* 70S ribosomes (or 50S subunits) at room temperature for 24-48 h. The efficiency of the method increases in proportion to alkynes tested, as compared to metal-catalyzed counterparts. For example, *in situ* click using 1000 alkynes screens 2000 analogs (i.e., 1000 1,4-triazoles and 1000 1,5-triazoles).

Figure 15 presents an overview of the validated 1x15 *in situ* click protocol used at positions N11, C9, O6, and N3’ (desosamine). Reactant

concentrations, reaction time, and temperature are systematically optimized. To increase efficiency the number of alkynes are increased. Results from the *in situ* click experiments are benchmarked against SOL (**1**) such that compounds possessing MC percent increases equal to or greater than **1** are characterized and confirmed by chemical synthesis after MIC evaluation.

Development of in situ click using pathogenic bacteria

While the isolation of ribosomes from varying sources is achievable, it can be time-consuming and tedious. Accordingly, the transfer of ribosome-templated *in situ* click from *E. coli* ribosomes is explored to those derived from pathogenic bacteria. For macrolides to exert their bacteriostatic effect, they need to inhibit a significant number of the approximately 10,000 ribosomes present in each growing bacterial cell 9 Tenson and Mankin, 2006, Mol Microbiol 59:1664-77). It is hypothesized that incubating live bacteria [i.e., 10^{2-12} colony forming units (CFU)] with azides such as **8** and libraries of alkynes will result in observable triazole formation by LC-MS, as in the case of isolated ribosomes. Bacteria are incubated overnight, diluted to the desired number of CFU, and incubated again in the presence of azide and alkyne partners (1-2 mL). Cells are lysed and filtered through 0.2 micron filters, solvent/media is removed by centrifugal evaporation, and the contents are re-dissolved in 100 µL of suitable solvent for injection on the LC-MS. Possible use of appropriate molecular weight cutoff spin filters are utilized to remove high molecular weight macromolecules (e.g., protein, RNA, DNA, lipids) that might interfere with the assay. This method allows for the accurate, fast, and, significantly, cost-effective analysis of large numbers of compounds and bacteria without the need for isolation of the ribosomes.

The application of *in situ* click methodology toward ribosomes derived from pathogenic bacteria is a major step in our antibiotic discovery process. It is a logical next step following the use of *E. coli* ribosomes as a model for pathogenic ribosomes strains. All side chains designed and optimized above are screened through the bacterial cell *in situ* click process. Bacteria include *S. aureus*, MRSA, *S. pneumoniae, E. faecalis,* and *H. influenzae*. The *in situ* click approach to antibiotic drug discovery greatly accelerates the pace of discovery while simultaneously cutting down the cost and time associated with the unattractive alternative (i.e., tedious and

expensive synthesis of all 1,4- and 1,5-regioisomers, purification, characterization, and evaluation of each possible azide/alkyne combination).

Differential uptake of alkyne fragments during the incubation growing bacterial cells (i.e., cell permeability) reduces fragment concentrations inside the cell. In this case, the bacterial cells are first lysed and then fragments are added such that the *in situ* click experiment is conducted with ribosomes in the cell lysate. Established chromatographic techniques for isolating pathogenic bacterial ribosomes by Xu and co-workers at Pfizer are able to overcome issues of sensitivity that arise from dilution by contents in the cellular milieu (Maguire et al., 2008, RNA 14:188-195).

Determination of K_d, protein synthesis inhibition and MIC values

SOL (**1**) and analogs **43-56** have been evaluated with MIC assays against *S. pneumoniae*, *S. aureus, and E. coli*, including wild-type (WT) and resistant strains. The MIC assays were run in triplicate on independent bacterial cell cultures. Furthermore, strains ATCC 29213 (*S. aureus*) and ATCC 49619 (*S. pneumoniae*) served as quality control strains with SOL (**1**) values matching those published by the Clinical and Laboratory Standards Institute. We have shown that thiophene analog **43** was two-fold more potent than SOL against *E. coli* DKpkk3535 and 2058G strains. Phenol analog **49** was two-fold more potent in the *S. pneumoniae* ATCC wild-type and *E. coli* mutant DK 2058G strains. Finally, analog **54** performed slightly better than **1** against a MRSA strain (Figure 16).

Evaluation of macrolide analogs

K_d values for SOL and analogs **43-56** are measured established competition binding experiments with [^{14}C]erythromycin and compared with published K_d values for SOL (Petropoulos et al., 2009, J Mol Biol 385:1179-92; Llano-Sotelo et al., 2010 Antimicrob Agents Chemother 54:4961-70). Analogs with bioactivities equal or superior to SOL in MIC assays are subjected to protein synthesis inhibition assays described above. In combination with K_d values, these serve to confirm analog MOA.

Altogether, these data show that the approach toward future-generation ketolides developed through the use of chemical synthesis, *in situ* click, and biological evaluation has great promise in discovering novel macrolides to combat resistance.

IND-enabling experiments

Compounds are ranked based upon the criteria described above. The best 3-5 compounds are selected for additional characterization including: (1) expanded MIC assay against 20 clinical isolates per species/phenotype of the potential target organisms; (2) determination of the maximum tolerated dose in mice versus SOL and ERY; (3) assessment of the pharmacokinetic profile of the agents in mice; and, (4) determination of the efficacy of each agent in a systemic lethal infection model in mice for one or more target pathogens (selection of organism based upon results of expanded MIC evaluation). The protective dose for 50% of animals (PD_{50}) is used as an estimation of *in vivo* efficacy. The *in vivo* efficacy of the two highest-ranking compounds in a mouse model of bacterial pneumonia are then assessed.

Example 2: Ribosome-templated azide−alkyne cycloadditions: synthesis of potent macrolide antibiotics by *in situ* click chemistry

Solithromyin (**1**), one of the most potent macrolide antibiotics discovered to date (Figure 17A), was prepared using the Cu(I)-catalyzed Huisgen [3+2] dipolar cyloaddition (i.e., click) reaction of azide **8** and 3-ethynylaniline (**9**) (Fernandes et al., 2011, Drug Future 36:751-8). Inspiration for **1** came from the erythromycin-derived ketolide antibiotic telithromycin (**6**), which possesses a structurally related pyridyl-imidazole side-chain (Figure 17B) (Bryskier, 2000, Clin Microbiol Infect 6:661-9). Over half of all antibiotics, including macrolides, target the bacterial ribosome (Tenson and Mankin, 2006, Mol Microbiol 59:1664-77). Macrolides reversibly bind near the peptidyl transferase center of the 50S subunit with low nanomolar affinity, thus blocking protein synthesis (Yan et al., 2005, Antimicrob Agents Chemother 49:3367-72; Spahn and Prescott, 1996, J Mol med 74:423-39). In 2010, the structure of solithromycin (**1**) bound to the *E. coli* 70S ribosome was solved, confirming both the location and mode of binding (Llano-Sotelo et al., 2010 Antimicrob Agents Chemother 54:4961-70). Like other macrolides, **1** interacts with specific 23S rRNA residues via the macrolactone ring and desosamine sugar; moreover, the biaryl side-chain attached at N11 engages in π-π interactions with the A752-U2609 base pair. It was hypothesized that the strong molecular recognition of macrolides by bacterial ribosomes could be leveraged in the templated synthesis of solithromycin (**1**) from fragments **8** and **9** (Figure 5) (Jencks, 1981, PNAS 78:4046-50).

The target-guided Huisgen cycloaddition reaction (i.e., *in situ* click chemistry) is predicated on the selective, proximal binding of azide- and alkyne-bearing fragments, which lowers the activation energy of irreversible 1,2,3-triazole ligation by co-localization (Mamidyala and Finn, 2010, Chem Soc Rev 39:1252-61).
5 Unlike the Cu-catalyzed click reaction that prepares exclusively the *syn* (1,4)-triazole or the Ru-catalyzed variant that prepares exclusively the *anti* (1,5)-triazole, the *in situ* click process results in the regioisomer that best establishes non-covalent interactions with the target (Rostovtsev et al., 2002, Agnew Chem Int Ed 41:2596-9; Boren et al., 2008, J Am Chem Soc 130:8923-30) (Figure 17). Accordingly, the resultant
10 cycloadduct possesses greater affinity for the target vis-à-vis the individual fragments (Jencks, 1981, PNAS 78:4046-50). In this regard, *in situ* click chemistry represents an extension of fragment-based drug design wherein the biological target participates in the synthesis of its own inhibitor (Rees et al., 2004, Nat Rev Drug Disc 3:660-72; Scott et al., 2012, Biochemistry 51:4990-5003). Moreover, *in situ* click chemistry is a
15 validated drug discovery platform having been successfully employed in the discovery of potent inhibitors for acetylcholine esterase (Manetsch et al., 2004, J Am Chem Soc 126: 12809-18; Lewis et al., 2002, Agnew Chem Int Ed 41:1053-7; Krasiński et al., 2005, J Am Chem Soc 127:6686-92; Grimster et al., 2012, J Am Chem Soc 134:6732-40), carbonic anhydrase (Mocharla et al., 2004, Agnew Chem Int
20 Ed 44:116-20), HIV-protease (Whiting et al., 2006, Agnew Chem Int Ed 45:1435-9), chitinase (Hirose et al., 2009, Antibiot 62:277-82), protein-protein interactions (Namelikonda and Manetsch, 2012, Chem Commun 48:1526-8), DNA-recognition (Poulin-Kerstien and Dervan, 2003, J Am Chem Soc, 125:15811-21), EthR (a transcriptional regulator in *M. tuberculosis*) (Mamidyala and Finn, 2010, Chem Soc
25 Rev 39:1252-61 ; Sharpless and Manetsch, 2006, Expert Opin Drug Discov 1:525-38), the creation of antibody-like protein capture agents (Millward et al., 2011, J. Am. Chem. Soc. 133:18280-18288; Agnew et al, 2009, Angew. Chem. Int. Ed. 48:4944-4948; Pfeilsticker et al., 2013, Plos One; Nag et al., 2013, Angew. Chem. Int. Ed. 52:13975-13979; Farrow et al., 2013, Acs Nano 7:9452-9460; Deyle et al., 2015,
30 Nature Chemistry 7:455-462), in addition to toxic RNA, which was targeted *in cellulo IMil* (Rzuczek et al., 2014, Angew. Chem. Int. Ed. 53:10956-10959).

The advent of the synthetic oxazolidinone class of antibiotics in 2000—like the sulfa drugs of the 1930s—showed that natural products are not the sole source of clinical antibiotics (Figure 1B). Flagship member linezolid (**2**) has been

used to treat drug-resistant Gram-positive pathogens including MRSA (Brickner et al., 2008, J Med Chem 51:1981-90). The emergence of resistance to **85** led to the development of 2nd-generation oxazolidinones torezolid (**87,** Trius/Cubist) and radezolid (**88**, Rib-X/Melinta), which are under FDA review and Phase II trials, respectively (Shaw and Barbachyn, 2011, Antimicrobial Therapeutics Reviews 1241:48-70; Barbachyn, 2012, Antibiotic Discovery and Development 1-2:271-99).

The materials and methods of the experimental example are now described.

General Methods

All reactions containing moisture or air sensitive reagents were performed in oven-dried glassware under nitrogen or argon. *N,N*-Dimethylformamide, tetrahydrofuran, toluene and dichloromethane were passed through two columns of neutral alumina prior to use. Water was double distilled prior to use in in situ click reactions. Buffers were filtered prior to use. All other reagents were purchased from commercial sources and used without further purification. All solvents for work-up procedures were used as received. Flash column chromatography was performed using an Isco Combiflash Rf 200 instrument with Isco Gold Silica Gel Columns with the indicated solvents. Thin layer chromatography was performed on Merck 60 F254 silica gel plates. Detection was performed using UV light, iodine, PMA stain and subsequent heating. 1H and 13C NMR spectra were recorded at the indicated field strength in $CDCl_3$ or MeOD at rt. Chemical shifts are indicated in parts per million (ppm) downfield from tetramethylsilane (TMS, $\delta = 0.00$) and referenced to the $CDCl_3$. Splitting patterns are abbreviated as follows: s (singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublet of doublets), t (triplet), q (quartet) and m (multiplet). Optical rotations were measured on a Perkin-Elmer 341 Polarimeter at room temperature, using the sodium D line.

In Situ click Experiments

In situ procedure for binary component Mixture of Azide 2 and Alkyne 3

***In situ* click:** Azide **8** [0.5 μL, 1 mM in aqueous buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol)] was added to 87 μL of a aqueous buffer [20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-

mercaptoethanol] in a 300 µL microcentrifuge tube. 50S subunits or 70S *E. coli* ribosomes [10 µL, 50 µM in aqueous buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol)] was added to the solution and incubated at 37 °C for 30 minutes. 3-ethynylaniline **9** [2.5 µL, 200 mM in dimethyl
5 sulfoxide (DMSO)] was added for a final volume of 100 µL and final concentrations of 5 µM azide **8**, 5 mM alkyne **9,** and 5 µM 50S subunits or 70S *E. coli* ribosomes. The solution was incubated at room temperature for 24 hours. LC-MS analysis of *in situ* click experiments with AZY and negative control are shown in table 3.

10 **Buffer-only control**: Azide **8** [0.5 µL, 1 mM in aqueous buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol)] was added to 97 µL of aqueous buffer [20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol] in a 300 µL microcentrifuge tube and incubated at 37 °C for 30 minutes. 3-ethynylaniline **9** [2.5 µL, 200 mM in dimethyl sulfoxide (DMSO)] was
15 added for final concentrations of 5 µM azide **8** and 5 mM alkyne **9**. The solution was incubated at room temperature for 24 hours.

BSA control: Azide **8** [0.5 µL, 1 mM in aqueous buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol)] was added to 97 µL of a
20 solution of Bovine Serum Albumin [5 µM in aqueous buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol)] in a 300 µL microcentrifuge tube and incubated at 37 °C for 30 minutes. 3-ethynylaniline **9** [2.5 µL, 200 mM in dimethyl sulfoxide (DMSO)] was added for final concentrations of 5 µM azide **8** and 5 mM alkyne **9**. The solution was incubated at room temperature for
25 24 hours.

30S *E. coli* subunit control: Azide **8** [0.5 µL, 1 mM in aqueous buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol)] was added to 87 µL of a aqueous buffer [20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM
30 NH_4Cl, 6 mM 2-mercaptoethanol] in a 300 µL microcentrifuge tube. 30S *E. coli* subunits [10 µL, 50 µM in aqueous buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol)] was added to the solution and incubated at 37 °C for 30 minutes. 3-ethynylaniline **9** [2.5 µL, 200 mM in dimethyl sulfoxide (DMSO)] was added for a final volume of 100 µL and final concentrations of 5 µM

azide **8**, 5 mM alkyne **9,** and 5 μM 50S subunits or 70S *E. coli* ribosomes. The solution was incubated at room temperature for 24 hours.

Azithromycin (AZY) inhibition experiment: Azide **8** [0.5 μL, 1 mM in aqueous buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol)] was added to 84.5 μL of a aqueous buffer [20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol] in a 300 μL microcentrifuge tube. AZY [2.5 μL, 1 mM in aqueous buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol)] and 50S or 70S *E. coli* ribosomes [10 μL, 50 μM in aqueous buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol)] were added to the solution and incubated at 37 °C for 30 minutes. 3-ethynylaniline **9** [2.5 μL, 200 mM in dimethyl sulfoxide (DMSO)] was added for a final volume of 100 μL and final concentrations of 5 μM azide **8** and 5 mM alkyne **9** and 5 μM 50S or 70S *E. coli* ribosomes. The solution was incubated at room temperature for 24 hours.

LC-MS Analysis: 90 μL of the above solutions were injected on an Agilent 6520B Q-TOF LC-MS instrument utilizing an Agilent Poroshell 120 4.6 mm x 30 mm (2.1 um particle size) C8 reverse phase column with a flow rate of 1 mL per minute and a 10 minute gradient from 0% ACN (0.1% formic acid)/100% water (0.1% formic acid) to 100% ACN (0.1% formic acid)/0% water (0.1% formic acid). Extracted ion chromatograms (EIC) were used to locate & quantify the amount of **1.**

General Procedure for 5-Alkyne *In Situ* click Competition Experiments

Azide **8** [1 μL, 1 mM in aqueous buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol)] was added to 74 μL of a aqueous buffer [20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol] in a 300 μL microcentrifuge tube. 50S *E. coli* ribosomes [20 μL, 50 μM in aqueous buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol)] were added to the solution and incubated at 37 °C for 30 minutes. 5 Alkynes [1 μL each, 200 mM in dimethyl sulfoxide (DMSO)] were added for a final volume of 100 μL and final concentrations of 10 μM azide **8**, 2 mM each alkyne, and 10 μM 50S or 70S *E. coli* ribosomes. The solution was incubated at room temperature for 24 hours. LC-MS analysis of *in situ* click experiments with azide **8**

and alkynes **9, 57, 67, 68,** and **62** are shown in table 4. LC-MS analysis of *in situ* click experiments with azide **8** and alkynes **66, 63, 59, 64,** and **68** are shown in table 5.

Buffer Control: Azide **8** [1 µL, 1 mM in aqueous buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol)] was added to 94 µL of a aqueous buffer [20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol] in a 300 µL microcentrifuge tube and incubated at 37 °C for 30 minutes. 5 Alkynes [1 µL each, 200 mM in dimethyl sulfoxide (DMSO)] were added for a final volume of 100 µL and final concentrations of 10 µM azide **8** and 2 mM each alkyne. The solution was incubated at room temperature for 24 hours.

LC-MS Analysis: 90 µL of the above solutions were injected on an Agilent 6520B Q-TOF LC-MS instrument utilizing an Agilent Poroshell 120 4.6 mm x 50 mm (2.1 um particle size) C8 reverse phase column with a flow rate of 1 mL per minute and a 10 minute gradient from 0% ACN (0.1%TFA)/100% water (0.1% TFA) to 100% ACN (0.1%TFA)/0% water (0.1%TFA). Extracted ion chromatograms (EIC) were used to locate and quantify the amount clicked products.

General Procedure for 15-Alkyne *In Situ* click Competition Experiments

***In situ* click:** Azide **8** [1 µL, 1 mM in aqueous buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol)] was added to 71.5 µL of a aqueous buffer [20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol] in a 300 µL microcentrifuge tube. 70S *E. coli* ribosomes [20 µL, 50 µM in aqueous buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol)] were added to the solution and incubated at 37 °C for 30 minutes. 15 Alkynes [0.5 µL each, 200 mM in dimethyl sulfoxide (DMSO)] were added for a final volume of 100 µL and final concentrations of 10 µM azide **8**, 1 mM each alkyne, and 10 µM 50S *E. coli* ribosomes. The solution was incubated at room temperature for 48 hours.

Buffer Control: Azide **8** [1 µL, 1 mM in aqueous buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol)] was added to 91.5 µL of a aqueous buffer [20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM 2-mercaptoethanol] in a 300 µL microcentrifuge tube and incubated at 37 °C for 30 minutes. 15 Alkynes [0.5 µL each, 200 mM in dimethyl sulfoxide (DMSO)] were

added for a final volume of 100 μL and final concentrations of 10 μM azide **8** and 1 mM each alkyne. The solution was incubated at room temperature for 48 hours.

Dissociation Constant (K_d) Determination

5 Competition Binding of BODIPY Erythromycin A with Triazoles **1**, Azide **8, 71-83** Utilizing Fluorescence Polarization (FP) was used to determine the Dissociation Constant (K_d)

Fluorescence Polarization (FP) Method

10 5.2 nM BODIPY-Erythromycin A was incubated with 37.8 nM 70S (12.9 nM active 70S as determined by binding assays) in buffer (20 mM HEPES pH 7.5, 50 mM NH_4Cl, 10 mM $MgCl_2$, 0.05% Tween 20) in a total volume of 96 μl in the wells of a 96-well plate for 30 min at room temp. 4 μl of 25X compound (1% DMSO final) or blank was added incubated at room temperature for one hour. The 96-well

15 plate was then scanned on a Tecan F200 (485 nm excitation / 535 nm emission) to determine milliPolarization (mP).

K_d fitting

Data was plotted as [compound] vs. mP units. The data was fitted to

20 Wang's cubic equation (Wang et al., 1995, FEBS Lett 360:111-4) in order to obtain the Kd of the competitive compound binding to 70S. Fitting parameters were Amax = 404, Amin = 82, Klig = 4.26e-9 (Kd of the BODIPY probe as determined by binding curves), [Lt] = 5.2 nM, [Rt] = 12.9 nM.

25 IC_{50} fitting

IC_{50} values were determined by extrapolating the compound concentration at which the mP signal was reduced to 50%.

Cell-Free Translation Inhibition

30 Cell-Free Translation Inhibition Method

The ability of triazoles **1** and **71-83** and **azide 8** to inhibit protein synthesis was evaluated by monitoring the expression of a superfolder EmGFP in one of two cell-free protein expression kits: 5Prime's RTS 100 *E. coli* HY kit and Invitrogen's Expressway mini cell-free expression kit (Rosenblum *et al*, Nucleic

Acids Res, 2011). Reactions were performed as per the manufacturers' instructions on a 10 µL scale and with a final triazole concentration of 1 µM. The synthesis of EmGFP was monitored on a Tecan M1000 plate reader (excitation 486 nm, emission 535 nm) at 30 °C for 100 min. Translation inhibition was calculated from the percent reduction in the maximal slope of each curve relative to a DMSO only control.

Minimum Inhibitory Concentration (MIC) Methods

E. coli

The *E. coli* strains used were DK, DK (pkk3535), DK (2058G), SQ171, and SQ171 (2058G). E. coli strains were inoculated from freezer stock into Lysogeny Broth containing 50 µg/mL of Ampicillin (LB-Amp) and incubated at 37 °C overnight (No Ampicillin used with DK strain). The culture was then diluted 1:100 and grown to an OD600nm = 0.6 (2-4 h). 75 µL of (LB-Amp) was added to wells in rows 1-11 of 96-well plates. 150 µL of a 64 µg/mL antibiotic solution for DK strains and 128 µg/mL for SQ strains in (LB-Amp) were then added to row 12. Serial dilutions were made from row 12 to 1 for a final volume of 75 µL in each well. The above prepared E. coli cultures were then diluted to OD600nm = 0.004 with (LB-Amp) and 75 µL of the above prepared *E. coli* culture was then added to all wells. The last column of the 96-well plate was reserved for negative controls (LB-Amp only) and positive controls (OD600nm = 0.004 *E. coli* culture). 96-well plates were covered and incubated at 37 °C for 18 h. Plates were visually inspected to determine MIC.

S. aureus.

The *S. aureus* strains used were UCN 14 (A2058U), UCN 17 (A2058G), UCN18 (A2059G), ATCC 29213 (wt), and ATCC 33591 (MRSA). *S. aureus* strains were inoculated from freezer stock into Brain Heart Infusion (BHI) Broth 37 °C overnight. The culture was then diluted 1:100 and grown to an OD_{600nm} = 0.6 (2-4 h). 75 µL of BHI Broth was added to wells in rows 1-11 of 96-well plates. 150 µL of a 256 µg/mL antibiotic solution in BHI Broth was then added to row 12. Serial dilutions were made from row 12 to 1 for a final volume of 75 µL in each well. The above prepared *S. aureus* cultures were then diluted to OD_{600nm} = 0.004 with BHI Broth and 75 µL of the above prepared *S. aureus* culture was then added to all wells. The last column of the 96-well plate was reserved for negative controls (BHI Broth

only) and positive controls (OD_{600nm} = 0.004 *S. aureus* culture). 96-well plates were covered and incubated at 37 °C for 18 h. Plates were visually inspected to determine MIC.

5 <u>*S. pneumonia*</u>

The *S. pneumoniae* strains used were ATCC 49619 (WT), 2196 (*erm* B), 655 (*mef* A), 1369 (*erm* B and *mef* A), 319 (A2059G), and 2634 (*mef* A and A2059G). *S. pneumoniae* strains were plated from freezer stock on Tryptic Soy (TS) agar plates containing 5% sheep's blood and incubated at 37 °C in the presence of 5%
10 CO_2 overnight. The *S. pneumoniae* was plated again from the above plates on new Tryptic Soy agar plates containing 5% sheep's blood and incubated at 37 °C in the presence of 5% CO_2 overnight. (Two growth cycles were needed to obtain a suitable amount of bacterial growth) Colonies were then added to Mueller Hinton (MH) Broth until an OD_{600nm} = 0.1 was reached. 75 µL of MH broth was added to wells in rows 1-
15 11 of 96-well plates. 150 µL of an 8 µg/mL antibiotic solution in MH broth was then added to row 12. Serial dilutions were made from row 12 to 1 for a final volume of 75 µL in each well. The above prepared *S. pneumoniae* cultures were then diluted to OD_{600nm} = 0.001 with MH broth and 75 µL was added to all wells. The last column of the 96-well plate was reserved for negative controls (MH broth only) and positive
20 controls (OD_{600nm} = 0.001 *S. pneumoniae* culture). 96-well plates were covered and incubated at 37 °C in the presence of 5% CO_2 for 20 h. Plates were visually inspected to determine MIC.

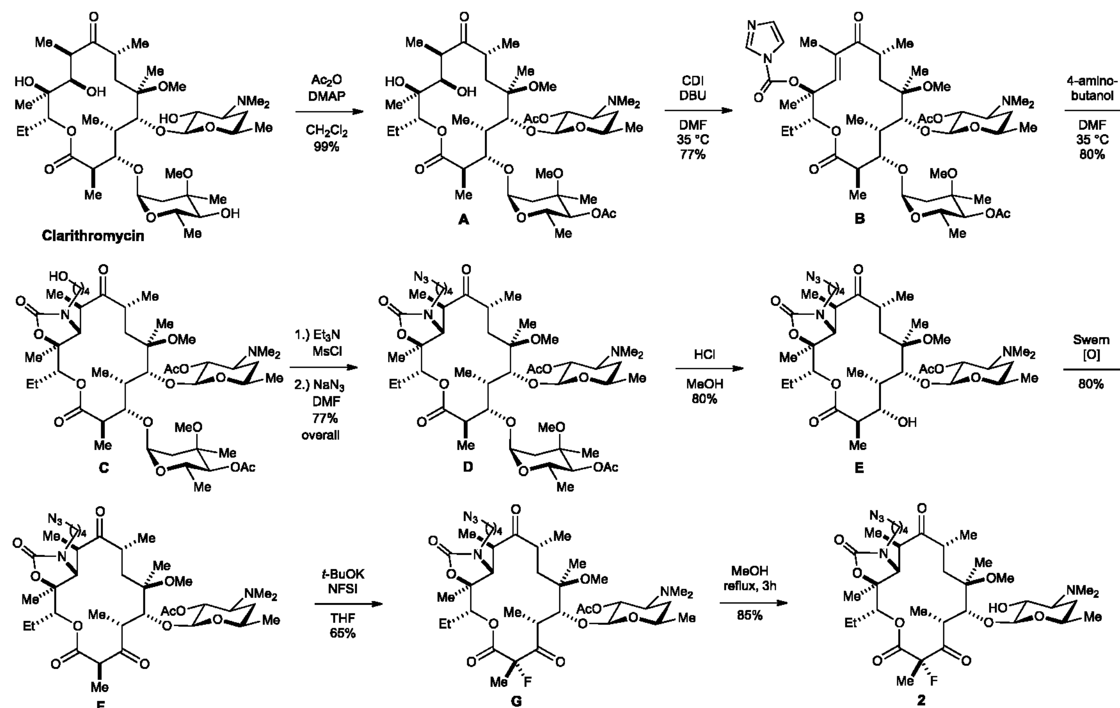
Synthetic Methods

25 <u>Experimental Procedure for Copper(I)-catalyzed click Reactions</u>

Azide **8** (45 mg, 0.062 mmol, 1 eq.), $CuSO_4$ (1.24 µmol, 0.02 eq.), (+)-Sodium L-ascorbate (6.2 µmol, 0.1 eq.), and alkyne **9, 57-70** (0.124 mmol, 2 eq.) in 1:1 water:*t*-BuOH (1.24 mL, 0.05 M) was stirred at rt for 24 hours. Water (2 mL) was added and the mixture extracted with EtOAC (3 x 5 mL). The combined organic
30 fractions were washed with brine (5 mL), dried over sodium sulfate, and the solvent removed under reduced pressure. The product was purified by flash chromatography with a Combiflash instrument (MeOH/DCM (1% NH_4OH) 0-10%) to give triazoles **1, 71-83** (70-90% yield).

General Scheme for Synthesis of Azide **8**

Scheme 1 shows the general synthesis of Azide **3**.



5 Scheme 1. Synthesis of Azide 2

Experimental Procedures for azide **8**, Solithromycin (**1**) and triazoles **71-83**

Synthesis and structural confirmation of intermediates **A-G**, azide **2,** Solithromycin (**1**) and triazoles **71-83** are described below. The chemical structures
10 for intermediates **A-G**, azide **8,** Solithromycin (**1**) and triazoles **71-83** are depicted in Figures 27-29.

Intermediate A. Ac_2O (3.42 g, 33.5 mmol) was added to a solution of Clarithromycin (5 g, 6.7mmol) and DMAP (205 mg, 1.67 mmol) in DCM (27 mL) under an inert
15 atmosphere. After 18 hours stirring at room temperature TLC analysis showed complete consumption of the starting material. Sat. Aq. NH_4Cl (15 mL) was added and the mixture was extracted with DCM (3 x 30 mL). The combined DCM fractions were washed with brine (10 mL), dried over sodium sulfate, and the solvent removed under reduced pressure to give 5.5 g (99%) of Bis-acetate protected Clarithromycin **A**
20 as a white foam. The product was sufficiently pure to carry on to the next step. $[\alpha]^{23}{}_D$ - 151.6 (c 1.5, $CHCl_3$); IR (neat) 2974, 1740, 1457, 1373, 1235, 1171, 1047, 1010, 986;

^{1}H NMR (500 MHz, $CDCl_3$) δ 5.06 (dd, *J* = 11.1, 2.2 Hz, 1H), 4.98 (d, *J* = 5.0 Hz, 1H), 4.74 (dd, *J* = 10.4, 7.5 Hz, 1H), 4.67 (dd, *J* = 12.5, 8.6 Hz, 2H), 4.29 (dd, *J* = 9.7, 6.2 Hz, 1H), 3.97 (s, 1H), 3.78 – 3.69 (m, 3H), 3.59 (d, *J* = 6.4 Hz, 1H), 3.34 (s, *J* = 10.7 Hz, 3H), 3.19 (s, 1H), 3.00 (s, 3H), 2.99 – 2.94 (m, 1H), 2.87 (dd, *J* = 9.5, 7.3 Hz, 1H), 2.72 (td, *J* = 12.3, 4.1 Hz, 1H), 2.60 – 2.50 (m, 1H), 2.40 (d, *J* = 15.2 Hz, 1H), 2.27 (s, 6H), 2.09 (s, 3H), 2.04 (s, 3H), 1.95 – 1.83 (m, 2H), 1.72 – 1.54 (m, 4H), 1.51 – 1.39 (m, 1H), 1.34 (s, 3H), 1.30 (d, *J* = 11.4 Hz, 1H), 1.20 (d, *J* = 6.1 Hz, 3H), 1.17 (d, *J* = 6.0 Hz, 3H), 1.12 (dt, *J* = 11.1, 4.7 Hz, 15H), 0.93 (d, *J* = 7.6 Hz, 3H), 0.83 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 221.14, 175.51, 170.44, 169.98, 99.93, 95.75, 80.16, 78.56, 78.23, 77.67, 76.58, 74.18, 72.68, 71.95, 69.10, 67.23, 63.13, 63.10, 50.50, 49.31, 45.25, 44.83, 40.74 (2C), 38.62, 38.56, 37.21, 35.14, 31.15, 21.57 (2C), 21.10, 21.04, 20.89, 19.73, 18.33, 17.93, 16.07, 15.95, 12.34, 10.56, 9.01; HRMS (ESI) calc'd for $C_{42}H_{73}NO_{15}$ + H = 832.5058, found 832.5069.

Intermediate B. Carbonyldiimidazole (5.4 g, 33.5 mmol), 1,8-Diazabicyclo[5.4.0]undec-7-ene (2.04 g, 13.4 mmol), and **A** in DMF (110 mL) under an inert atmosphere were heated to 35 °C for 72 h. The solution was then cooled to 0 °C and water (110 mL) was added. The solid precipitate formed was then filtered off and washed with water (3 x 50 mL). The white solid was then dissolved in DCM and filtered over sodium sulfate to remove in residual water. The DCM was removed under reduced pressure to give 4.7g (77%) of **B** as a white solid. $[\alpha]^{23}_D$ -66.3 (c 0.79, $CHCl_3$); IR (neat) 2976, 2939, 2831, 1763, 1740, 1670, 1465, 1380, 1292, 1240, 1163, 1047, 1000, 752, 667; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.07 (s, 1H), 7.35 (s, 1H), 7.06 (s, 1H), 6.64 (s, 1H), 5.81 (dd, *J* = 10.4, 2.8 Hz, 1H), 4.96 (s, 1H), 4.75 – 4.64 (m, 2H), 4.60 (d, *J* = 7.5 Hz, 1H), 4.34 (dd, *J* = 9.5, 6.1 Hz, 1H), 3.88 – 3.76 (m, 1H), 3.70 – 3.60 (m, 1H), 3.58 (d, *J* = 6.8 Hz, 1H), 3.33 (s, *J* = 5.6 Hz, 3H), 3.27 – 3.18 (m, 1H), 3.13 (s, 3H), 2.97 – 2.80 (m, 1H), 2.79 – 2.60 (m, 1H), 2.40 (d, *J* = 15.1 Hz, 1H), 2.26 (s, *J* = 9.6 Hz, 6H), 2.11 (s, 3H), 2.02 (s, *J* = 5.9 Hz, 3H), 1.93 – 1.87 (m, 1H), 1.84 (s, *J* = 5.0 Hz, 3H), 1.77 (s, *J* = 11.3 Hz, 3H), 1.74 – 1.59 (m, 4H), 1.41 – 1.30 (m, 3H), 1.25 (d, *J* = 5.3 Hz, 6H), 1.18 (d, *J* = 6.2 Hz, 6H), 1.14 (d, *J* = 5.0 Hz, 6H), 0.96 – 0.87 (m, *J* = 13.7, 6.7 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 204.61, 174.37, 170.50, 169.83, 145.76, 138.75, 137.82, 136.97, 130.82, 117.09, 100.46, 96.28, 84.43, 80.08, 78.51, 78.26, 77.21, 75.22, 72.65, 71.84, 67.56, 63.25, 63.03, 50.76, 49.42,

45.11, 40.68, 39.99, 39.25, 35.30, 30.82, 29.67, 22.33, 21.68, 21.49, 21.44, 21.06, 20.90, 19.91, 18.36, 18.13, 16.14, 13.32, 10.21, 9.26; HRMS (ESI) calc'd for $C_{46}H_{73}N_3O_{15}$ + H = 908.5114, found 908.5111.

5 **Intermediate C.** A solution of **B** (4.7 g, 5.2 mmol) and 4-aminobutanol (2.3 g, 26 mmol) in DMF (17 mL) was heated to 35 °C for 48 h under an inert atmosphere. The solution was then cooled to 0 °C and water (17 mL) was added. The solid precipitate formed was then filtered off and washed with water (3 x 10 mL). The white solid was then dissolved in DCM and filtered over sodium sulfate to remove in residual water.
10 The DCM was removed under reduced pressure to give 3.7g (80%) of **C** as a white solid. $[\alpha]^{23}_D$ -65.9 (c 1.2, $CHCl_3$); IR (neat) 2974, 2938, 1740, 1457, 1372, 1234, 1167, 1048, 1011, 754; ^1H NMR (400 MHz, $CDCl_3$) δ 5.02 – 4.92 (m, 2H), 4.79 – 4.70 (m, 1H), 4.69 – 4.62 (m, 2H), 3.76 – 3.55 (m, 8H), 3.33 (s, *J* = 3.4 Hz, 3H), 3.14 (s, *J* = 5.8 Hz, 1H), 3.07 (q, *J* = 6.9 Hz, 1H), 3.01 (s, 3H), 2.90 (dd, *J* = 9.7, 7.2 Hz,
15 1H), 2.78 – 2.67 (m, 1H), 2.62 – 2.49 (m, 1H), 2.40 (d, *J* = 15.2 Hz, 1H), 2.27 (s, *J* = 6.1 Hz, 6H), 2.10 (s, 3H), 2.04 (s, 3H), 1.98 – 1.76 (m, 4H), 1.76 – 1.45 (m, 8H), 1.39 (s, *J* = 3.6 Hz, 3H), 1.34 (s, *J* = 8.9 Hz, 3H), 1.21 (d, *J* = 7.3 Hz, 3H), 1.17 (d, *J* = 6.0 Hz, 3H), 1.15 – 1.08 (m, 9H), 1.00 (d, *J* = 6.9 Hz, 3H), 0.94 (d, *J* = 7.6 Hz, 3H), 0.82 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.56, 176.20, 170.42, 169.96,
20 157.46, 99.96, 95.81, 82.58, 79.45, 78.64, 78.44, 77.14, 76.33, 72.63, 71.90, 67.32, 63.14, 63.07, 62.07, 60.08, 50.55, 49.32, 45.56, 45.02, 43.14, 40.72 (2C), 38.97, 38.36, 35.08, 31.08, 30.01, 29.68, 23.19, 21.99, 21.57, 21.53, 21.07, 20.88, 20.07, 18.83, 18.30, 15.97, 14.36, 14.17, 10.32, 8.98; HRMS (ESI) calc'd for $C_{47}H_{80}N_2O_{16}$ + H = 929.5581, found 929.5572.

25

Intermediate D. Et_3N (739 mg, 7.3 mmol) was added slowly to a solution of **C** and mesylchloride (722 mg, 6.3 mmol) in DCM (26 mL) under an inert atmosphere at 0 °C. The solution was allowed to warm to room temperature stirring overnight for 18 hours. Water (13 mL) was added and the mixture extracted with DCM (3 x 30 mL).
30 The combined organic fraction was washed with brine (10 mL), filtered over sodium sulfate, and the solvent removed under reduced pressure. The crude material was dissolved in DMF (125 mL) and NaN_3 (410 mg, 6.3 mmol) was added. The solution was heated at 80 °C under an inert atmosphere for 15 hours. The solution was cooled to 0 °C and water (125 mL) was added. The white precipitate was filtered and washed

with water (3 x 50 mL). The white solid was then dissolved in DCM (50 mL) and filtered over sodium sulfate to remove residual water. The solvent was removed to give 4.6 g (77% over 2 steps) of **D** as a white solid. $[\alpha]^{23}_{D}$ -66.385.5 (c 0.99, $CHCl_3$); IR (neat) 2978, 2098, 1741, 1455, 1372, 1234, 1165, 1105, 1047, 1010, 752, 666; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.02 – 4.88 (m, 2H), 4.80 – 4.71 (m, 1H), 4.71 – 4.61 (m, 2H), 4.36 – 4.24 (m, 1H), 3.76 – 3.66 (m, 3H), 3.65 – 3.52 (m, 4H), 3.34 (s, 3H), 3.32 – 3.28 (m, 1H), 3.05 (q, *J* = 6.8 Hz, 1H), 2.99 (s, 3H), 2.89 (dd, *J* = 9.5, 7.2 Hz, 1H), 2.80 – 2.65 (m, 1H), 2.61 – 2.48 (m, 1H), 2.40 (d, *J* = 15.2 Hz, 1H), 2.27 (s, 6H), 2.10 (s, *J* = 6.9 Hz, 3H), 2.04 (s, 3H), 1.98 – 1.44 (m, 12H), 1.38 (s, 3H), 1.34 (s, 3H), 1.21 (d, *J* = 7.1 Hz, 3H), 1.18 (d, *J* = 6.0 Hz, 3H), 1.15 – 1.10 (m, 9H), 0.99 (d, *J* = 6.8 Hz, 3H), 0.94 (d, *J* = 7.6 Hz, 3H), 0.82 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.14, 175.98, 170.41, 169.94, 157.37, 99.96, 95.80, 82.60, 79.50, 78.64, 78.44, 77.15, 76.09, 72.62, 71.87, 67.32, 63.13, 63.05, 60.18, 50.40, 49.32, 45.52, 45.00, 44.44, 42.79, 40.70 (2C), 38.89, 38.38, 38.33, 35.07, 31.08, 30.22, 24.66, 21.96, 21.56, 21.52, 21.06, 20.87, 20.02, 18.79, 18.32, 15.94, 14.33, 14.16, 10.33, 8.96; ; HRMS (ESI) calc'd for $C_{47}H_{79}N_5O_{15}$ + H = 954.5645, found 954.5630.

Intermediate E. Intermediate **D** (3.2 g, 3.4 mmol) was added to a mixture of 20% HCl (40 mL) and MeOH (10 mL) in small portions allowing each portion to mix thoroughly before adding more. The resulting suspension was stirred at room temperature for 20 h. The solution was then basified to pH 10-12 with 2 N NaOH. This solution was extracted with EtOAc (3 x 100 mL). The combined organic fraction was washed with brine (100 mL), filtered over sodim sulfate, and solvent removed under reduced pressure. The product was purified by flash chromatography with a Combiflash instrument (MeOH/DCM(1 % NH_4OH) 0-10%) to give 2.0 g (80%) of cladinose removed product **E**. $[\alpha]^{23}_{D}$ -30.7 (c 2.0, $CHCl_3$); IR (neat) 2972, 2096, 1735, 1456, 1375, 1235, 1164, 1055, 755, 667; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.05 – 4.96 (m, 1H), 4.78 – 4.70 (m, 1H), 4.58 (d, *J* = 7.6 Hz, 1H), 3.71 (d, *J* = 2.6 Hz, 1H), 3.65 (s, 1H), 3.62 – 3.54 (m, 2H), 3.50 – 3.43 (m, 1H), 3.39 (d, *J* = 10.5 Hz, 1H), 3.36 – 3.22 (m, 2H), 3.04 (q, *J* = 6.8 Hz, 1H), 2.92 (s, 3H), 2.74 – 2.63 (m, 2H), 2.58 – 2.45 (m, 1H), 2.23 (s, 6H), 2.16 (s, 1H), 2.04 (s, 3H), 2.00 (d, *J* = 7.3 Hz, 1H), 1.95 – 1.84 (m, 1H), 1.75 – 1.57 (m, 6H), 1.56 – 1.48 (m, 1H), 1.48 – 1.42 (m, 1H), 1.39 (s, 3H), 1.36 – 1.28 (m, 1H), 1.25 (s, 3H), 1.23 (d, *J* = 6.7 Hz, 3H), 1.20 (d, *J* = 6.1 Hz, 3H), 1.09 (d, *J* = 7.1 Hz, 3H), 1.00 (d, *J* = 6.8 Hz, 3H), 0.93 (d, *J* = 7.5 Hz, 3H), 0.80 (t, *J* =

7.3 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 215.75, 175.11, 169.90, 157.33, 99.80, 82.78, 80.43, 78.34, 77.37, 76.05, 71.37, 68.78, 63.11, 60.44, 50.95, 49.66, 45.71, 44.11, 42.98, 40.60 (2C), 38.78, 38.21, 35.79, 30.89, 26.31, 24.32, 22.11, 21.42, 21.06, 19.34, 18.81, 15.13, 14.26, 14.16, 10.20, 7.73; HRMS (ESI) calc'd for
5 $C_{37}H_{63}N_5O_{11}$ + H = 754.45.97, found 754.4602.

Intermediate F. Oxalylchloride (1.2 g, 9.6 mmol) and DCM (30 mL) were added to a flamed dried flask under an inert atmosphere. The solution was cooled to -78 °C and DMSO (1.5 g, 19.2 mmmol) was added dropwise. The solution was allowed to stir for
10 45 minutes and then a solution of **E** (1.8 g, 2.4 mmol) in DCM (20 mL) was cannulated into the flask. This solution was then stirred for 1.5 hours at -78 °C. Triethylamine (1.9 g, 19.2 mmol) was added and the solution stirred for 30 minutes at -78 °C and then allowed to slowly warm to room temperature over 1.5 hours. Water (25 mL) was added to the solution and the mixture extracted with DCM (3 x 50 mL).
15 The combined organic fraction was washed with brine (50 mL), dried over sodium sulfate, and the solvent removed under reduced pressure. The product was purified by flash chromatography on a Combiflash instrument (MeOH/DCM 0-10%) to give 1.3 g (70%) of C3-ketone **F**. $[\alpha]^{23}_D$ +17.5 (c 1.2, $CHCl_3$); IR (neat) 2979, 2100, 1744, 1455, 1376, 1215, 1163, 1061, 748, 668; ^1H NMR (400 MHz, $CDCl_3$) δ 4.90 (d, J = 8.7 Hz,
20 1H), 4.71 (dd, J = 10.4, 7.7 Hz, 1H), 4.33 (d, J = 7.6 Hz, 1H), 4.19 (d, J = 8.1 Hz, 1H), 3.79 (q, J = 6.7 Hz, 1H), 3.71 – 3.61 (m, 1H), 3.60 – 3.44 (m, 5H), 3.08 (q, J = 6.8 Hz, 1H), 3.04 – 2.93 (m, 1H), 2.69 – 2.62 (m, 1H), 2.62 (s, J = 13.0 Hz, 3H), 2.60 – 2.50 (m, 1H), 2.21 (s, J = 7.9 Hz, 6H), 2.03 (s, 3H), 1.98 – 1.87 (m, 1H), 1.82 – 1.58 (m, 7H), 1.57 – 1.48 (m, 2H), 1.45 (s, 3H), 1.33 (d, J = 6.7 Hz, 3H), 1.29 (s, 3H), 1.22
25 (d, J = 6.0 Hz, 3H), 1.13 (t, J = 6.4 Hz, 6H), 0.97 (d, J = 6.8 Hz, 3H), 0.82 (t, J = 7.3 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 216.00, 203.75, 169.70, 169.40, 157.10, 101.43, 82.03, 78.22, 78.05, 77.39, 71.45, 69.08, 63.30, 60.44, 51.15, 49.62, 46.95, 44.82, 44.30, 42.67, 40.54 (2C), 39.01, 38.93, 30.32, 29.99, 24.59, 22.24, 21.32, 20.89, 19.62, 18.29, 15.45, 14.66, 14.02, 13.84, 10.35; HRMS (ESI) calc'd for
30 $C_{37}H_{61}N_5O_{11}$ + H = 752.4440, found 752.4438.

Intermediate G. KO-*t*Bu (1.7 mL of 1 M in THF, 206.1 mg, 1.7 mmol) was added dropwise to a solution of **F** in THF (16 mL) at 0 °C under an inert atmosphere and stirred for 30 minutes. NFSI (410 mg, 1.3 mmol) was then added and the solution

allowed to warm to room temperature stirring for 2 hours. Sat. aq. NH_4Cl (10 mL) was added and the mixture extracted with EtOAC (3 x 20 mL). The combined organic fractions were washed with brine (20 mL) and dried over sodium sulfate. The solvent was removed under reduced pressure and the product purified by flash
5 chromatography on a Combiflash instrument (MeOH/DCM 0-10%) to give 650 mg (65%) of **G**. $[\alpha]^{23}_D$ -3.2 (c 0.78, $CHCl_3$); IR (neat) 2980, 2100, 1744, 1372, 1214, 1108, 1062, 1001, 748, 668; ^1H NMR (400 MHz, $CDCl_3$) δ 4.87 (dd, *J* = 10.2, 2.1 Hz, 1H), 4.74 (dd, *J* = 10.6, 7.6 Hz, 1H), 4.37 (d, *J* = 7.6 Hz, 1H), 4.05 (d, *J* = 10.6 Hz, 1H), 3.72 – 3.42 (m, 4H), 3.42 (s, 1H), 3.34 – 3.21 (m, 2H), 3.09 (q, *J* = 7.0 Hz, 1H),
10 2.72 – 2.63 (m, 1H), 2.59 (d, *J* = 7.9 Hz, 1H), 2.56 (s, 3H), 2.25 (s, *J* = 9.9 Hz, 6H), 2.08 (s, 3H), 2.01 – 1.94 (m, 1H), 1.93 – 1.87 (m, 1H), 1.78 (d, *J* = 21.5 Hz, 3H), 1.73 – 1.56 (m, 8H), 1.49 (s, 3H), 1.32 (s, 3H), 1.24 (d, *J* = 6.1 Hz, 3H), 1.20 – 1.14 (m, 6H), 1.00 (d, *J* = 7.0 Hz, 3H), 0.88 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.54, 202.47 (d, *J* = 28.2 Hz), 169.77, 166.18 (d, *J* = 23.1 Hz), 157.05, 101.78,
15 97.88 (d, *J* = 205.8 Hz), 81.94, 79.58, 78.63, 78.58, 71.57, 69.22, 63.22, 60.88, 50.96, 49.14, 44.53, 43.01, 40.60 (2C), 39.28, 39.20, 30.44, 26.19, 25.16 (d, *J* = 22.3 Hz), 24.33, 22.41, 22.15, 21.39, 20.93, 19.67, 17.96, 14.69, 14.60, 13.73, 10.41; HRMS (ESI) calc'd for $C_{37}H_{60}FN_5O_{11}$ + H = 770.4346, found 770.4345.

20 **Azide 8.** Intermediate **G** (650 mg, 0.85 mmol) was stirred in refluxing MeOH (26 mL) under an inert atmosphere for 3h. The solution was cooled to rt and the solvent removed under reduced pressure. The product was purified by flash chromatography on a Combiflash instrument (MeOH/DCM 0-10%) to give 520 mg (85%) of **2**. $[\alpha]^{23}_D$ +12.1 (c 1.3, $CHCl_3$); IR (neat) 2975, 2098, 1749, 1457, 1381, 1261, 1215, 1052,
25 1002, 749, 667; ^1H NMR (500 MHz, $CDCl_3$) δ 4.86 (dd, *J* = 10.3, 2.0 Hz, 1H), 4.29 (d, *J* = 7.3 Hz, 1H), 4.08 – 4.04 (m, 1H), 3.66 (dd, *J* = 13.8, 6.6 Hz, 1H), 3.60 – 3.47 (m, 3H), 3.41 (s, 1H), 3.33 – 3.22 (m, 2H), 3.17 (dd, *J* = 10.2, 7.3 Hz, 1H), 3.09 (dd, *J* = 13.9, 6.9 Hz, 1H), 2.63 – 2.58 (m, 1H), 2.57 (s, 3H), 2.47 – 2.39 (m, 1H), 2.25 (s, 6H), 1.96 (ddd, *J* = 14.5, 7.5, 2.5 Hz, 1H), 1.87 (dd, *J* = 14.5, 2.7 Hz, 1H), 1.77 (d, *J* =
30 21.4 Hz, 3H), 1.69 – 1.56 (m, 6H), 1.53 (d, *J* = 12.7 Hz, 1H), 1.48 (s, 3H), 1.34 (s, 3H), 1.29 (d, *J* = 7.1 Hz, 3H), 1.22 (d, *J* = 6.1 Hz, 3H), 1.19 (s, 1H), 1.17 (d, *J* = 6.9 Hz, 3H), 0.99 (d, *J* = 7.0 Hz, 3H), 0.87 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.93, [203.42, 203.14 (d, *J* = 28.3 Hz)], [166.78, 166.55 (d, *J* = 23.2 Hz)], 157.45, 104.57, [99.12, 97.07 (d, *J* = 206.1 Hz)], 82.35, 81.01, 78.91, 78.87, 70.70,

70.00, 66.15, 61.23, 51.33, 49.52, 44.98, 43.38, 41.13, 40.60 (2C), 39.92, 39.58, 28.45, 26.56, [25.72, 25.50 (d, J = 22.4 Hz)], 24.68, 22.50, 21.52, 20.10 18.28, 15.40, 15.08, 14.13, 10.77; HRMS (ESI) calc'd for $C_{35}H_{58}FN_5O_{10}$ + H = 728.4240, found 728.4231.

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Solithromycin (1). $[\alpha]^{23}_D$ +12.6 (c 1.5, $CHCl_3$); IR (neat) 2980, 2360, 2341, 1750, 1457, 1374, 1261, 1162, 1109, 1078, 1051, 1003, 754, 668; ^1H NMR (500 MHz, $CDCl_3$) δ 7.80 (s, 1H), 7.27 (d, J = 1.7 Hz, 1H), 7.20 – 7.14 (m, 2H), 6.66 – 6.61 (m, 1H), 4.87 (dd, J = 10.4, 2.0 Hz, 1H), 4.41 (td, J = 7.1, 1.4 Hz, 2H), 4.29 (d, J = 7.3
10 Hz, 1H), 4.03 (ddd, J = 18.4, 11.3, 3.7 Hz, 2H), 3.80 – 3.70 (m, 2H), 3.68 – 3.58 (m, 1H), 3.57 – 3.46 (m, 2H), 3.42 (s, 1H), 3.17 (dd, J = 10.2, 7.3 Hz, 1H), 3.10 (q, J = 6.9 Hz, 1H), 2.65 – 2.56 (m, 1H), 2.54 (s, 3H), 2.48 – 2.41 (m, 1H), 2.26 (s, 6H), 2.02 – 1.92 (m, 3H), 1.87 (dd, J = 14.5, 2.7 Hz, 1H), 1.77 (d, J = 21.4 Hz, 3H), 1.72 – 1.61 (m, 5H), 1.52 (d, J = 14.1 Hz, 1H), 1.49 (s, 3H), 1.33 (s, J = 8.6 Hz, 3H), 1.30 (d, J =
15 7.1 Hz, 3H), 1.23 (d, J = 6.1 Hz, 3H), 1.17 (d, J = 6.8 Hz, 3H), 1.00 (d, J = 7.0 Hz, 3H), 0.87 (t, J = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.56, [202.97, 202.68 (d, J = 28.3 Hz)], [166.56, 166.33 (d, J = 23.2 Hz)], 157.20, 147.82, 146.82, 131.70, 129.63, 119.68, 116.13, 114.72, 112.35, 104.21, [98.79, 96.74 (d, J = 206.1 Hz)], 82.11, 80.70, 78.57, 78.52, 70.33, 69.62, 65.79, 61.03, 49.70, 49.20 (2C), 44.56,
20 42.75, 40.83, 40.22, 39.54, 39.18, 28.11, 27.58, [25.31, 25.09 (d, J = 22.5 Hz)], 24.25, 22.12, 21.14, 19.74, 17.88, 15.02, 14.69, 13.75, 10.45; HRMS (ESI) calc'd for $C_{43}H_{65}FN_6O_{10}$ + H = 845.4824, found 845.4817.

Triazole 71. $[\alpha]^{23}_D$ +12.7 (c 1.07, $CHCl_3$); IR (neat) 2972, 2978, 2361, 2341, 1750,
25 1559, 1489, 1260, 1163, 1078, 1052, 1003, 725, 668; ^1H NMR (500 MHz, $CDCl_3$) δ 7.86 (s, 1H), 7.42 – 7.39 (m, 2H), 7.29 (t, J = 8.1 Hz, 1H), 6.88 – 6.84 (m, 1H), 4.88 (dd, J = 10.2, 2.1 Hz, 1H), 4.53 – 4.33 (m, 2H), 4.29 (d, J = 7.3 Hz, 1H), 4.02 (d, J = 9.5 Hz, 1H), 3.81 (dt, J = 13.8, 6.7 Hz, 1H), 3.68 – 3.60 (m, 1H), 3.60 – 3.48 (m, 3H), 3.43 (s, 1H), 3.18 (dd, J = 10.2, 7.3 Hz, 1H), 3.11 (q, J = 6.9 Hz, 1H), 2.64 – 2.57 (m,
30 J = 12.2, 6.9, 2.8 Hz, 1H), 2.51 (s, 3H), 2.50 – 2.41 (m, 1H), 2.27 (s, 6H), 2.03 – 1.92 (m, 3H), 1.88 (dd, J = 14.5, 2.7 Hz, 1H), 1.79 (d, J = 21.4 Hz, 3H), 1.72 – 1.59 (m, 5H), 1.54 – 1.40 (m, 4H), 1.33 – 1.28 (m, 6H), 1.22 (d, J = 6.1 Hz, 3H), 1.17 (d, J = 6.9 Hz, 3H), 1.00 (d, J = 7.0 Hz, 3H), 0.86 (t, J = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.98, [204.75, 204.46 (d, J = 28.5 Hz)], [166.98, 166.75 (d, J = 22.8 Hz)],

157.63, 156.90, 148.04, 132.44, 130.52, 120.13, 118.36, 115.57, 113.31, 104.59, [99.15, 97.10 (d, J = 206.3 Hz)], 82.52, 81.19, 79.14, 79.01, 70.69, 70.00, 66.17, 61.74, 50.29, 49.57, 44.91, 43.23, 41.38, 40.59 (2C), 39.96, 39.64, 28.53, 27.73, [25.71, 25.48 (d, J = 22.9 Hz)], 24.98, 22.51, 21.51, 20.07, 18.26, 15.49, 15.15, 14.17,
5 10.85; HRMS (ESI) calc'd for $C_{43}H_{64}FN_5O_{11}$ + H = 846.4664, found 846.4665.

Triazole 72 $[\alpha]^{23}_D$ +8.5 (c 1.0, $CHCl_3$); IR (neat) 2972, 2361, 2341, 1751, 1489, 1323, 1261, 1165, 1126, 1072, 1052, 1003, 763, 668; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.10 – 8.05 (m, 2H), 7.96 (s, 1H), 7.60 – 7.50 (m, 2H), 4.85 (dd, J = 10.4, 2.0 Hz, 1H), 4.45
10 (t, J = 7.4 Hz, 2H), 4.29 (d, J = 7.3 Hz, 1H), 4.05 (dd, J = 10.6, 1.3 Hz, 1H), 3.79 – 3.72 (m, 1H), 3.68 – 3.60 (m, 1H), 3.57 – 3.47 (m, 2H), 3.42 (s, 1H), 3.18 (dd, J = 10.2, 7.3 Hz, 1H), 3.10 (q, J = 6.9 Hz, 1H), 2.64 – 2.57 (m, 1H), 2.52 (s, 3H), 2.48 – 2.41 (m, 1H), 2.26 (s, 6H), 2.02 – 1.93 (m, 3H), 1.87 (dd, J = 14.5, 2.8 Hz, 1H), 1.74 (d, J = 21.4 Hz, 3H), 1.71 – 1.62 (m, 4H), 1.54 – 1.51 (m, 1H), 1.50 – 1.48 (m, 3H),
15 1.32 (s, 3H), 1.30 (d, J = 7.1 Hz, 3H), 1.23 (d, J = 6.1 Hz, 3H), 1.22 – 1.19 (m, 1H), 1.17 (d, J = 6.9 Hz, 3H), 1.00 (d, J = 7.0 Hz, 3H), 0.85 (t, J = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 217.02, δ 202.99 (d, J = 28.1 Hz), 166.90 (d, J = 23.3 Hz), 157.59, 146.77, 132.03, 129.63, 129.31, 124.44 (q, J = 273.2 Hz), 124.88, 122.88, 120.69 (2C), 104.58, 98.15 (d, J = 206.2 Hz), 82.51, 81.01, 78.95, 78.89, 70.70,
20 69.99, 66.19, 61.27, 50.24, 49.56, 44.95, 43.05, 41.21, 40.60 (2C), 39.91, 39.55, 28.51, 27.98, 25.52 (d, J = 22.5 Hz), 24.57, 22.49, 21.51, 20.11, 18.26, 15.37, 15.05, 14.12, 10.82; HRMS (ESI) calc'd for $C44H63F_4N5O11$ + H = 898.4589, found 898.4640.

25 **Triazole 73** $[\alpha]^{23}_D$ +8.7 (c 1.06, $CHCl_3$); IR (neat) 2972, 2941, 2360, 2341, 1751, 1457, 1262, 1161, 1109, 1078, 1052, 759, 668; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.25 (s, 1H), 7.97 (d, J = 7.6 Hz, 1H), 7.94 (s, 1H), 7.70 (s, 1H), 7.49 (t, J = 7.8 Hz, 1H), 4.88 (dd, J = 10.4, 2.1 Hz, 1H), 4.47 (t, J = 7.4 Hz, 2H), 4.32 (d, J = 7.3 Hz, 1H), 4.07 (dd, J = 10.7, 1.3 Hz, 1H), 3.82 – 3.73 (m, 1H), 3.70 – 3.61 (m, 1H), 3.54 (qd, J = 10.5,
30 4.9 Hz, 3H), 3.44 (s, 1H), 3.20 (dd, J = 10.2, 7.3 Hz, 1H), 3.12 (q, J = 7.0 Hz, 1H), 2.67 – 2.59 (m, J = 12.0, 7.2 Hz, 1H), 2.55 (s, 3H), 2.51 – 2.42 (m, 1H), 2.29 (s, 6H), 2.05 – 1.95 (m, 3H), 1.93 – 1.86 (m, 1H), 1.77 (d, J = 21.4 Hz, 3H), 1.73 – 1.61 (m, 5H), 1.58 – 1.52 (m, J = 14.5 Hz, 1H), 1.51 (s, 3H), 1.35 (s, 3H), 1.32 (d, J = 7.1 Hz, 3H), 1.25 (d, J = 6.1 Hz, 3H), 1.19 (d, J = 6.9 Hz, 3H), 1.03 (d, J = 7.0 Hz, 3H), 0.88

(t, J = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.61, 202.66 (d, J = 28.2 Hz), 166.46 (d, J = 23.2 Hz), 157.22, 146.94, 133.09, 131.20, 128.95, 128.12, 126.14, 124.07, 120.11 (2C), 104.14, 97.75 (d, J = 206.0 Hz), 82.13, 80.64, 78.56, 78.48, 70.29, 69.55, 65.85, 60.92, 49.80, 49.18, 44.56, 42.67, 40.81, 40.23 (2C), 39.52, 39.17, 28.24, 27.59, 25.17 (d, J = 22.3 Hz), 24.18, 22.11, 21.13, 19.73, 17.87, 15.00, 14.66, 13.74, 10.45; HRMS (ESI) calc'd for $C_{44}H_{63}FN_6O_{10}$ + H = 855.4668, found 855.4652.

Triazole 74 $[\alpha]^{23}_D$ +17 (c 0.92, $CHCl_3$); IR (neat) 2978, 2943, 2356, 2341, 1750, 1710, 1455, 1251, 1167, 1052, 1003, 755, 668; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.00 – 7.96 (m, J = 3.8, 2.6 Hz, 2H), 7.91 (s, 1H), 7.54 – 7.43 (m, 2H), 6.69 (t, J = 56.4 Hz, 1H), 4.87 (dd, J = 10.2, 2.3 Hz, 1H), 4.45 (t, J = 7.3 Hz, 2H), 4.30 (d, J = 7.3 Hz, 1H), 4.06 (dd, J = 10.6, 1.4 Hz, 1H), 3.80 – 3.71 (m, 1H), 3.69 – 3.61 (m, 1H), 3.57 – 3.48 (m, 922H), 3.44 (s, 1H), 3.19 (dd, J = 10.2, 7.3 Hz, 1H), 3.11 (q, J = 7.0 Hz, 1H), 2.65 – 2.58 (m, 1H), 2.53 (s, 3H), 2.51 – 2.44 (m, 1H), 2.29 (s, 6H), 2.04 – 1.93 (m, 3H), 1.87 (dd, J = 14.5, 2.8 Hz, 1H), 1.75 (d, J = 21.4 Hz, 3H), 1.72 – 1.61 (m, 4H), 1.57 – 1.51 (m, 1H), 1.50 (s, 3H), 1.33 (s, 3H), 1.31 (d, J = 7.1 Hz, 3H), 1.24 (d, J = 6.1 Hz, 3H), 1.22 – 1.20 (m, 1H), 1.18 (d, J = 6.9 Hz, 3H), 1.01 (d, J = 7.0 Hz, 3H), 0.87 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 216.62, 202.68 (d, J = 28.2 Hz), 166.49 (d, J = 23.2 Hz), 157.21, 146.77, 134.89, 131.42, 129.20, 127.94, 124.83, 123.00, 120.13, 114.62 (t, J = 238.9 Hz), 104.20, 97.76 (d, J = 206.0 Hz), 82.12, 80.64, 78.56, 78.49, 77.25, 77.00, 76.75, 70.32, 69.63, 65.78, 60.91, 49.82, 49.18, 44.56, 42.68, 40.83, 40.22 (2C), 39.52, 39.17, 28.08, 27.59, 25.17 (d, J = 22.4 Hz), 24.20, 22.10, 21.14, 19.73, 17.88, 15.01, 14.67, 13.75, 10.45, -0.03, -18.36; HRMS (ESI) calc'd for $C_{44}H_{64}F_3N_5O_{11}$ + H = 880.4653, found 880.4654.

Triazole 75 $[\alpha]^{23}_D$ +9.8(c 0.61, $CHCl_3$); IR (neat) 2980, 2360, 2341, 1756, 1559, 1261, 1162, 1109, 1078, 1052, 668; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.72 – 7.69 (m, 3H), 6.79 – 6.74 (m, 2H), 4.88 (dd, J = 10.4, 2.1 Hz, 1H), 4.40 (t, J = 7.5 Hz, 2H), 4.30 (d, J = 7.3 Hz, 1H), 4.06 (dd, J = 10.7, 1.2 Hz, 1H), 3.79 – 3.70 (m, 1H), 3.68 – 3.59 (m, 1H), 3.59 – 3.46 (m, 2H), 3.43 (s, 1H), 3.18 (dd, J = 10.2, 7.3 Hz, 1H), 3.10 (q, J = 6.9 Hz, 1H), 2.98 (s, 6H), 2.66 – 2.59 (m, 1H), 2.56 (s, 3H), 2.49 – 2.40 (m, 1H), 2.26 (s, 6H), 2.02 – 1.92 (m, 3H), 1.88 (dd, J = 14.5, 2.8 Hz, 1H), 1.78 (d, J = 21.4 Hz, 3H), 1.73 – 1.59 (m, 4H), 1.56 – 1.51 (m, 1H), 1.50 (s, 3H), 1.34 (s, 3H),

1.31 (d, J = 7.0 Hz, 3H), 1.23 (d, J = 6.1 Hz, 3H), 1.20 (s, J = 6.0 Hz, 1H), 1.18 (d, J = 6.9 Hz, 3H), 1.00 (d, J = 7.0 Hz, 3H), 0.88 (t, J = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.52, 202.71 (d, J = 28.2 Hz), 166.43 (d, J = 23.3 Hz), 157.20, 150.30, 148.18, 126.68 (2C), 119.19, 118.19, 112.50 (2C), 104.19, 97.78 (d, J = 206.3 Hz), 82.09, 80.68, 78.57, 70.33, 69.60, 65.84, 60.95, 49.62, 49.24, 44.57, 42.78, 40.83, 40.51 (2C), 40.23 (2C), 39.55, 39.19, 34.10, 28.16, 27.66, 25.22 (d, J = 22.6 Hz), 24.22, 22.13, 21.15, 19.76, 17.89, 15.02, 14.69, 13.76, 10.47; HRMS (ESI) calc'd for $C_{45}H_{69}FN_6O_{10}$ + 2H = 437.2607, found 437.2605.

Triazole 76 $[\alpha]^{23}_D$ +5.6 (c 1.5, $CHCl_3$); IR (neat) 2972, 2940, 2360, 2341, 1755, 1709, 1457, 1379, 1250, 1162, 1108, 1078, 1051, 1003, 755, 668; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.79 – 7.74 (m, 3H), 6.97 – 6.92 (m, 2H), 4.87 (dd, J = 10.4, 2.0 Hz, 1H), 4.41 (t, J = 7.4 Hz, 2H), 4.29 (t, J = 6.6 Hz, 1H), 4.07 – 4.04 (m, 1H), 3.83 (s, 3H), 3.79 – 3.70 (m, 1H), 3.67 – 3.59 (m, 1H), 3.58 – 3.47 (m, 2H), 3.43 (s, 1H), 3.18 (dd, J = 10.2, 7.3 Hz, 1H), 3.10 (q, J = 6.9 Hz, 1H), 2.65 – 2.57 (m, 1H), 2.54 (s, 3H), 2.49 – 2.41 (m, 1H), 2.26 (s, 6H), 2.01 – 1.92 (m, 3H), 1.88 (dd, J = 14.5, 2.7 Hz, 1H), 1.77 (d, J = 21.4 Hz, 3H), 1.73 – 1.60 (m, 4H), 1.55 – 1.51 (m, 1H), 1.49 (s, 3H), 1.33 (s, 3H), 1.30 (d, J = 7.0 Hz, 3H), 1.25 (d, J = 1.7 Hz, 1H), 1.23 (d, J = 6.1 Hz, 3H), 1.17 (d, J = 6.9 Hz, 3H), 1.00 (d, J = 7.0 Hz, 3H), 0.89 – 0.83 (m, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.56, 202.65 (d, J = 28.2 Hz), 166.45 (d, J = 23.1 Hz), 159.41, 157.19, 147.56, 127.04 (2C), 123.54, 118.91, 114.12 (2C), 104.01, 97.76 (d, J = 206.0 Hz), 82.10, 80.63, 78.54, 70.22, 69.40, 65.92, 60.95, 55.28, 49.66, 49.20, 44.55, 42.73, 40.80, 40.25 (2C), 39.50, 39.17, 29.67, 28.48, 27.59, 25.19 (d, J = 22.3 Hz), 24.21, 22.11, 21.09, 19.73, 17.87, 15.00, 14.67, 13.74, 10.45; HRMS (ESI) calc'd for $C_{44}H_{66}FN_5O_{11}$ + 2H = 430.7450, found 430.7451.

Triazole 77 $[\alpha]^{23}_D$ +9.0 (c 3.6, $CHCl_3$); IR (neat) 2972, 2940, 2360, 2341, 1752, 1708, 1457, 1379, 1261, 1109, 1078, 1051, 1003, 754, 668; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.03 (s, 1H), 8.56 (s, 1H), 8.24 (dt, J = 7.9, 1.7 Hz, 1H), 7.96 (s, 1H), 7.37 (dd, J = 7.6, 5.0 Hz, 1H), 4.86 (dd, J = 10.5, 2.1 Hz, 1H), 4.52 – 4.41 (m, 2H), 4.30 (d, J = 7.3 Hz, 1H), 4.07 – 4.03 (m, 1H), 3.80 – 3.73 (m, 1H), 3.68 – 3.59 (m, 1H), 3.57 – 3.48 (m, 2H), 3.42 (s, 1H), 3.19 (dd, J = 10.1, 7.3 Hz, 1H), 3.10 (q, J = 7.0 Hz, 1H), 2.65 – 2.56 (m, 1H), 2.51 (s, 3H), 2.48 (d, J = 9.7 Hz, 1H), 2.29 (s, 6H), 2.03 – 1.93 (m, 3H), 1.87 (dd, J = 14.5, 2.8 Hz, 1H), 1.75 (d, J = 21.4 Hz, 3H), 1.72 – 1.59 (m, 4H), 1.54 –

1.50 (m, 1H), 1.50 (s, 3H), 1.32 (s, 3H), 1.30 (d, J = 7.1 Hz, 3H), 1.24 (d, J = 6.1 Hz, 3H), 1.21 (d, J = 6.0 Hz, 1H), 1.18 (d, J = 6.9 Hz, 3H), 1.01 (d, J = 7.0 Hz, 3H), 0.87 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 216.62, 202.56 (d, J = 28.2 Hz), 166.49 (d, J = 23.4 Hz), 157.17, 148.99, 147.08, 144.62, 133.04, 123.66, 120.17,
5 104.06, 97.73 (d, J = 206.2 Hz), 82.11, 80.56, 78.52, 78.43, 70.24, 69.48, 65.80, 60.90, 49.86, 49.11, 44.53, 42.65, 40.77, 40.21 (2C), 39.46, 39.12, 28.27, 27.54, 25.16 (d, J = 22.2 Hz), 24.19, 22.07, 21.10, 19.69, 17.84, 14.94, 14.63, 13.72, 10.44; HRMS (ESI) calc'd for $C_{42}H_{63}FN_6O_{10}$ + H = 831.4647, found 831.4668.

10 **Triazole 78** $[\alpha]^{23}_D$ +7.1 (c 0.21, $CHCl_3$); IR (neat) 2972, 2940, 2360, 2341, 1754, 1709, 1457, 1379, 1260, 1233, 1162, 1108, 1077, 1051, 1003, 759, 668; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.33 – 8.26 (m, 1H), 7.96 (t, J = 3.8 Hz, 1H), 7.31 – 7.21 (m, 2H), 7.14 – 7.07 (m, 1H), 4.86 (dd, J = 10.4, 2.0 Hz, 1H), 4.44 (t, J = 7.5 Hz, 2H), 4.30 (d, J = 7.3 Hz, 1H), 4.06 (dd, J = 10.7, 1.3 Hz, 1H), 3.79 – 3.69 (m, 1H), 3.68 –
15 3.59 (m, 1H), 3.57 – 3.47 (m, 2H), 3.42 (s, 1H), 3.18 (dd, J = 10.2, 7.3 Hz, 1H), 3.10 (q, J = 7.0 Hz, 1H), 2.65 – 2.57 (m, 1H), 2.54 (s, 3H), 2.48 – 2.41 (m, 1H), 2.26 (s, 6H), 2.03 – 1.94 (m, 3H), 1.87 (dd, J = 14.5, 2.8 Hz, 1H), 1.74 (d, J = 21.4 Hz, 3H), 1.71 – 1.60 (m, 4H), 1.55 – 1.51 (m, 1H), 1.49 (s, 3H), 1.33 (s, 3H), 1.30 (d, J = 7.0 Hz, 3H), 1.24 (d, J = 6.1 Hz, 3H), 1.20 (t, J = 3.0 Hz, 1H), 1.17 (d, J = 6.9 Hz, 3H),
20 1.00 (d, J = 7.0 Hz, 3H), 0.87 (t, J = 7.4 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 216.51, 202.63 (d, J = 28.2 Hz), 166.34 (d, J = 23.4 Hz), 159.16 (d, J = 247.8 Hz), 157.15, 141.03 (d, J = 2.5 Hz), 129.00 (d, J = 8.3 Hz), 127.78 (d, J = 3.6 Hz), 124.44 (d, J = 3.0 Hz), 122.75 (d, J = 12.6 Hz), 118.72 (d, J = 13.1 Hz), 115.52 (d, J = 21.8 Hz), 104.04, 97.73 (d, J = 206.2 Hz), 82.04, 80.60, 78.50, 78.44, 70.23, 69.45, 65.83,
25 60.78, 49.76, 49.16, 44.50, 42.71, 40.77, 40.21(2C), 39.47, 39.12, 28.29, 27.67, 25.09 (d, J = 22.2 Hz), 24.12, 22.05, 21.08, 19.70, 17.84, 14.96, 14.62, 13.69, 10.34; HRMS (ESI) calc'd for $C_{43}H_{63}F_2N_5O_{10}$ + H = 848.4621, found 848.4615.

Triazole 79 $[\alpha]^{23}_D$ +2.0 (c 0.4, $CHCl_3$); IR (neat) 2971, 2360, 2341, 1750, 1653, 1464,
30 1260, 1078, 1052, 668; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (s, 1H), 7.39 (dd, J = 3.6, 1.1 Hz, 1H), 7.28 (dd, J = 5.1, 1.1 Hz, 1H), 7.07 (dd, J = 5.0, 3.6 Hz, 1H), 4.87 (dd, J = 10.3, 2.1 Hz, 1H), 4.42 (t, J = 7.4 Hz, 2H), 4.30 (d, J = 7.3 Hz, 1H), 4.06 (dd, J = 10.7, 1.3 Hz, 1H), 3.81 – 3.70 (m, 1H), 3.63 (dd, J = 14.9, 7.2 Hz, 1H), 3.58 – 3.48 (m, 2H), 3.43 (s, 1H), 3.19 (dd, J = 10.2, 7.3 Hz, 1H), 3.10 (q, J = 7.1 Hz, 1H), 2.67 –

2.57 (m, 1H), 2.54 (s, 3H), 2.51 – 2.42 (m, 1H), 2.28 (s, 6H), 1.97 (ddt, J = 14.9, 9.6, 4.9 Hz, 3H), 1.88 (dd, J = 14.5, 2.8 Hz, 1H), 1.77 (d, J = 21.4 Hz, 3H), 1.64 (ddd, J = 14.4, 12.6, 9.5 Hz, 4H), 1.53 (s, 1H), 1.50 (s, 3H), 1.34 (s, 3H), 1.30 (d, J = 7.0 Hz, 3H), 1.24 (d, J = 6.2 Hz, 3H), 1.21 (s, 1H), 1.18 (d, J = 6.9 Hz, 3H), 1.01 (d, J = 7.0
5 Hz, 3H), 0.88 (t, J = 7.4 Hz, 3H); HRMS (ESI) calc'd for $C_{41}H_{62}FN_5O_{10}S$ + H = 836.4279, found 836.4258.

Triazole 80 $[\alpha]^{23}{}_D$ +11.8 (c 0.69, $CHCl_3$); IR (neat) 2971, 2940, 2359, 1754, 1457, 1380, 1283, 1262, 1109, 1078, 1051, 1003, 763; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.63
10 (s, 1H), 7.42 (s, 1H), 7.05 (d, J = 11.2 Hz, 2H), 5.34 (q, J = 15.6 Hz, 2H), 4.79 (dd, J = 10.3, 2.1 Hz, 1H), 4.49 – 4.40 (m, 1H), 4.31 – 4.24 (m, 2H), 4.02 (dd, J = 10.7, 1.2 Hz, 1H), 3.76 – 3.68 (m, 1H), 3.63 – 3.49 (m, 3H), 3.35 (s, 1H), 3.18 (dd, J = 10.2, 7.3 Hz, 1H), 3.07 (q, J = 7.0 Hz, 1H), 2.63 – 2.54 (m, 1H), 2.50 – 2.42 (m, 1H), 2.33 (s, 3H), 2.27 (s, 6H), 1.97 (ddd, J = 14.5, 7.5, 2.5 Hz, 1H), 1.92 – 1.83 (m, 3H), 1.79
15 (d, J = 21.3 Hz, 3H), 1.70 – 1.52 (m, 5H), 1.48 (s, 3H), 1.32 (s, 3H), 1.30 (d, J = 7.1 Hz, 3H), 1.25 (d, J = 6.1 Hz, 3H), 1.21 (s, 1H), 1.17 (d, J = 6.9 Hz, 3H), 0.98 (d, J = 7.0 Hz, 3H), 0.89 (t, J = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.61, 202.87 (d, J = 28.4 Hz), 166.57 (d, J = 23.4 Hz), 157.14, 143.74, 137.21, 129.84, 122.19, 119.09, 104.25, 97.64 (d, J = 206.8 Hz), 82.11, 80.74, 78.57, 78.49, 70.31, 69.63,
20 65.83, 60.76, 50.06, 48.97, 44.55, 42.52, 42.40, 40.73, 40.24 (2C), 39.54, 39.14, 28.17, 27.53, 25.41 (d, J = 22.5 Hz), 24.33, 22.14, 21.17, 19.69, 17.83, 15.04, 14.65, 13.76, 10.56; HRMS (ESI) calc'd for $C_{41}H_{64}FN_7O_{10}$ + H = 834.4777, found 834.4755.

Triazole 81 $[\alpha]^{23}{}_D$ -2.5 (c 13.6, $CHCl_3$); IR (neat) 3384, 2971, 2939, 2360, 2340,
25 1750, 1457, 1262, 1161, 1106, 1078, 1051, 1003, 761, 668; ^{1}H NMR (400 MHz, MeOH) δ 8.01 (s, 1H), 5.04 (d, J = 12.5 Hz, 1H), 4.87 – 4.80 (m, 1H), 4.54 – 4.35 (m, 6H), 4.10 (d, J = 10.5 Hz, 1H), 3.88 (t, J = 10.4 Hz, 2H), 3.82 – 3.63 (m, 4H), 3.63 – 3.48 (m, 2H), 3.45 – 3.34 (m, 2H), 3.28 – 3.14 (m, 2H), 2.87 (s, 1H), 2.71 (s, 6H), 2.57 (s, 1H), 2.34 (s, 3H), 2.04 – 1.83 (m, 5H), 1.78 (d, J = 21.5 Hz, 3H), 1.74 – 1.57
30 (m, 3H), 1.54 (s, 3H), 1.51 – 1.39 (m, 2H), 1.37 – 1.25 (m, 9H), 1.21 (d, J = 6.7 Hz, 3H), 0.98 (d, J = 6.8 Hz, 3H), 0.92 (t, J = 7.3 Hz, 3H); ^{13}C NMR (101 MHz, MeOH) δ 218.03, 203.85 (d, J = 28.4 Hz), 167.80 (d, J = 23.2 Hz), 159.23, 145.77, 125.53, 104.24 (d, J = 99.4 Hz), 102.08, 99.27 (d, J = 205.4 Hz), 84.03, 81.20, 80.06, 79.75 (d, J = 8.5 Hz), 78.02 (d, J = 6.3 Hz), 76.20, 75.06, 74.86, 71.61, 70.81, 69.76, 66.43,

62.77 (dd, J = 47.5, 40.8 Hz), 56.52, 50.97, 49.96, 49.64, 49.43, 49.21, 49.00, 48.79, 48.57, 48.36, 45.84, 43.85, 42.03, 40.52, 40.40, 40.08, 31.25, 30.73, 28.70, 25.55 (d, J = 25.9 Hz), 25.42, 23.34, 21.24, 20.42, 18.04, 15.50, 14.93, 14.06, 11.00; HRMS (ESI) calc'd for $C_{44}H_{72}FN_5O_{16} + H$ = 946.5036, found 946.5017.

5

Triazole 82 $[\alpha]^{23}_{D}$ +14.4 (c 0.9, $CHCl_3$); IR (neat) 2971, 2939, 2880, 2360, 2341, 1751, 1457, 1375, 1261, 1161, 1109, 1078, 1052, 1003, 970, 753, 668; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.45 (s, 1H), 4.82 (dd, J = 10.4, 2.1 Hz, 1H), 4.45 – 4.37 (m, 1H), 4.36 – 4.27 (m, 2H), 4.07 – 4.02 (m, 1H), 3.74 – 3.66 (m, 1H), 3.61 – 3.46 (m, 3H),
10 3.38 (s, 1H), 3.19 (dd, J = 10.0, 7.4 Hz, 1H), 3.07 (q, J = 6.8 Hz, 1H), 2.64 – 2.57 (m, 1H), 2.52 – 2.47 (m, 1H), 2.45 (s, 3H), 2.28 (s, 6H), 1.99 – 1.82 (m, 8H), 1.77 (d, J = 21.4 Hz, 3H), 1.73 – 1.51 (m, 5H), 1.48 (s, 3H), 1.32 (s, 3H), 1.29 (d, J = 7.0 Hz, 3H), 1.24 (d, J = 5.9 Hz, 3H), 1.22 – 1.20 (m, 1H), 1.16 (d, J = 6.9 Hz, 3H), 0.99 (d, J = 7.0 Hz, 3H), 0.87 (t, J = 7.4 Hz, 3H), 0.83 (t, J = 7.3 Hz, 6H); HRMS (ESI) calc'd for
15 $C_{42}H_{70}FN_5O_{11} + H$ = 840.5134, found 840.5152.

Triazole 83 $[\alpha]^{23}_{D}$ +8.5 (c 1.77, $CHCl_3$); IR (neat) 2970, 2941, 2361, 2341, 1752, 1457, 1379, 1261, 1162, 1108, 1078, 1052, 1003, 755, 668; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.49 (s, 1H), 4.79 (dd, J = 10.4, 2.1 Hz, 1H), 4.46 (dt, J = 12.8, 6.2 Hz, 1H),
20 4.31 – 4.20 (m, 2H), 4.02 (dd, J = 10.7, 1.2 Hz, 1H), 3.75 – 3.66 (m, 1H), 3.62 – 3.55 (m, 1H), 3.55 – 3.46 (m, 2H), 3.34 (s, J = 19.3 Hz, 1H), 3.16 (dd, J = 10.2, 7.3 Hz, 1H), 3.06 (q, J = 6.9 Hz, 1H), 2.63 – 2.53 (m, 1H), 2.48 – 2.38 (m, 1H), 2.31 (s, 3H), 2.26 (s, J = 6.8 Hz, 6H), 2.23 – 2.13 (m, 3H), 2.11 – 2.03 (m, 1H), 2.03 – 1.91 (m, 4H), 1.91 – 1.83 (m, 4H), 1.77 (d, J = 21.4 Hz, 3H), 1.63 (ddt, J = 17.7, 14.6, 5.2 Hz,
25 4H), 1.52 – 1.42 (m, 1H), 1.47 (s, 3H), 1.30 (s, 3H), 1.29 (d, J = 7.1 Hz, 3H), 1.24 (d, J = 6.1 Hz, 3H), 1.20 (s, 1H), 1.16 (d, J = 6.9 Hz, 3H), 0.98 (d, J = 7.0 Hz, 3H), 0.88 (t, J = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.55, 202.98 (d, J = 28.4 Hz), 166.65 (d, J = 23.1 Hz), 157.05, 154.52, 120.06, 104.20, 97.54 (d, J = 206.7 Hz), 82.04, 80.57, 78.81, 78.54, 70.30, 69.63, 65.78, 60.76, 49.91, 48.92, 44.55, 42.64,
30 41.15 (2C), 40.70, 40.21(2C), 39.49, 39.10, 28.12, 27.61, 25.36 (d, J = 22.5 Hz), 24.60, 23.66 (2C), 22.07, 21.12, 19.64, 17.80, 14.95, 14.59, 13.74, 10.52; HRMS (ESI) calc'd for $C_{42}H_{68}FN_5O_{11} + H$ = 838.4977, found 838.4997.

Triazole 84 $[\alpha]^{23}{}_D$ +10.4 (c 1.44, $CHCl_3$); IR (neat) ^1H NMR (500 MHz, $CDCl_3$) δ 7.50 (s, J = 8.5 Hz, 1H), 4.84 (dd, J = 10.3, 2.0 Hz, 1H), 4.43 – 4.31 (m, 2H), 4.29 (t, J = 7.0 Hz, 1H), 4.05 (dd, J = 10.6, 1.1 Hz, 1H), 3.72 – 3.69 (m, 4H), 3.68 (s, 2H), 3.62 – 3.57 (m, 1H), 3.57 – 3.48 (m, 3H), 3.40 (s, 1H), 3.17 (dd, J = 10.2, 7.3 Hz, 1H), 3.09 (q, J = 7.0 Hz, 1H), 2.64 – 2.57 (m, 1H), 2.51 (s, 4H), 2.48 (s, 3H), 2.46 – 2.41 (m, 1H), 2.26 (s, 6H), 2.01 – 1.83 (m, 4H), 1.77 (d, J = 21.3 Hz, 3H), 1.70 – 1.57 (m, 4H), 1.49 (s, 3H), 1.54 – 1.44 (m, 1H), 1.33 (s, 3H), 1.30 (d, J = 7.0 Hz, 3H), 1.24 (d, J = 6.2 Hz, 3H), 1.20 (s, J = 4.3 Hz, 1H), 1.17 (d, J = 6.9 Hz, 3H), 0.99 (d, J = 7.0 Hz, 3H), 0.88 (t, J = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 216.58, 202.74 (d, J = 28.2 Hz), 166.42 (d, J = 23.0 Hz), 157.17, 144.02, 122.68, 104.22, 97.73 (d, J = 206.3 Hz), 82.10, 80.64, 78.56, 78.45, 70.33, 69.64, 66.87 (2C), 65.79, 60.85, 53.66, 53.38 (2C), 49.71, 49.12, 44.57, 42.70, 40.79, 40.21(2C), 39.53, 39.15, 29.67, 27.62, 25.26 (d, J = 22.6 Hz), 24.23, 22.13, 21.16, 19.74, 17.87, 15.03, 14.66, 13.74, 10.48; HRMS (ESI) calc'd for $C_{42}H_{69}FN_6O_{11}$ + H = 853.5086, found 853.5105.

The results of the experimental example are now described.

To test the hypothesis that bacterial ribosomes can template the Huisgen reaction, azide **8** was synthesized using known methods, while 3-ethynylaniline (**9**) was obtained from commercial sources (Liang et al., 2005, Biorg Med Chem Lett 15:1307-10). Ribosomes from *Escherichia coli* (70S, 50S and 30S) were isolated using known procedures (Grigoriadou et al., 2007, J. Mol. Biol. 373:562-572). After varying concentrations of ribosome, azide **8**, and alkyne **9** in tris(hydroxymethyl)aminomethane (Tris) buffer (20 mM Tris-HCl (pH 27.25), 10 mM $MgCl_2$, 150 mM NH_4Cl, 156 mM 2-mercaptoethanol), it was found that 5 mM ribosome, 5 mM azide and 5 mM alkyne at room temperature for 24-48 h resulted in the formation of **1** and its *syn* (1,5)-regioisomer (~2:1 ratio) in 8- to 16-fold greater amounts than in the absence of 70S or 50S ribosomal subunits (e.g., buffer, BSA, or 30S subunit). Analysis was performed on an Agilent 6520B Q-TOF LC-MS instrument wherein extracted ion chromatograms were used to locate and quantify the masses of interest (Figure 7, normalized to highest value).

Retention times of both *anti* (1,4)- and *syn* (1,5)-regioisomers were confirmed by independent chemical synthesis via thermal cycloaddition; moreover, solithromycin (**1**) was exclusively prepared by Cu(I)-catalysis (Kolb et al., 2001,

Agnew Chem Int Ed 40:2004-27). Several lines of evidence strongly support the ribosome-templated *in situ* click process: (1) in the absence of 70S or 50S ribosomal subunits (i.e., only buffer), there was 16-fold less product formation showing only mass counts due to the thermal cycloaddition background reaction; (2) the 30S subunit, which does not possess a macrolide binding site, also displayed an ion profile similar to background; (3) in the presence of ribosomal inhibitor azithromycin (AZY, 25 μM), which competes for the binding site with azide **8**, blocks 70S ribosome-dependent product formation; (4) replacing ribosomes with bovine serum albumin (BSA), a standard negative control used to rule out non-competitive binding, resulted in ion counts similar to those of the background cycloaddition; and finally, (5) the ratio of regioisomers in all negative control reactions (i.e., 30S, BSA, and buffer) and the inhibition experiment was 1:1 whereas in the presence of 70S ribosome and 50S subunits, the product ratio was 2:1 favoring **1**, which is a hallmark of selectivity resulting from the vectorial nature of *in situ* click chemistry (Sharpless and Manetsch, 2006, Expert Opin Drug Discov 1:525-38).

Having established the viability of *in situ* click chemistry using bacterial ribosomes in binary experiments (i.e., one azide, one alkyne) for the synthesis of solithromycin (**1**), the scope of the method in terms of the alkyne fragment (i.e., what functionalities could be screened and how many) and its capacity to discover novel, potent antibiotics was explored. To this end, a training set of fifteen alkynes (Figure 18A) containing both aromatic (e.g., **9**, **57-66**) and non-aromatic (e.g., **67-70**) functionalities was selected, including 3-ethynyl aniline (**9**) used in the synthesis of solithromycin (**1**). The aromatic alkynewas selected based on the ability of each fragment to engage in π-stacking interactions with the 23S rRNA A752-U2609 Watson-Crick base-pair, in addition to probing the impact of a hydrogen bonding network established between the aniline in **1** and A752 of the *E. coli* ribosome (PDB 3ORB) (Llano-Sotelo et al., 2010 Antimicrob Agents Chemother 54:4961-70). The non-aromatic group included structural motifs that could bind rRNA via hydrogen bond donors (e.g., **67-69**), acceptors (e.g., **67-70**), or by forming electrostatic interactions (i.e., salt bridges) between the protonated amine in *N*-propargyl morpholine (**70**) and proximal, negatively-charged phosphates.

Alkynes **9**, **57**, **58**, **61**, **68-69** were purchased from commercial sources. Compounds **69** and **65** were prepared from their commercially available, TMS-protected alkyne variants by reaction with KOH in MeOH. Alkynes **66** and **70** were

prepared by the reaction of imidazole and morpholine, respectively, with propargyl bromide. Alkyne **67** was prepared from the commercially available, peracetylated glucoside by deprotection with NaOMe in MeOH. Compound **70** was prepared in a three-step sequence starting with the Pd-catalyzed Sonogashira coupling of commercial meta-bromobenzaldehyde with TMS-acetylene, transformation of the aldehyde to the difloromethyl with commercial Deoxo-Fluor, and TMS deprotection with potassium carbonate in MeOH.

As diagrammed in Figure 7, triazoles from the *in situ* click process between azide **8** and the alkynes (Figure 18A) can yield *anti* (1,4)- and/or *syn* (1,5)-regioisomers depending on optimal positioning of rRNA recognition elements on the alkyne fragment (Figure 18B, represented as 'R').

Before conducting *in situ* click experiments with multiple alkyne partners, it was important to determine the binding affinity of both azide **8** and the triazole cycloadducts (Figure 18B) for the ribosome. Products derived from the *in situ* click method possess greater affinity for their targets vis-à-vis individual fragments due to the additivity of binding energies (Figure 5) (Jencks, 1981, PNAS 78:4046-50). It follows that the triazoles formed in the greatest amounts (i.e., highest ion counts) should possess higher affinity. To quantify affinity, dissociation constants (K_d) of triazoles **1**, **71-84** and azide **8** for 70S *E. coli* ribosomes were measured by an established fluorescence polarization competition assay using BODIPY-functionalized erythromycin (Table 1) (Yan et al., 2005, Antimicrob Agents Chemother 49:3367-72). *Anti*-triazoles **1**, **71-84** were prepared by Cu(I)-catalysis (Kolb et al., 2001, Agnew Chem Int Ed 40:2004-27).

Inspection of Table 1 showed that *anti*-triazoles **1**, **71**, **73-77**, **79**, **80**, and **84** bound the ribosome tighter than azide fragment **8**; moreover, *anti*-triazoles **72**, **78**, **81**, **82**, and **83** were weaker binders, though the range of K_d values only varied by a factor of 8. Further analysis revealed several important structure-activity relationships within the training set.

Detailed analysis revealed several important structure-activity relationships within the training set. Specifically, *meta*- or 3-substituted aromatic and/or heteroaromatic groups with the ability to engage in hydrogen bonding provided the best boost in potency (e.g., **1**, **71**, **73**, **74**, **77**). In contrast, the 3-substituted trifloromethylphenyl triazole **72** and 2-fluorophenyl triazole **78**, with no capacity for hydrogen bonding, failed to increase binding. In addition, the nonaromatic triazoles

81, **82**, and **83** all showed decreased binding as compared to **8,** indicating that moieties that participate primarily in hydrogen bond interactions but cannot participate in π-stacking do not stabilize macrocycle+desosamine-rRNA interactions. Although not wishing to be bound by any particular theory, these results suggest that
5 the ability of the side-chain to participate in both π-stacking and hydrogen bonding leads to stabilization of macrolactone+desosamine-rRNA interactions. It is hypothesized that the relatively high binding activity of the nonaromatic morpholine-containing triazole **84**, which bound only slightly less tightly than solithromycin (**1**, SOL), may be attributed to the presence of a basic amine that can interact
10 electrostatically with rRNA. Lastly, the five-membered heteroaromatics **79** and **80** showed increased binding and thus represent an interesting, novel structural class to explore.

With the K_d data in hand, two *in situ* click experiments were designed wherein azide **8** was incubated with five different alkynes in the presence of 50S *E.*
15 *coli* ribosomes. The proof-of-concept experiments (Figure 7) established that either 70S or 50S ribosomes effectively templated the *in situ* click reaction. The first experiment included 3-ethynyl aniline (**9**), which is the precursor to solithromycin (**1**), as one of the five alkyne partners whereas the second experiment did not. The rationale was to test whether the ribosome could differentiate between triazoles with
20 K_d's lower than azide **8** and those with higher K_d's; in other words, how selective was this method in discovering novel, potent macrolide antibiotics.

The first five-alkyne *in situ* click experiment was carried out by incubating a mixture of alkynes **9**, **57**, **67**, **68**, and **62** (2 mM each; 10 mM total), 5 μM azide **8**, and 5 μM 50S *E. coli* ribosomes at room temperature for 48 h. The data
25 in Figure 19 showed that **1** gave the greatest combined mass counts, with the *anti*-regioisomer (solithromycin, **1**) being preferred over *syn*-**1**, which is consistent with the K_d data above. Phenol-functionalized triazole **71**, which possessed a low K_d, or the *anti*-regioisomer, was also formed in significant amounts. This result established the importance of aromatic fragments with the capacity for hydrogen bonding with rRNA
30 at the *meta*-position, again drawing an analogy to **1**. Triazole formation from glycosyl alkyne **67** resulted in small amounts of both *syn*- and *anti*-**81**. Aliphatic compound **82** was not formed in significant amounts, which could be attributed to the absence of π-stacking interactions. Triazole **76** possessing a K_d lower than azide **8** and capable of π-

stacking was formed in the lowest amount. It was hypothesized that this phenomenon is most likely due to competitive product inhibition arising from **1** and **71**, which are two of the tightest binders in the training set (Jencks, 1981, PNAS 78:4046-50; Lewis et al., 2002, Agnew Chem Int Ed 41:1053-7).

5 The second five-alkyne *in situ* click experiment featured alkynes bearing a range of functional groups such as imidazole **66**, pyridine **63**, nitrile **59**, and fluoride **64**. The rationale here was to test how the ribosome-templated reaction would perform in the presence alkynes that results in triazoles that bind weaker than **1**. The results from the experiment are shown in Figure 20. Imidazole-functionalized triazole 10 **80**, as a mixture of *syn*- and *anti*-regioisomers, was detected in the greatest amount followed by **77** then **73**. Alternatively, triazoles **78** and **82** were not detected in significant quantities. Taken together, the five-alkyne *in situ* click experiments demonstrate that the ribosome is templating the formation of tighter binding molecules, specifically those with increased affinity vis-à-vis azide **8**, in greater 15 quantity as per LC-MS analysis and that these results are consistent with K_d determination. Thus, the *in situ* click method represents a powerful tool in the triaging and prioritization of drug candidates by obviating the onerous need to independently synthesize, characterize, and evaluate both *syn*- and *anti*-triazoles.

The successful execution of five-alkyne *in situ* click experiments with 20 ribosomes justified a greater exploration of chemical space while expanding the scope of the method. To this end, experiments were initiated with fifteen alkynes, which would yield thirty congeners (Figure 21). Whereas the five-alkyne reaction with azide **8** represents a screening of ten triazoles (i.e., five *syn*- and five *anti*-triazoles), a fifteen-membered alkyne library would yield thirty congeners.

25 To facilitate alkyne solubility, the concentration of each member was decreased from 2 mM used in the five-alkyne experiments to 1 mM. Azide **8** and 70S *E. coli* ribosome concentrations were both increased from 5 µM to 10 µM. The fifteen-membered alkyne mixture (15 mM total) was separately sonicated for 1-5 min to obtain a homogenous solution prior to the addition of azide **8** and ribosomes. The 30 reaction mixture was incubated at room temperature for 48h, and the results are shown in Figure 21. Consistent with previous *in situ* click reactions, the formation of triazoles with K_d values lower than **8** were detected (i.e., better binders than the azide fragment) including solithromycin (**1**), **71**, **73-77**, **79**, and **80** (Figure 21). All of these cycloadducts were derived from aromatic alkynes, again underscoring the significance

of π-stacking interactions with the A752-U2609 base pair. The only aromatic triazole that was not detected in appreciable quantity was trifluoromethyl congener **72**. However, inspection of Table 2 reveals its K_d value was the second lowest of the training set further illustrating selectivity in the *in situ* click process. Non-aromatic triazoles **72**, **81**, **82**, and **83** failed to be detected in significant quantities. In addition, morpholine-functionalized **84** failed to was not detected in significant quantities despite the fact that it binds ribosomes as well as **1**. With a depressed pK_a value of 5.55, *N*-propargyl morpholine (**70**) will not be protonated in the buffer solution (pH=7.5) (Mocharla et al., 2004, Agnew Chem Int Ed 44:116-20). Thus, speculation that the conjugate acid of **70** could be electrostatically sequestered by phosphate residues is ruled out. Competitive product inhibition, which was observed in both five-alkyne competition experiments (*vide supra*), may account for the modest formation of triazoles **72**, **81-84** (Table 2).

The acquisition of dissociation constants and LGFE's for the *in situ* click-derived triazoles was critical in determining how these compounds interact with the bacterial ribosome. However, it was necessary to confirm the mechanism of action of these cycloadducts and evaluate their antibiotic activity. To this end, (1) *in vitro* protein synthesis assays using a cell-free system (Rosenblum et al., 2012, Nucleic Acids res. 40:10) and (2) minimum inhibitory concentration (MIC) assays for azide **8** and triazoles **1**, **71-84** were conducted (Reller et al., 2009, Clin. Infect. Dis. 49:1749-1755).

For the *in vitro* translation inhibition studies, all of the compounds were assayed at 1 μM. Given that the 70S concentration in the cell-free protein synthesis (CFPS) reactions are approximately 1.5-2 μM, we would expect these low- to sub- nM affinity compounds to bind stoichiometrically, negating any differences in affinity and yielding a theoretical inhibition around 50%. Indeed, all of the compounds, including the azide, inhibited the CFPS reaction in the range of 48 ± 16 %. For the MIC assays, solithromycin (**1**, SOL), azide **8**, and triazoles **71-84** were tested against *E. coli*, *S. pneumoniae*, and *S. aureus* strains in triplicate. Strains ATCC 29213 (*S. aureus*) and ATCC 49619 (*S. pneumoniae*) served as quality control strains with values for solithromycin (**1**) matching those published by the Clinical and Laboratory Standards Institute (Reller et al., 2009, Clin Infect Dis 49:1749-55). Results of the MIC analsyis are summarized in tables 7-9.

Analysis of the data reveals that the poorest-performing compounds (i.e., **84**, **72**, and **81** shown in red) against both strains tracked with the binding data; in fact, **72** and **81** had the highest K_d values. Although not wishing to be bound by any particular theory, these results suggest that the polarity of **81** and **84** may be contributing to poor uptake and/or permeability. Taken together, these results indicate satisfactory levels of selectivity in the ribosome-templated *in situ* click process. In addition, thiophene-functionalized triazole **79** was two-fold more potent than SOL against *E. coli* DKpkk3535 and 2058G strains, whereas phenol-functionalized triazole **71** was two-fold more potent in the *S. pneumoniae* ATCC wild-type and *E. coli* mutant DK 2058G strains. Altogether these data validate this approach for the discovery of novel, potent antibiotics that obviates the need to synthesize, purify, and characterize both *syn*- and *anti*-triazole regioisomers derived from a library of azide and alkyne fragments.

In conclusion, an *in situ* click chemistry method that employs 70S *E. coli* ribosomes and 50S ribosomal subunits as platforms has been developed, with the ribosome-templated synthesis of solithromycin (1) serving as proof-of-concept. The method was applied in five- and fifteen-alkyne competition experiments. Consistent with other kinetic, target-guided *in situ* click processes, the extent of triazole formation correlated with ribosome binding affinity, as revealed by K_d values. Interestingly, LGFEs associated with the macrolactone and desosamine moieties, rather than the full triazoles, were correlated to dissociation constants for the congeners. Although not wishing to be bound by any particular theory, this result suggests that the chemical nature of the side-chain alters macrocycle-ribosome interactions, thereby indirectly impacting affinity. The inclusion of bacterial ribosomes in the repertoire of targets represents a powerful drug discovery platform that obviates the onerous need to independently synthesize, characterize, and evaluate both *syn*- and *anti*-triazoles. Significantly, the use of ribosomes possessing known mechanism of resistance (e.g., ribonucleotide modification or mutation) can lead to the discovery of antibiotics that selectively target resistant over wild-type bacterial strains. Protein synthesis inhibition experiments confirmed the mechanism of action of these congeners. Finally, MIC evaluation of the *in situ* click products quantified antibiotic activity and firmly established this method as efficacious in the triaging and prioritization of potent antibiotic candidates targeting the bacterial ribosome.

Table 2. Evaluation of triazoles **1**, **71-84** and azide **8** using minimum inhibitory concentration (MIC) in μg/mL against *E. coli* and *S. pneumoniae* strains and protein synthesis inhibition assays at 1 μM.

Compound	MIC *E. coli* DK pKK3535	MIC *E. coli* DK 2058G	MIC *S. pneumoniae* ATCC 49619	MIC *S. pneumoniae* 655 *mefA*	% Translation Inhibition
79	1	1	0.004	0.5	47 ± 12
71	2	1	≤ 0.002	0.5	46 ± 3
80	2	2	0.032	4	49 ± 12
SOL (1)	2	2	0.004-0.008	0.25-0.5	47 ± 11
Azide 8	2	2	2	0.25	55 ± 4
76	2	2	0.002-0.008	1	37 ± 5
78	2	4	≤ 0.002	0.5	47 ± 8
77	4	2	0.016	0.5	57 ± 10
74	2	4	0.004	1	34 ± 1
75	4	4	0.008	1	33 ± 4
73	4	4	0.016	1	42 ± 10
83	4	4	0.016	2	43 ± 2
82	4	4	4	2	47 ± 15
72	4	4	4	2	36 ± 9
84	8	8	8	4	64 ± 14
81	>32	>32	>32	>4	32 ± 5

5 Compounds are rank-ordered by potency in MIC assays against *E. coli* then *S. pneumoniae* strains. MIC values determined in triplicate; translation values in duplicate.

Table 3. LC-MS Analysis of In situ click experiments with E. coli 70S ribosomes,50S subunits, 70S with inhibitor azithromycin (AZY, 25 μM) and negative controls (30S subunits, BSA, or buffer only). Mass counts (normalized) correspond to 1 (solithromycin) and syn-1 regioisomer ions.

	1 [counts]	syn-1 [counts]	1 (normalized)	syn-1 (normalized)
70S	1204889	540970	100	45
50S	1161665	534718	96	44
25 μM AZT	145321	131192	12	11
30S	116592	100953	10	8
BSA	73313	73634	6	6

10

Table 4. LC-MS Analysis of *in situ* click experiment with azide **2** and alkynes 3, 5, 15, 16, and 10 with 50S E. coli ribosomal subunits.

Cmpd#	Experiment 1			Experiment 2			average % Increase	S.E.
	50S [counts]	Buffer [counts]	% Increase	50S [counts]	Buffer [counts]	% Increase		
81	8183798	5204776	57	4523500	3667171	23	40	12

81	3460609	2589691	34	2142196	1734690	23	29	4
anti-**71**	344598	45127	664	199476	33450	496	580	59
syn-**71**	91950	38066	142	48111	24115	100	121	15
82	25505	18505	38	15147	11347	33	36	2
anti-**76**	41568	46620	-11	26366	28255	-7	-9	1
syn-**76**	45157	45637	-1	41163	38932	6	2	2
anti-**1**	155901	18739	732	116629	23851	389	560	121
syn-**1**	376312	73916	409	220358	36426	505	457	34

Table 5. LC-MS Analysis of *in situ* click experiment with azide **2** and alkynes 14, 11, 7, 12, and 16 with 50S E. coli ribosomal subunits

Cmpd#	Experiment 1			Experiment 2			average % Increase	S.E.
	50S [counts]	Buffer [counts]	% Increase	50S [counts]	Buffer [counts]	% Increase		
73	951389	181150	425	997031	500926	99	262	115
80	372947	26902	1286	256826	20157	1174	1230	40
82	20670	13304	55	14304	10958	31	43	9
78	56089	34909	61	164476	60282	173	117	40
anti-77	169688	28874	488	133002	38919	242	365	87
syn-77	72822	17498	316	51502	18367	180	248	48

Table 6. LC-MS Analysis of *in situ* click experiment with azide 2 and alkynes 3, 5-18 with 50S E. coli ribosomal subunits

Cmpd #	EXPT 1 70S [counts]	EXPT 1 buffer [counts]	EXPT 1 % increase	EXPT 2 70S [counts]	EXPT 2 buffer [counts]	EXPT 2 % increase	EXPT 3 70S [counts]	EXPT 3 buffer [counts]	EXPT 3 % increase	EXPT 4 70S [counts]	EXPT 4 buffer [counts]	EXPT 4 % increase	EXPT 5 70S [counts]	EXPT 5 buffer [counts]	EXPT 5 % increase	average % increase	standard error
anti-1	463037	76755	503	118233	25635	361	100750	29069	247	n.d.	n.d.	n.a.	106379	16103	561	418	71
syn-1	260046	89803	190	92108	26940	242	110991	21354	420	149603	47372	216	129513	17964	621	338	81
anti-19	334455	75062	346	93425	20104	365	84460	18048	368	138184	39584	249	190028	29501	544	374	48
syn-19	28030	40647	-31	25565	11720	118	15678	9327	68	mix	mix	n.d.	mix	mix	n.a.	52	44
20	3339059	2375207	41	270886	n.d.	n.a.	15335	n.d.	n.a.	109453	n.d.	n.a.	n.d.	n.d.	n.a.	41	n.a.
anti-21	1.1E+07	990922	972	675368	115034	487	351172	55474	533	616637	56470	992	690927	47342	1359	869	162
syn-21	653900	528261	24	133470	48857	173	63290	26766	136	118718	28097	323	59960	20996	186	168	54
22	1357140	507611	167	63912	11312	465	12867	n.d.	n.a.	33620	7103	373	28459	15851	80	271	89
anti-23	379646	159900	137	45533	n.d.	n.a.	13948	n.d.	n.d.	27115	n.d.	n.a.	n.d.	n.d.	n.a.	137	n.a.
syn-23	80121	196266	-59	48738	n.d.	n.a.	15483	n.d.	n.d.	30546	n.d.	n.a.	n.d.	n.d.	n.a.	-59	n.a.
anti-24	385567	104936	267	29101	n.d.	n.a.	n.d.	n.d.	n.a.	15994	n.d.	n.a.	17590	n.d.	n.a.	267	n.a.
syn-24	114707	250380	-54	32893	n.d.	n.a.	n.d.	n.d.	n.a.	15238	n.d.	n.a.	mix	mix	n.a.	-54	n.a.
anti-25	1281462	117355	992	82743	21437	286	64659	17056	279	88966	18384	384	135215	24693	448	478	132
syn-25	39670	42782	-7	27179	10132	168	28215	10169	177	27971	11252	149	46838	11623	303	158	49
26	979659	558900	75	76031	15347	395	13429	n.d.	n.a.	38958	9194	324	n.d.	n.d.	n.a.	265	97
27	4493512	356756	1160	88613	25438	248	25438	8785	190	56723	18166	212	28947	10644	172	396	191
28	530923	290417	83	231555	49310	370	196859	44047	347	247967	45560	444	449801	41600	981	445	147
anti-29	4451605	3266657	36	2720360	2681522	1	1939756	22219644	-91	2318501	2264299	-90	1854383	1590452	17	-25	27
syn-29	1754470	1424862	23	1219698	1087280	12	881023	1078854	-18	1005284	1080967	-7	704196	725805	-3	1	7
30	44877	25727	74	n.d.	n.d.	n.a.	10665	7223	48	n.d.	n.d.	n.a.	13955	7070	97	73	14
31	59862	28030	114	14881	n.d.	n.a.	11829	n.d.	n.a.	12630	n.d.	n.d.	27388	n.d.	n.a.	114	n.a.
anti-32	53813	36319	48	13183	n.d.	n.a.	12073	n.d.	n.a.	12117	n.d.	n.a.	24001	105578	-77	-15	63
syn-32	11514	10721	7	10266	n.d.	n.a.	6857	n.d.	n.a.	7092	n.d.	n.a.	15702	5371	192	100	93

Table 7. MIC analysis of 1, 2, 19-32 against Escherichia coli DK, DK (pkk3535), DK (2058G), SQ171, SQ171 (2058G)

MIC (µg/mL)	***Escherichia coli***				
Compound	***DK***	**DK pkk3535**	**DK 2058G**	**SQ 171**	**SQ171 A2058G**

SOL (1)	2	2	2	32	>64
Azide (8)	2	2	2	64	>64
71	2	2	1	32	>64
72	4	4	4	64	>64
73	8	4	4	>64	>64
74	4	2	4	64	>64
75	4	4	4	64	>64
76	2	2	2	64	>64
77	4	2	2	32	>64
78	2	2	2	32	>64
79	2	1	1	32	>64
80	2	2	2	64	>64
81	>32	>32	>32	>64	>64
82	8	4	4	>64	>64
83	4	4	4	>64	>64
84	8	8	8	>64	>64

Table 8. MIC analysis of 1, 2, 19-32 against Staphylococcus aureus UCN 14 (A2058U), UCN 17 (A2058G), UCN18 (A2059G), ATCC 29213 (wt), ATCC 33591 (MRSA).

MIC (μg/mL)	*Staphylococcus aureus*				
Compound	ATCC 29213 (WT)	ATCC 33591 MRSA	UCN14 A2058U	UCN17 A2058G	UCN18 A2059G
SOL (1)	<0.0625	32-64	8	4	4
Azide (8)	< 0.0625	>64	>128	>128	>128
71	<0.0625	>128	16	8	8
72	<0.0625	32	32	32	32
73	0.5	>128	>128	128	128
74	<0.0625	128	32-64	16	8
75	<0.0625	128	128	32	32
76	<0.0625	>128	>128	32	32
77	<0.0625	>128	32-64	16	16
78	<0.0625	128	32	8	8
79	<0.0625	>128	32	8	8
80	0.25	>128	>128	128	128
81	128	>128	>128	>128	>128
82	0.25	>128	>128	>128	>128
83	0.125	>128	>128	128	>128
84	0.25	>128	128	>128	>128

Table 9. MIC analysis of 1, 2, 19-32 against Streptococcus pneumoniae ATCC 49619 (WT), 2196 (ermB), 655 (mefA), 1369 (ermB and mefA), 319 (A2059G), and 2634 (mefA and A2059G).

MIC (μg/mL)	*Streptococcus pneumoniae*

Compound	ATCC 49619 (WT)	2196 *ermB*	655 *mefA*	1369 *ermB* & *mefA*	319 A2059G	2634 *mefA* & A2059G
SOL (1)	0.002-0.008	0.5-1	0.25-0.5	0.5	0.008	0.125-0.25
Azide (8)	≤ 0.002	>4	0.25	>4	1	1
71	≤ 0.002	2	0.5	4	0.016	0.25
72	0.016	>4	2	>4	0.5	1
73	0.016	>4	1	>4	0.25	0.5
74	0.004	4	1	>4	0.0625	0.5
75	0.008	4	1	>4	0.0625	0.5
76	0.002-0.008	4	1	>4	0.0625	0.25
77	0.016	1	0.5	>4	0.031	0.25
78	≤ 0.002	2	0.5	4	0.031	0.25
79	0.004	2	0.5	0.5	0.008	0.125
80	0.031	>4	4	>4	0.25	1
81	4	>4	>4	>4	>4	>4
82	0.031	4	2	>4	0.5	2
83	0.016	>4	2	>4	1	2
84	0.0625	>4	4	>4	2	4

Example 3: *In situ* click identification of novel macrolide and oxazolidinone anitbiotics

The ribosome is a complex molecular machine composed by weight of 2/3 RNA and 1/3 protein whose singular purpose is to synthesize proteins by decoding mRNA in concert with aminoacylated tRNAs (Tenson and Mankin, 2006, Mol Microbiol 59:1664-77; Spahn and Prescott, 1996, J Mol Med 74:423-39). The bacterial ribosome, whose molecular weight is 2.5M Daltons, represents the largest macromolecule to template the azide-alkyne Huisgen [3+2] cycloaddition reaction (i.e., *in situ* click reaction). This novel method is summarized in Figure 5 wherein the tighter binder docks first to the ribosome (i.e., macrolide azide, **A**) followed by a second fragment (i.e., alkyne, **B**). The scope of *in situ* click is expanded herein by using two azides (Figure 22). This has not been demonstrated before and enables the exploration of $4n^2$ (# of bis-clicked) + 4n (# of mono-clicked) congeners where n=number of alkynes.

The data presented herein, shows the preparation of bis-azide cores that anchor to the ribosome due to their affinity (Figure 22, first step). A mixture of alkynes sequentially bind and ‘click’ to the extent they favorably interact with

proximal rRNA; the end result will be a more potent, selective antibiotic lead. These data demonstrate the viability of mono-azide ISCC and bis-azide ISCC. A second side chain is used to ensure sufficient affinity for resistant ribosomes derived from mutation and/or modification, which are anticipated following clinical use.

5 The data presented herein demonstrates a novel method that directly employs the bacterial ribosome in the synthesis of its own inhibitors. Specifically, it is demonstrated that the ribosome can template the irreversible Huisgen 1,3-dipolar cycloaddition of azide- and alkyne-functionalized fragments that bind in proximity (Figure 5). The target-guided *in situ* click chemistry (ISCC) approach to novel 10 antibiotic leads has been validated by "rediscovering" solithromycin (**1**), the best-in-class fluoroketolide antibiotic. Significantly resistant ribosomes are effective templates for ISCC, providing an opportunity to develop antibiotics that selectively target resistant bacteria over wild-type and commensal bacterial strains.

There is a need to identify novel lead candidates derived from three 15 different compound classes that can subsequently be developed into next-generation antibiotics addressing the rising problem of bacterial resistance.

Identification of novel macrolide-based antibiotics to address resistance

These experiments focused on the design of ketolide analogs bearing 20 new side chains that improve upon those in TEL, CET, and SOL, including the extension to a novel class of congeners bearing two side chains. The location (blue spheres) and composition (shown in red) of rRNA recognition elements within **1** are systematically evaluated. Both mono- (Figure 5) and bis-azide ISCC are applied to the discovery of macrolides bearing one side-chain (e.g., **1**) or two side-chains, which 25 heretofore has not been reported. Lead compounds are evaluated for minimum inhibitory concentration (MIC), binding (K_d), and *in vitro* translation inhibition to confirm mode-of-action (MOA).

E. coli 70S ribosomes and 50S subunits reproducibly template the [3+2] cycloaddition reaction between macrolide azide **8** and 3-ethynylaniline (**9**) to 30 prepare SOL (**1**, Figure 7), thus demonstrating that bacterial ribosomes perform *in situ* click chemistry (Sharpless and Manetsch, 2006, Expert Opin Drug Discov 1:525-38). Since ERY (**2**) and its congeners bind *E. coli* ribosomes with nM K_d values (Yan et al., 2005, Antimicrob Agents Chemother 49:3367-72), the macrolide-tethered azide precursor **8** of SOL would be readily "anchored" to the ribosomal 50S subunit (Figure

3); addition of excess 3-ethynylaniline (**9**), which has a markedly lower affinity for the ribosome, would sample various binding regions including those corresponding to SOL's side chain. Accordingly, the ribosome would make favorable interactions with the transition state leading to **1** and pay the entropic penalty of bringing **8** and **9**
5 together, ultimately resulting in irreversible formation of triazole **1** (Sharpless and Manetsch, 2006, Expert Opin Drug Discov 1:525-38; Mocharla et al., 2004, Agnew Chem Int Ed 44:116-20). After varying concentrations of ribosome, azide, and alkyne in buffer, it was deteremined that 5 μM ribosome, 5 μM azide and 5 mM alkyne at room temperature for 24-48 h resulted in the formation of **1** and its 1,5-regioisomer
10 (~2:1 ratio) in 8- to 16-fold greater amounts than in the absence of 70S or 50S ribosomal subunits (e.g., buffer, BSA, or 30S subunit). To locate and quantify the masses of interest an Agilent 6520B Q-TOF LC-MS instrument was used and extracted ion chromatograms were analyzed. (Figure 7, normalized to highest value). Retention times of both triazole products were confirmed by independent synthesis
15 via thermal cycloaddition, and SOL (**1**) was prepared by Cu-catalysis.

Several lines of evidence demonstrate that macrolide derivative synthesis occurs via the *in situ* click process: (1) in the absence of 70S or 50S ribosomal subunits (i.e., only buffer), there was 16-fold less product formation showing only mass counts due to the thermal cycloaddition background reaction; (2)
20 the 30S subunit, which does not have a macrolide binding site, also displayed an ion profile similar to background; (3) in the presence of ribosomal inhibitor azithromycin (AZY, 25 μM), which competes with **8** for the binding site, 70S ribosomes were unable to template the process; (4) replacing ribosomes with bovine serum albumin (BSA), a standard negative control used to rule out non-competitive binding, also
25 resulted in ion counts similar to those of the background cycloaddition; and finally, (5) the ratio of regioisomers in all negative controls (i.e., 30S, BSA, and buffer reactions) and the inhibition experiment was 1:1 whereas in the presence of 70S ribosome and 50S subunits, the product ratio was 2:1 favoring **1** (i.e., a hallmark of selectivity) (Sharpless and Manetsch, 2006, Expert Opin Drug Discov 1:525-38).
30 Sharpless demonstrated that in general mass counts from the *in situ* click process correlated with target inhibition, allowing classification of inhibitors into good, medium, and poor binders (Manetsch et al., 2004, J Am Chem Soc 126: 12809-18). The same has been observed with the ribosome-templated process.

The ribosome-templated *in situ* click method with azide **8** has been reproducibly extended from a single alkyne (Figure 7), to a five-alkyne, and most recently to a fifteen-alkyne competition experiment (Figure 13). K_d values of SOL (**1**) and *anti*-triazoles **43-56** (made by Cu-catalysis) were measured for *E. coli* 70S
5 ribosomes by FP using established competition binding experiments with BODIPY-functionalized ERY (**2**) (Yan et al., 2005, Antimicrob Agents Chemother 49:3367-72). The K_d values were consistent with published data (Petropoulos et al., 2009, J Mol Biol 385:1179-92; Llano-Sotelo, 2010, Antimicrob Agents Chemother 54:4961-70). Much like Sharpless' findings, the K_d values largely correlated with both mass
10 count (MC) % increase and potency (Figure 13). However, two outliers in Table 1 were nitrile **44** and morpholine **46**. The latter may be rationalized by considering sequestration of the protonated morpholine fragment by ribosomal phosphate residues. The former (869% increase vs 418% for **1**) is being investigated with computational chemistry. Notwithstanding these outliers, the utility of the method is
15 its ability to reproducibly prioritize analogs (in blue) as per MIC against *E. coli* and *S. pneumoniae*. The method's selectivity is particularly pronounced in the latter, clinically relevant strain.

For the fifteen-alkyne ISCC competition experiment, both ribosome and azide **8** concentrations were increased from 5 μM to 10 μM, and 0.5 mM of each
20 of the 15 alkynes were used for a total alkyne concentration of 7.5 mM. A negative control containing no ribosomes was also set up and incubated in the same way to provide data for the background reaction. Assays were incubated at room temperature for 24 h before LC-MS analysis. Extracted ion chromatograms $(M+H)^+$ were generated for all expected triazole products, and peaks were integrated to obtain MC.
25 Values in Figure 13 are reported as MC percent increase of product formed in the presence of ribosomes relative to the respective background reaction in the absence of ribosomes (i.e., $|\Delta MC|$/background MC x 100). It is important to compare these values relative to the background reaction since background rates can differ markedly from analog to analog. In competition *in situ* click experiments, Sharpless reported
30 background reaction rates were similar amongst analogs tested; thus, calculating MC difference and normalizing to the best performer (highest MC) was sufficient for rank-ordering compounds (Manetsch et al., 2004, J Am Chem Soc 126: 12809-18; Lewis et al., 2002, Agnew Chem Int Ed 41:1053-7; Krasiński et al., 2005, J Am Chem Soc 127:6686-92; Grimster et al., 2012, J Am Chem Soc 134:6732-40; Mocharla et

al., 2004, Agnew Chem Int Ed 44:116-20). This approach was used in the binary experiments (Figure 7). To confirm MOA, percent translation inhibition data was obtained on SOL (**1**) and analogs **43-56** using a cell-free assay, which fell in the range of 48±16% consistent with ribosomal inhibition (Rosenblum et al., 2012, NAR 5 40:10).

Co-crystal structures of bacterial ribosomes (e.g., *E. coli, H. marismortuii, D. radiodurans*) with ketolides (TEL, SOL, CET) have shown that although the ribosomes of these different species are highly conserved in their binding of ketolides, side chain orientations of bound ketolides vary greatly (Figure 8) 10 (Ippolito et al., 2008, J Med Chem 51:3353-6).

The impact of ribosomal changes on drug efficacy is not always proportional amongst compounds (i.e., the binding/activity of a set of compounds does not retain its rank order when tested against a resistant bacterium or ribosome). It is possible for a compound with low activity against wild-type bacteria to be a top-15 performing compound against resistant bacteria. For this very reason, resistant ribosomes were targeted for *in situ* click assays. Isolated mutant 70S *E. coli* ribosomes dimethylated at position A2058 were obtained and using cell free protein synthesis inhibition demonstrated that SOL (**1**) has an IC_{50} of 30 μM, which is thirty-fold greater than wild-type 70S ribosomes. Thus, binary *in situ* click experiment were 20 designed with these ribosomes to make SOL (**1**) and its 1,5 isomer (Figure 14). Due to reduced affinity, the concentration of azide **8** was increased from 5 to 50 μM, whereas other parameters remained the same as the wild-type binary experiment (Figure 7), which explains the higher background numbers with resistant ribosomes.
The results, like the wild-type binary experiment (Figure 7), showed an increase of 25 both SOL (**1**) and its 1,5-isomer over the background (i.e., no ribosomes). Remarkably, the ratio of **1** to its 1,5-isomer switched, suggesting the latter is more active against *E. coli* (pikR2) and possibly other *erm*-modified resistant bacteria. 1,5-SOL in pure form is synthesized by using the TBS-acetylene derivative of 3-ethynylaniline followed by desilylation (Coats et al., 2005, Org lett 7:1469-72) and is 30 evaluated using the methods described above (i.e., K_d, inhibition, MIC). Alkyne competition experiments are carried out with resistant pikR2 ribosomes (dimethylated at A2058) in order to prepare compounds that are selective for resistant strains.

SOL (**1**) and analogs **43-56** were evaluated using MIC assays against *S. pneumoniae (Sp)*, *S. aureus, and E. coli (Ec)*, including wild-type and resistant strains,

in collaboration with Prof. Buttaro (see letter of support). The MIC assays were run in triplicate on independent bacterial cell cultures (Reller et al. 2009, Clin Infect Dis 49:1749-55). Thiophene analog **43** was two-fold more potent than SOL against *E. coli* DKpkk3535 and 2058G strains. Phenol analog **49** was two-fold more potent in the *Sp* ATCC wild-type and *Ec* mutant DK 2058G strains.

Synthesis of mono- and bis-azide macrolides and alkyne fragments

The application of *in situ* click chemistry toward the discovery of novel macrolide antibiotics first requires the synthesis of suitable azide and aryl alkyne reactants (Figure 8). Procurement of alkyne partners identified by modeling are through commercial vendors or chemical synthesis employing established alkynylation reactions of commercial aryl halide, triflate or aldehyde precursors (including but not limited to., Sonogashira coupling (Sonogashira, 2002, J Organomet Chem 653:46-9), Corey-Fuchs (Corey and Fuchs, 1972, Tetrahedron Lett 13:3769-72), Seyferth-Gilbert (Gilbert and Weerasooriya, 1982, J Org Chem 47:1837-45; Seyferth et al., 1971 J Org Chem 36:1379-86) homologations). Aromatic substrates include, but are not limited to, mono- and fused carbo- and heterocyclic ring systems that can recognize rRNA (Thomas et al., 2008, Chem Rev 108:1171-1224; Foloppe et al., 2006, Drug Dicov Today 11:101-27; Aboul-Ela, 2012, Fut Med Chem 2:93-119). Four logical, validated positions were targeted to tether the side chains, specifically N11, C9, and O6 on the macrolactone and N3' of desosamine. Of the three sites, the first (N11) has been the most utilized (e.g., TEL, SOL); moreover, extensive structure-activity relationships (SAR) has revealed a four-carbon tether is ideal (Xu et al., 2012, Antibiotic Discovery and Development, pp 181-228). To date, the use of a two side chain strategy has not been reported.

Figure 8 shows the 9-step, gram-scale synthesis of macrolide-tethered azide **8** at N11 used in the *in situ* click synthesis of SOL (**1**) from commercial CLA (**3**). The reaction of **18** with 4-aminobutanol afforded oxazolidinone **19**. Two-step azide installation, cladinose removal, oxidation, installation of the 2-fluoro group with t-BuOK and N-fluorobenzenesulfonimide (NFSI), and finally removal of the 2'-OAc (Des) delivered **8**. Mono-azide ISC of **12** is pursued with wild-type and mutant ribosomes. To access the requisite bis-azides for ISC (see Figure 2), a tactic developed by Abbott was used; namely, the oxidative demethylation of desosamine with NIS to afford **89** in 70%

yield (Stenmark t al., 2000, J Org chem 65:3875-6). Alkylation with ω-azido mesylates and i-Pr_2NEt with a variable linker length furnishes bis-azides **90**.

The synthesis of C9-tethered macrolide azides **21-24** and **91-92** focuses on the oxime functionality (Figure 9). Roxithromycin (**5**), a 2nd generation
5 macrolide derived from ERY (**2**), features an (*E*)-oxime ether at C9 (Gasc et al., 1991, J Antibiot 44:313-330). For this series, both carbonate **21** and oxazolidinone **22** are used as substrates for diversity. Nucleophiles react site-selectively at the C9 keto over C3 (Beebe et al., 2004, Bioorg Med Chem Lett 14:2417-21). ω-azido hydroxylamine (in red) is reacted with **21-22** to access (*E*)-oxime ether mono-azides **23-24**. Oxidation
10 with NIS and *N*-alkylation with ω-azido mesylates and *i*-Pr_2NEt furnishes bis-azides **91-92.**

The synthesis of O6-tethered macrolide azides **29-30 and 93-24** starts from readily available CET (**7**) precursors **27-28** on mulitgram scale (Figure 10) (Cao et al., 2013, J Chem Res, 37:107-9). Results from molecular modeling determine the
15 optimal linker length. Accordingly, one of two approaches are taken to procure the requisite azide. Butyl and greater azides ($n \geq 4$) will employ olefin cross-metathesis of **27-28** and the appropriate ω-alkenyl azide and phosphine-free Hoveyda-Grubbs 2nd generation catalyst (HG-II) (Garber et al., 2000, J Am Chem Soc 122:8168-79). Subsequent and precedented chemoselective reduction of the olefin in the presence of
20 the azide by diimide reduction with Myers' *o*-nitrobenzenesulfonyl hydrazide (NBSH)[95] and Et_3N affords **29-30** after fluorination and methanolysis (Haukaas and O'Doherty, 2002, Org Lett 4:1771-4). The propyl series (n=3) employs hydroboration of the terminal olefin and two-step conversion of the alcohol to the azide. To access the ethyl (n=2) series (not shown), the allyl group is subjected to ozonolysis and
25 reduced to the alcohol (Ma et al., 2001, J Med Chem 44:4137-4156). Mesylation of the alcohol, substitution with NaN_3, and steps shown in Figure 10 procures the material. Bis-azides **93-94** are prepared using tactics outlined in Figures 8 and 9.

<u>*In situ* click preparation of novel macrolide antibiotics</u>

30 The current mono-azide ISCC protocol (Figure 13) consists of incubating 10 μM azide, 15 different alkynes (0.5 mM each), and 10 μM *E. coli* 70S ribosomes (or 50S subunits) at room temperature for 24-48 h followed by LC-MS analysis of reaction mixtures as compared to the ribosome-free control reaction. Conditions are optimized for the bis-azide variant first using a five-alkyne mixture. The number of products in

the bis-azide *in situ* click ($4n^2+4n$) where n=# of alkynes is markedly higher than the mono-azide. That said, efficient routes described herein (Figures 8-10) enable rapid synthesis and validation of hits based on LC-MS analysis.

Figure 15 presents an overview of (A) 1x15 mono-azide *in situ* click protocol used at
5 positions N11, C9, O6, and (B) the bis-azide variant, which targets desosamine. Wild-type and resistant pikR2 ribosomes are used (dimethylated at A2058) from Figure 4. Results are benchmarked against SOL (**1**) such that compounds possessing MC percent increases equal to or greater than **1** are characterized and confirmed by synthesis and subjected to biological evaluation.
10

Optimization of best analogs via Hofmann reaction

Desosamine in CLA (**3**) can be modified with secondary amines (Figures 6 and 11).

Evaluation of macrolide analogs

15 Analogs with potency equal or superior to SOL (**1**) in MIC assays with the panel of resistant and wild-type organisms (e.g., *S. pneumoniae*, *S. aureus, and E. coli*) are subjected to FP binding assays with BODIPY-functionalized ERY (**2**) to measure K_d and *in vitro* percent translation inhibition to confirm MOA. To obtain a more extensive bioactivity profile, expanded *in vitro* evaluations of anticipated lead
20 candidates are conducted using a panel of wild-type and resistant pathogens for evaluation including *S. pneumoniae*, *H. influenzae*, *S. aureus*, *M. catarrhalis*, *S. pyogenes*, *N. gonorrhoeae*, *E. coli* and *L. pneumophila.*

Identification of novel oxazolidinone -based antibiotics to address resistance

25 The chiral *N*-fluoroaryl oxazolidinone scaffold (in black) of linezolid (**85**) is essential for binding rRNA whilst the acetamide and morpholine moieties (in red) can be modified. Accordingly, azides are installed at both termini of **85** and perform ISCC with wild-type and resistant ribosomes using both mono- and bis-azides. GCMC/MD is employed to identify the optimal alkyne-functionalized fragments. Leads derived
30 from are evaluated using the same assays as described above.

Synthesis of mono- and bis-azide oxazolidinones and alkyne fragments

The morpholine nucleus in LIN (**85**) can be replaced by an aromatic ring; in fact, 2nd-generation biaryl oxazolidinones include TOR (**87**) and RAD (**88**) (Skripkin et al.,

2008, Antimicrob Agents Chemother 52:3550-7; Zhou et al., 2008, Biorg Med Chem Lett 18:6175-8). The acetamide can be replaced with an alcohol or a tetrazole; thus, the data presented herein include cores having –OH and $-N_3$ moieties.

To prepare the cores, commercial aniline **97** are converted to azide **98** in two steps and feature Cu-mediated cross-coupling (Stacy et al., 2013, Org Biomol Chem 11:938-54). The Manninen reaction of **97**, *n*-BuLi, and (*R*)-(−)-glycidyl butyrate access oxazolidinone core **99** (Brickner et al., 2008, J Med Chem 51:1981-90). Acetamide **100** is prepared in two steps from alcohol **99**. Reaction of the latter with DBU and diphenyl phosphoroazidate (DPPA) delivers bis-azide core **101** (Figure 23A) (Thompson et al., 1993, J Org Chem 58:5886-8).

The synthesis of core **104** requires LIN precursor **103** (Figure 23B) (Willand et al., 2010, ACS Chem Biol 5:1007-13). Following the Brickner route, cores **105-107** employs S_NAr with ω-azidoalkyl piperazines (Figure 23C). Biaryl cores **108** and **110** are prepared by the Suzuki-Miyaura coupling of suitable *N*-iodoaryl oxazolidinone precursors derived from **97** as reported to make **109** (Figure 23D) (Zhou et al., 2008, Biorg Med Chem Lett 18:6175-8; Zhou et al., 2008, Biorg Med Chem Lett 18:6175-8).

<u>*In situ* click for preparation of novel oxazolidinone analogs</u>

Figure 24 shows an overview of the *in situ* click reactions of oxazolidinone cores **99-101** and **104-110** with wild-type and resistant ribosomes. Experience from ISCC using macrolides cores from preliminary (Figure 13) and experiments (Figure 15) inform the approach taken, particularly for the bis-azide variant. Hits from the LC-MS analyses of mono- and bis-azide ISCC experiments (i.e., **111-120**) are confirmed by chemical synthesis as previously described and subjected to biological evaluation.

<u>Evaluation of oxazolidinone analogs</u>

Confirmed hits are first be subjected to MIC assays against a small panel of organisms. LIN (**85**), TOR (**87**) and RAD (**88**) are used as comparators. Those hits with potencies equal to or superior to **85**, **87**, or **88** are tested in FP binding assays to measure K_d and *in vitro* percent translation inhibition to confirm MOA. Since the binding sites for macrolides and oxazolidinones overlap, BODIPY-ERY probe may be used (Figure 13). Alternatively, synthesize a BODIPY-labeled oxazolidinone

intermediate is synthesized from Figure 23 and compare with linezolid (**85**) to ensure efficacy.

Example 4: *In cellulo* and *ex cellulo* ribosomal click chemistry

5 New inhibitors that are species-selective or resistance-selective are identified using *in cellulo* (intact bacterial cells) or *ex cellulo* (bacterial cell componets or bacterial lysate) bacteria. This method enables rapid phenotypic screening (kill vs no kill) by a synthetic lethal mechanism to discover new antibiotics. Moreover, using *in cellulo* or *ex cellulo* methodology allows for novel antibiotics 10 which need not be restricted to ribosomal targeting or mode-of-action (Figure 30). Other bacterial targets include, but are not limited to, inhibition of cell wall synthesis, membrane disruption, inhibition of protein synthesis, inhibition of folate synthesis, and inhibition of DNA or RNA synthesis.

Click chemistry synthesis of antibiotics can be performed *in cellulo* 15 with resistant bacterial strains using traditional MIC assays, enabling rapid evaluation of antibiotic potency (Figure 34). The *in cellulo* click experiment is based on the principle of a MIC assay to determine the minimal inhibitory concentration (MIC) of antibiotics. In this assay, a bacterial culture is diluted (1:100) into a series of wells of a microtiter plate containing fresh medium with a two-fold dilution series of 20 antibiotics. When the bacteria are inoculated into the medium, they have a slight lag phase and then will start to grow exponentially in the presence of the fresh medium. This exponential growth is heavily dependent on ribosome activity. The MIC is the lowest concentration of a compound that inhibits growth. The *in cellulo* click plates, which are run in a 96-well format, are set up as diagrammed in a 48-well format 25 (Figure 35). The strain used in the proof-of-concept was the Gram-positive *S. aureus* UCN14 strain carrying a point-mutation (A2058T) *S. aureus*. Each starting compound may have a MIC, so they are tested individually. In this case, the azide precursor to solithromycin has an MIC of 256 mg/mL (row A, Figure 35) where as the MIC of the alkyne is 4 mg/mL (row C, Figure 35). The MIC of solithromycin is 2 mg/mL. The *in* 30 *cellulo* click reaction is shown in row B with the negative control (i.e., no bacteria) in Row D wherein cells are lysed and the contents analyzed on an LC-MS for the detection and quantification of product. Using azithromycin (AZY), a macrolide antibiotic that binds the ribosome (albeit weakly since UCN14 is resistant to

macrolides), a 1:20 ratio of azide:AZY was found to effectively shut down the process.

The *in cellulo* click can also be run with concentration gradients are set up to test different ratios of the azide and alkyne to allow for the appropriate ratios of both compounds to bind a majority of the ribosomes. In this manner, one need not determine the MIC of the fragment being varied (i.e., the alkyne). The low number of bacterial cells used to inoculate the MIC assays increases the chance that compounds will be available to bind a majority of the ribosomes. If the click reaction occurs and the clicked compound has a higher activity than the individual components the MIC will decrease. Three different scenarios will result in a lower MIC; additive effects of the two compounds, synergistic effects of the two compounds or click chemical compounds. A positive in cellulo score is a 4-fold change in MIC, this helps to eliminate additive effects of the two compounds. To distinguish between synergistic effects and click chemistry LC-MS is performed to detect clicked products using authentic samples. If clicked compounds are present in reasonable concentrations (nM) they are considered candidate compounds with appropriate levels of activity against growing bacterial cells in an MIC assay. The activity of the compound can be verified by synthesis and the use of the purified compounds in a traditional MIC assay.

Example 5: Procurmnet of alkyne fragments and fragment libraries

Various commercial vendors provide access to either the alkyne-functionalized fragments directly or precursors bearing a handle (e.g., halide, triflate or aldehyde) that can be easily converted into terminal alkynes by well-established Sonogashira, Corey-Fuchs, or Seyferth-Gilbert reactions (Sonogashira, 2002, J Organomet Chem 653:46-9; Corey and Fuchs, 1972, Tetrahedron Lett 13:3769-72; Gilbert and Weerasooriya, 1982, J Org Chem 47:1837-45; Seyferth et al., 1971 J Org Chem 36:1379-86). Described herein is a library of 50 alkynes. Exampes of alkynes include those bearing a mixture of aliphatic, alicyclic, aromatic, and heteroaromatic functionalities. Some of the targeted alkynes (e.g., 5- and 6-membered arene/heterocycles) are shown in figure 32. The synthesis of all the alkynes is straightforward. Many only require alkylation of the nucleophilic nitrogen with propargyl bromide, which may be employed to access various building blocks. The entire alkyne fragment library may be composed of 300 to 500 compounds.

Inspection of the peptidyl transferase center (PTC) and exit tunnel within the 50S ribosomal subunit (Figure 33) reveals it is a rich region wherein many antibiotics bind, suggesting the use of ISCC employing a vast array of alkyne building blocks leads to novel, potent antibiotic leads. A group of 15 alkynes represents an acceptable amount for fragments per ISCC reaction mixture, and this number of fragments is consistent with studies by Sharpless (Sharpless and Manetsch, 2006, Exp Opin Drug Discov 1:525-38). Ribosome and azide concentrations run well at 10 μM with 0.5 mM of each of the 15 alkynes (i.e. total alkyne concentration of 7.5 mM).

Example 6: Identification of novel aminoglycoside antibiotics and novel peptide antibiotics to address resistance

The 3rd-generation neoglycoside plazomicin (Figure 36) is the best-in-class aminoglycoside antibiotic that effectively targets Gram-negative bacteria. Azides moieties are installed at two sites and mono- and bis- in situ and in cellulo methods are applied using both wild-type and resistant ribosomes. SILCS is then employed to identify optimal alkyne-functionalized fragments, which will be purchased or synthesized.

Negamycin (Figure 36) is a peptide natural product possessing antibacterial activity against a broad range of Gram-negative pathogens. Structure-activity studies confirm the terminal amine (in red) can be modified to enhance activity whereas other functional groups, including stereochemistry, cannot. Thus, the in situ and in cellulo click method descriebd herein are applied at this site guided by SILCS analysis. Leads derived from Aim 3 will be evaluated using the same assays as Aim 1.

The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety. While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include all such embodiments and equivalent variations.

CLAIMS

What is claimed is:

1. A method of identifying a compound, the method comprising:

reacting a first fragment and a second fragment using a click chemistry reaction, wherein the click chemistry reaction is catalyzed by a biological target to form a compound; and

identifying the compound.

2. The method of claim 1, the method comprises:

obtaining a first library of fragments and a second library of fragments;

selecting the first fragment from the first library of fragments, wherein the first fragment exhibits affinity for the biological target;

selecting a plurality of fragments from the second library of fragments, wherein at least one fragment of the plurality of fragments exhibits affinity for the biological target;

contacting the first fragment and the plurality of fragments with the biological target,

forming a complex comprising the biological target, the first fragment, and the second fragment from the at least one fragment of the plurality of fragments;

reacting the first fragment and the second fragment using the click chemistry reaction to form the compound, wherein the click chemistry reaction is catalyzed by a biological target to form the compound;

isolating the compound; and

identifying the compound.

3. The method of claim 2, wherein the first library of fragments comprises an at least one fragment, wherein the at least one fragment comprises at least one azide.

4. The method of claim 2, wherein the second library of fragments comprises an at least one fragment, wherein the at least one fragment comprises at least one alkyne.

5. The method of claim 2, wherein the biological target is selected from the group consisting of a biomolecule, a bacterial cell and a bacterial cell lysate.

6. The method of claim 5, wherein the biomolecule is a bacterial organelle.

7. The method of claim 6, wherein the bacterial organelle is at least a part of a ribosome.

8. The method of claim 7, wherein the at least a part of a ribosome is selected from the group consisting of an *E. coli* ribosome, a *S. aureus* ribosome, a *S. pneumonia* ribosome, a *H. influenzae* ribosome, a *M. catarrhalis* ribosome, a *S. pyogenes* ribosome, a *N. gonorrhoeae* ribosome, and a *L. pneumophila* ribosome.

9. The method of claim 7, wherein at least a part of a ribosome is a drug resistant ribosome.

10. The method of claim 9, wherein the drug resistant ribosome is selected from the group consisting of a *E. coli* DK A2058G ribosome, *E. coli* SQ171 A2058G ribosome, *S. aureus* UCN 14 ribosome, *S. aureus* UCN 17 ribosome, *S. aureus* UCN18 ribosome, *S. aureus* ATCC 33591 ribosome, *S. pneumoniae* 2196 ribosome, *S. pneumoniae* 655 ribosome, *S. pneumoniae* 1369 ribosome, *S. pneumoniae* 319 ribosome, and *S. pneumoniae* 2634 ribosome.

11. The method of claim 7, wherein the at least a part of a ribosome is selected from the group consisting of a 70S ribosomal subunit and the 50S ribosomal subunit.

12. The method of claim 2, wherein isolating the at least one compound further comprises separating the compound from the biological target by a method selected from the group consisting of size exclusion chromatography, chomolith chromatography, and monolith chromatography.

13. The method of claim 2 wherein identifying the compound further comprises subjecting the compound to Q-TOF LC-MS.

14. A compound identified by the method of claim 1 or a pharmaceutically acceptable salt or prodrug thereof.

15. The compound of claim 14, wherein the compound is an antibiotic.

16. The compound of claim 15, wherein the antibiotic is selected from the group consisting of a macrolide, an oxazolidinone, an aminoglycoside, and a peptide antibiotic.

17. A pharmaceutical composition comprising one or more compounds identified by the method of claim 1 and a pharmaceutically acceptable carrier.

18. A method of treating or preventing an infection, disease or disorder associated with a microbial infection in a subject in need thereof, the method comprising the step of administering a therapeutically effective amount of a composition comprising at least one compound identified by the method of claim 1 to the subject.

19. The method of claim 18, wherein the microbial infection is associated with a pathogen from the group consisting of: *Streptococcus pneumonia*, *Haemophilus influenzae*, and *Mycoplasma pneumoniae* and *Legionella*.

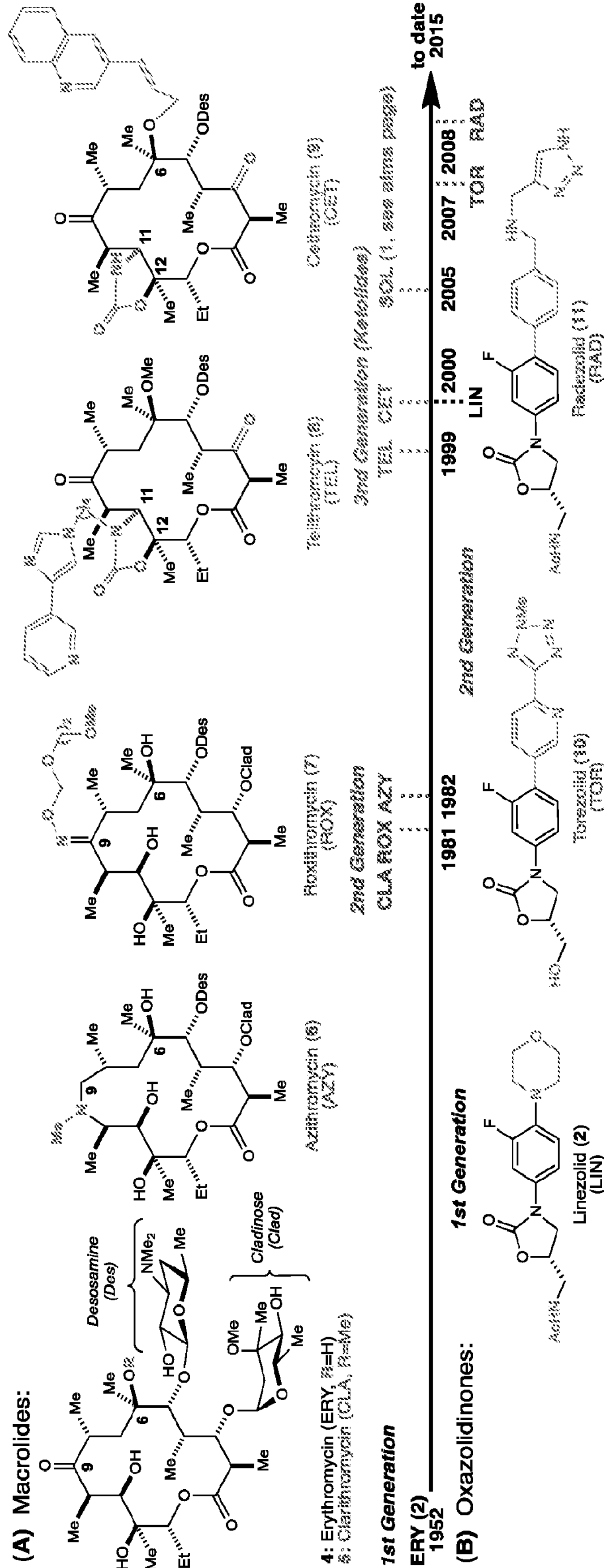
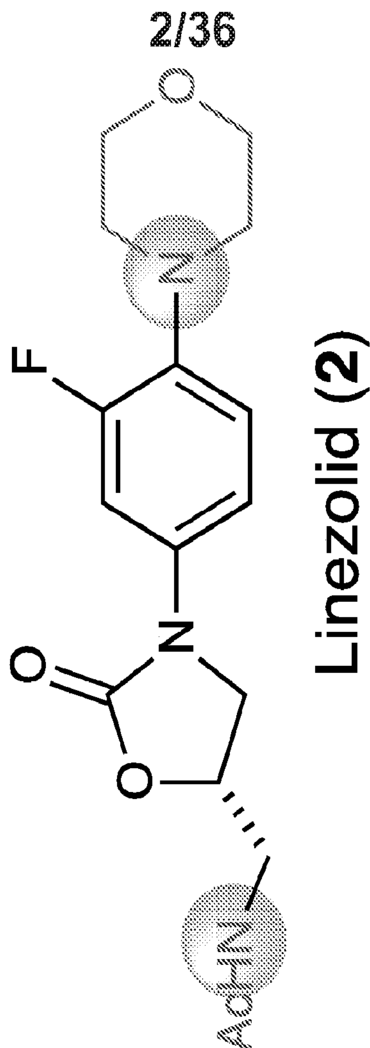


Figure 1



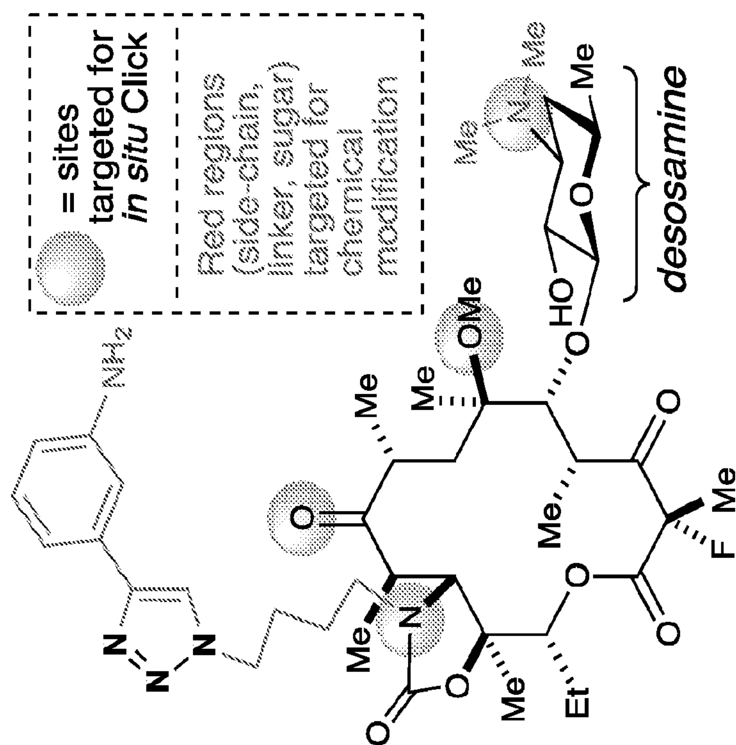


Figure 2

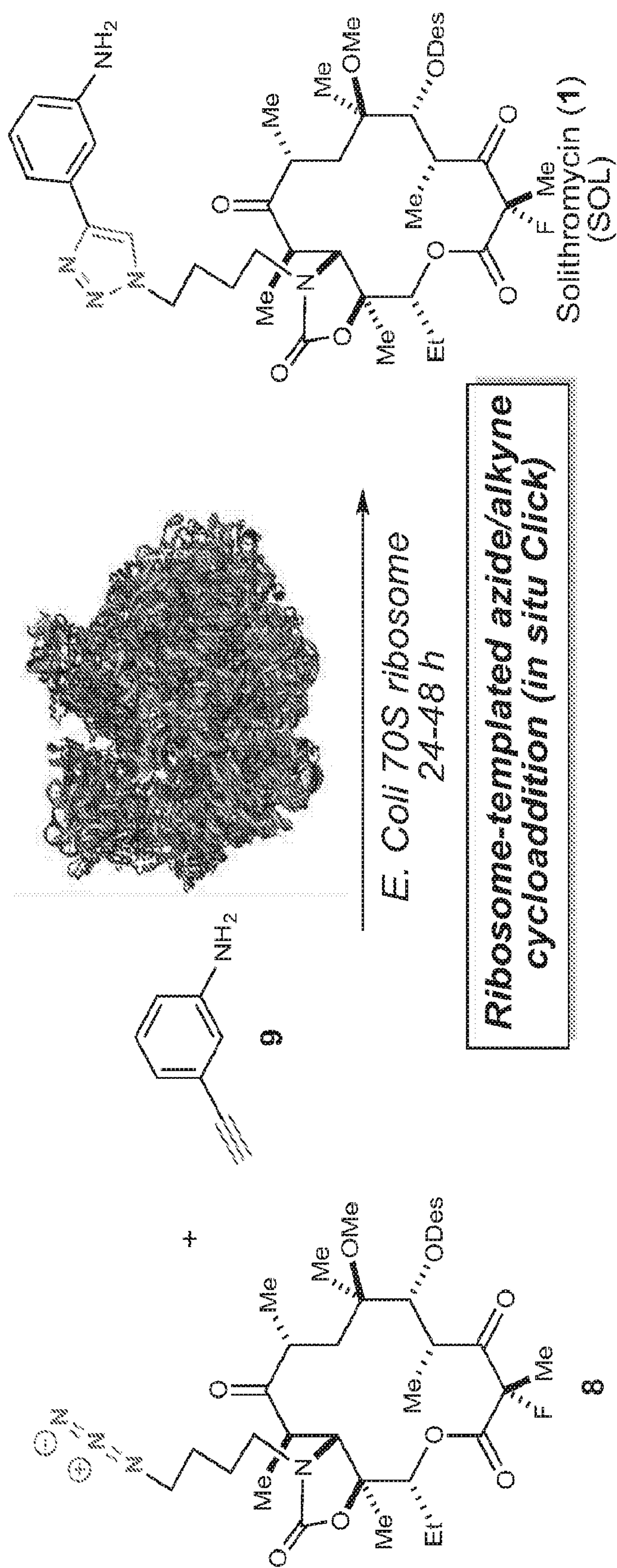
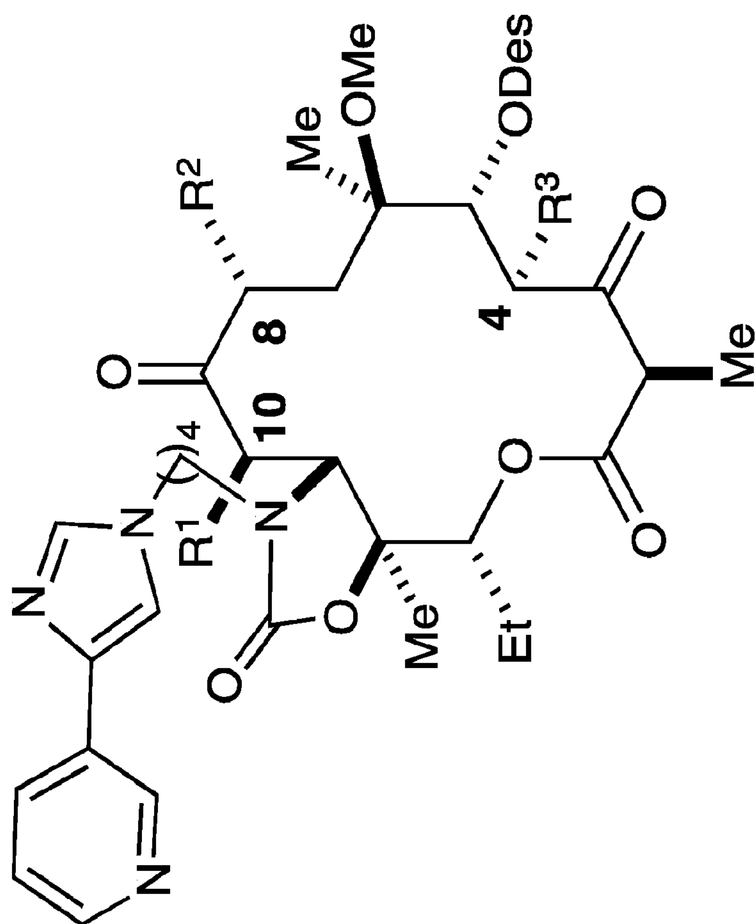


Figure 3



R^1=R^2=R^3=Me: TEL (**6**)
R^1=R^2=R^3=H: 4,8,10-TridesMe TEL (**10**)
R^1=R^3=H,R^2=Me: 4,10-DidesMe TEL (**11**)
R^1=Me,R^2=R^3=H: 4,8-DidesMe TEL (**12**)
R^1=R^2=Me, R^3=H: 4-DesMe TEL (**13**)

Figure 4

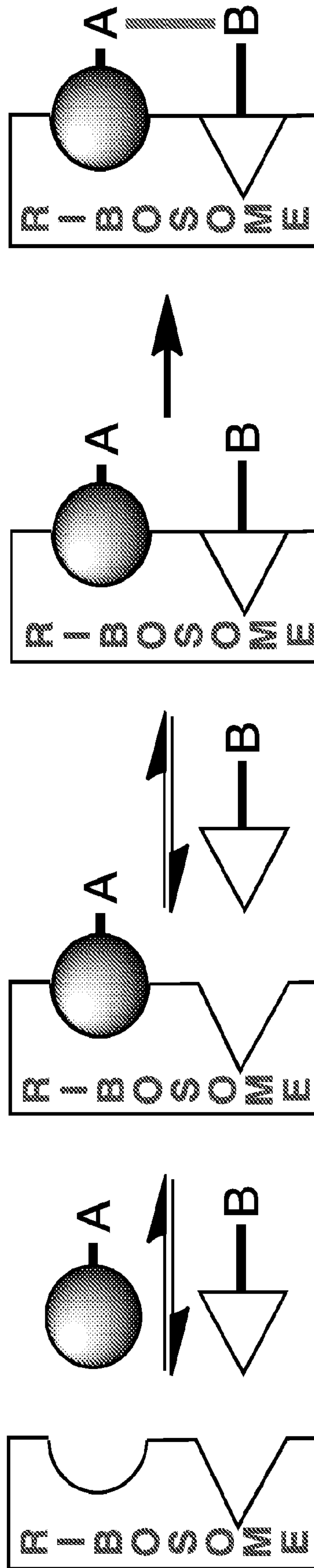


Figure 5

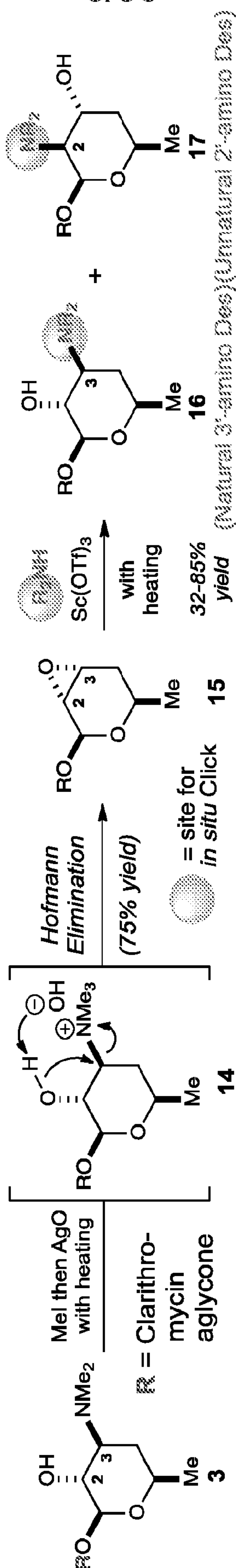


Figure 6

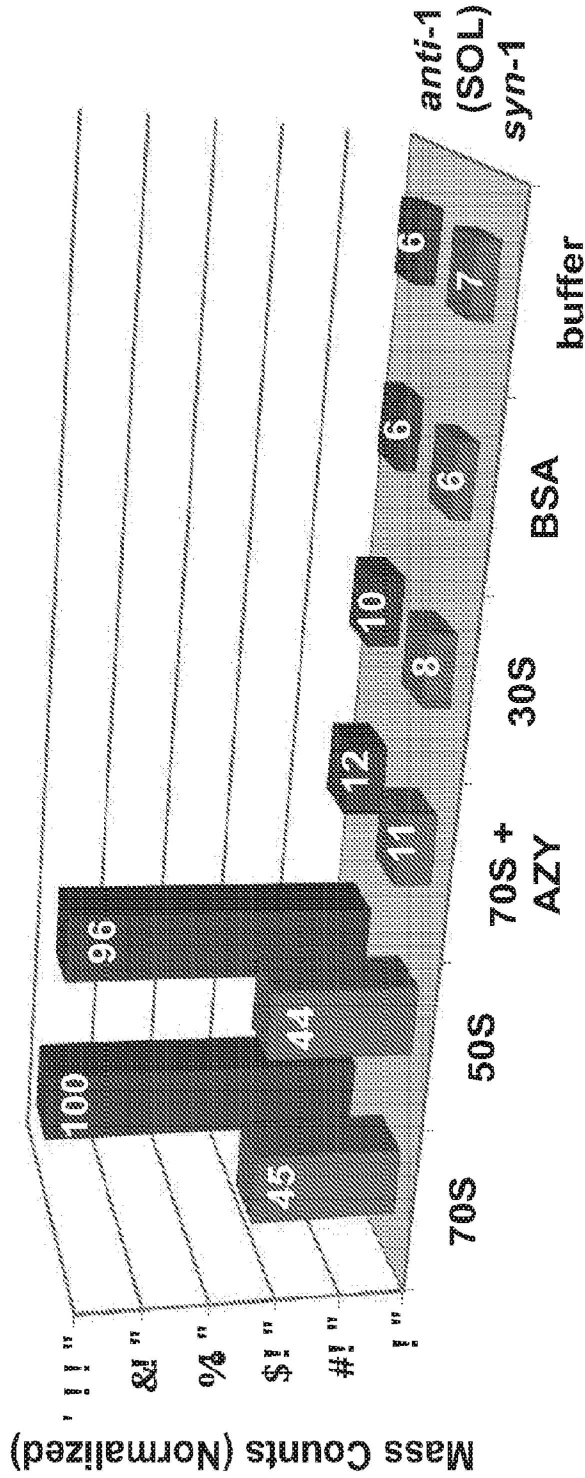


Figure 7

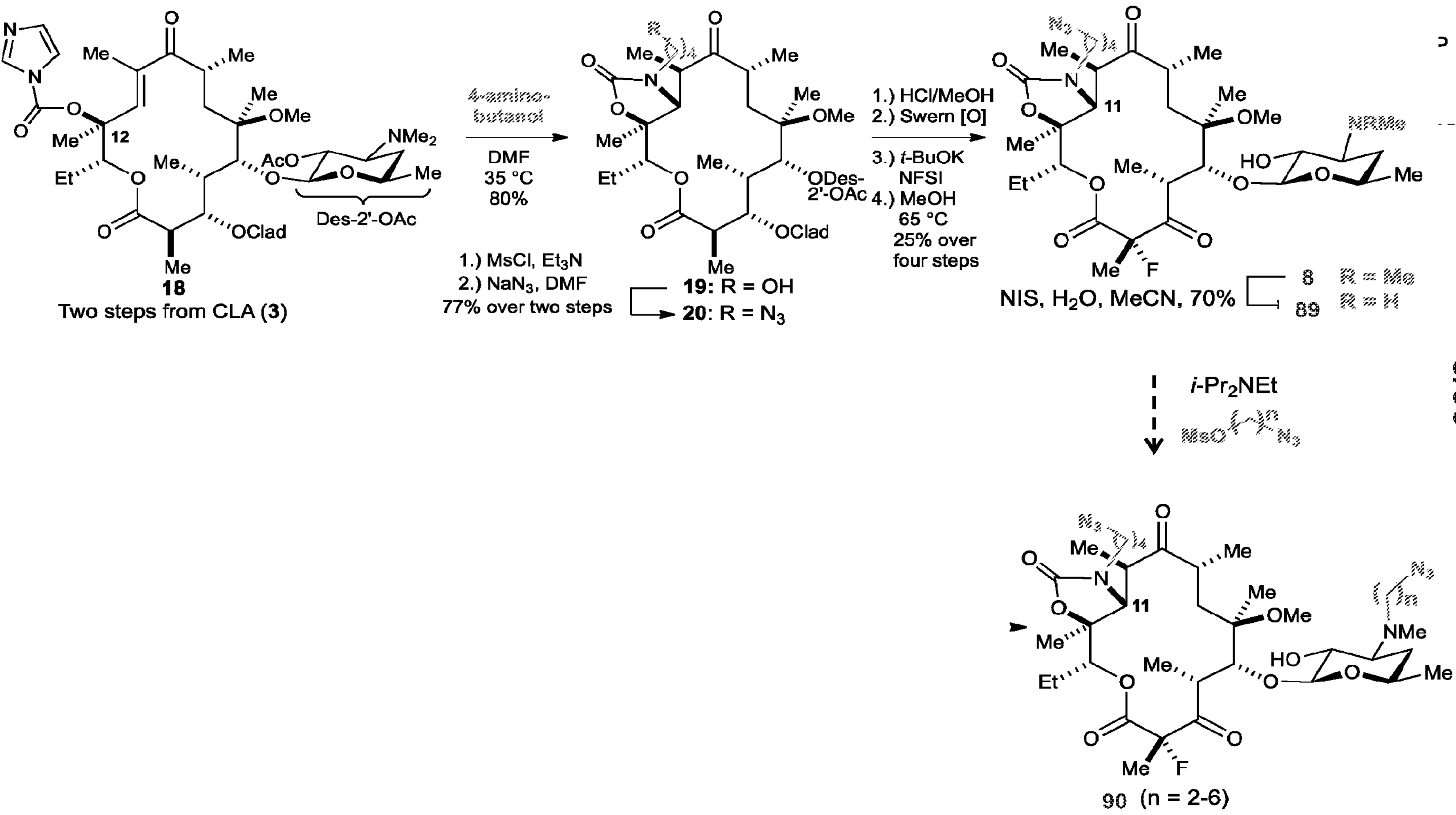


Figure 8

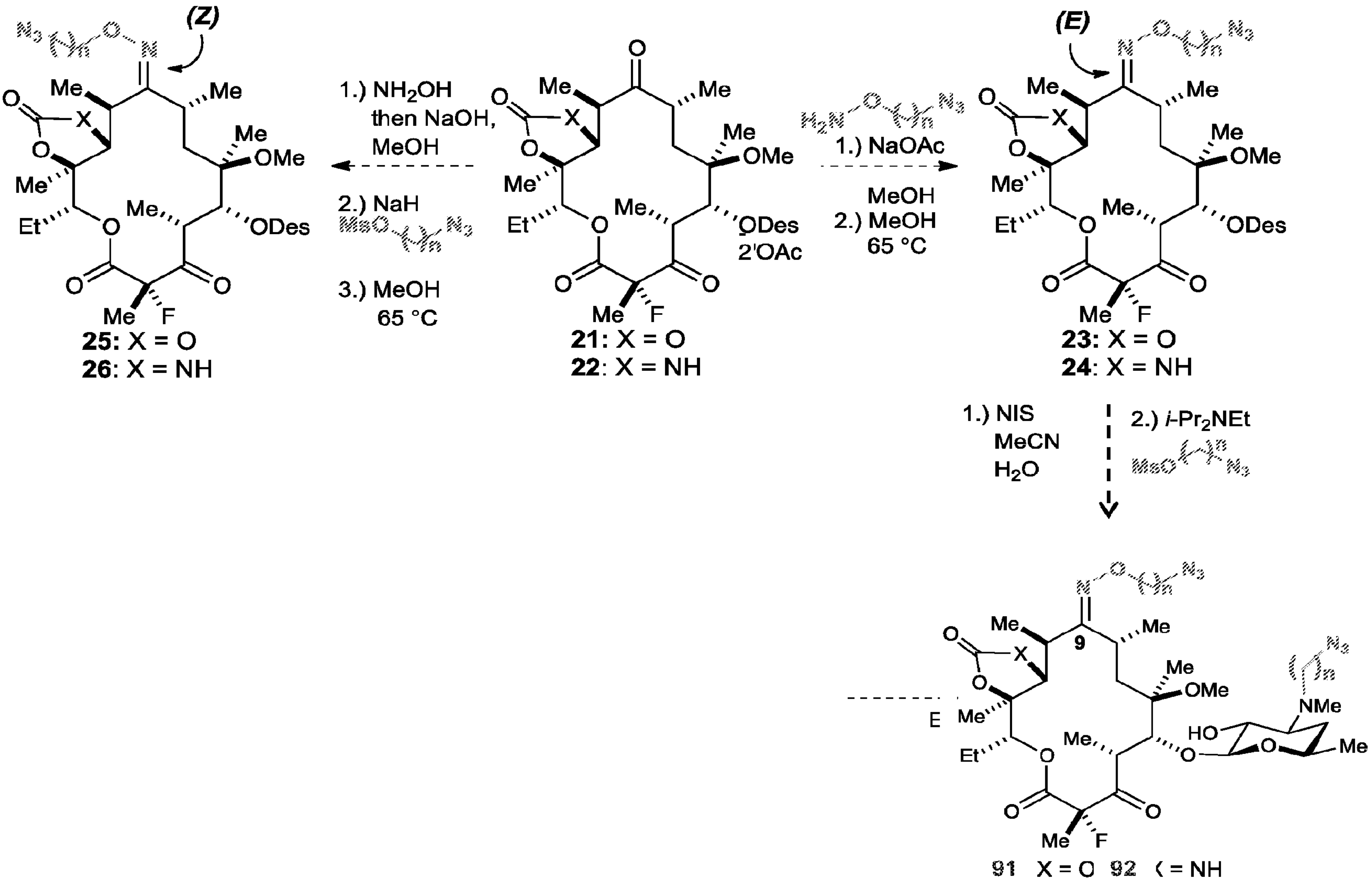


Figure 9

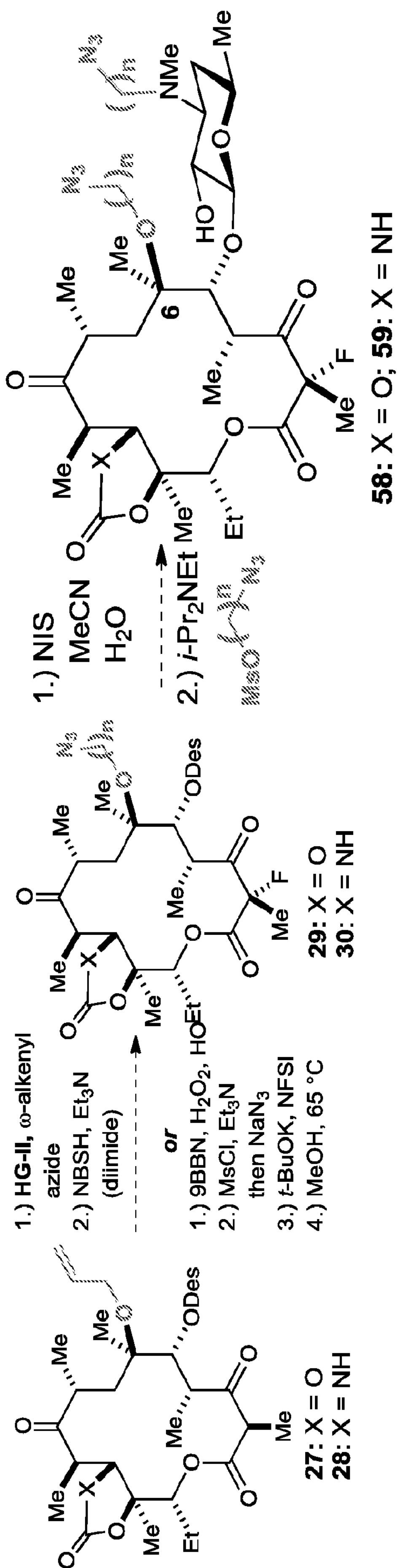


Figure 10

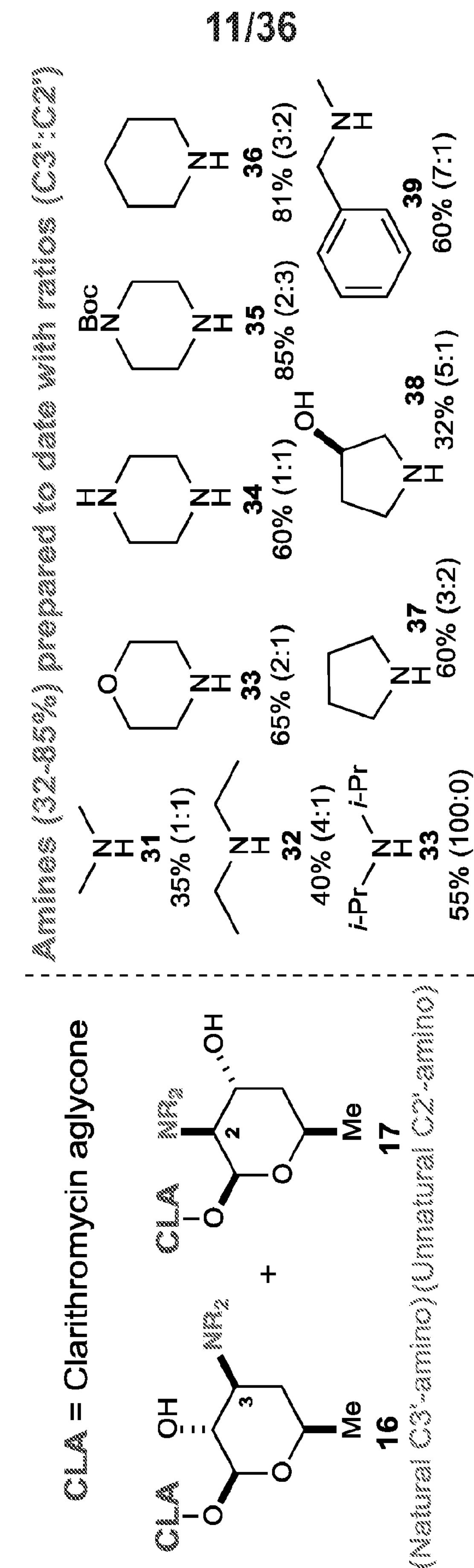
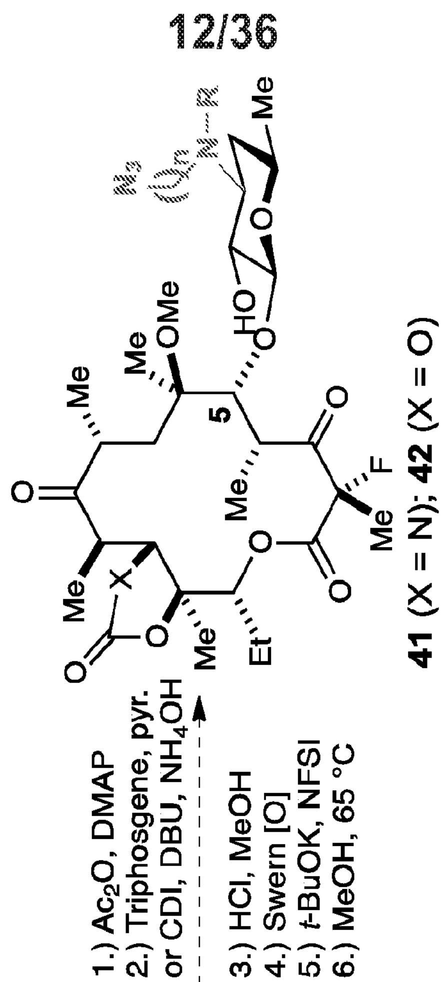


Figure 11



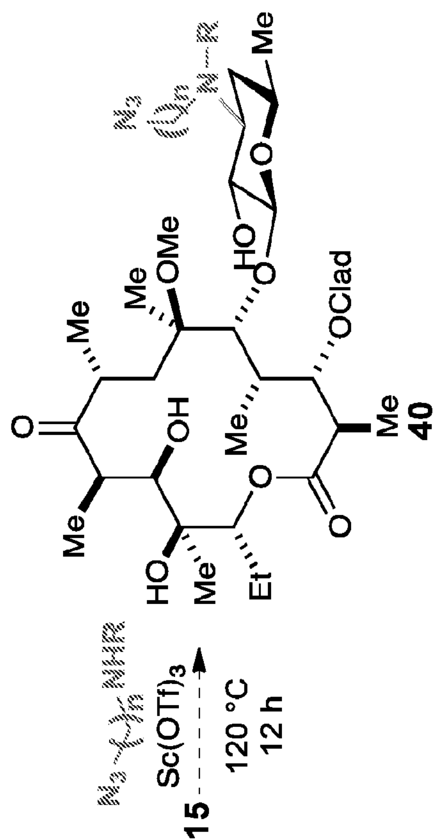
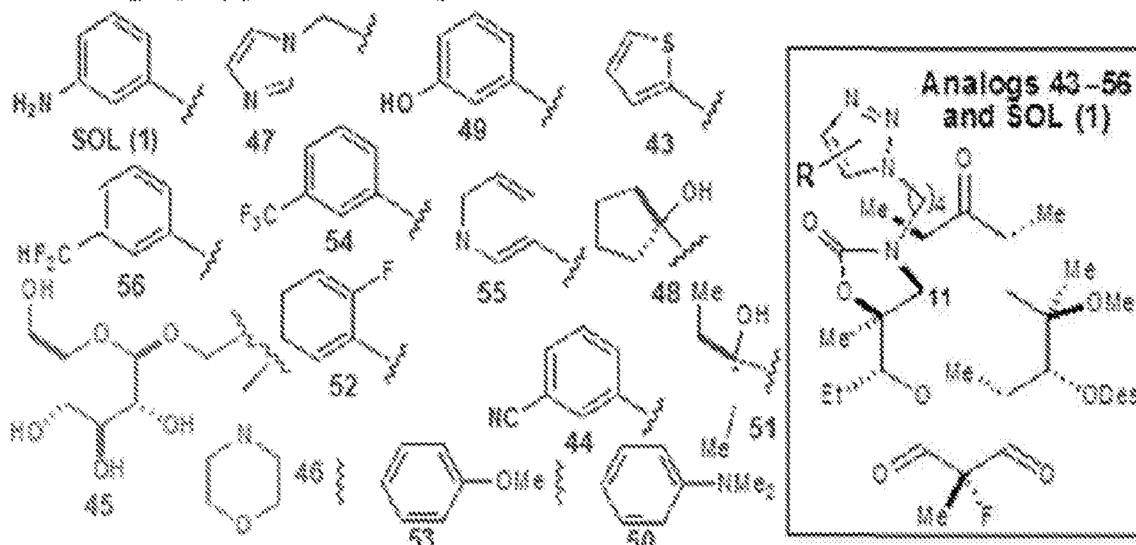


Figure 12



Analog	*In situ* Click MC % increase	SILCS LE	% Transl. (1 µM)	MIC (µg/mL) *E. coli*	MIC (µg/mL) *S. pneumoniae*
43	1160 (mix)	-0.64	53 ± 12	1	**0.004**
55	992 (1,4)	-0.58	44 ± 10	4	0.016
44	972 (1,4)	-0.59	58 ± 10	4	0.016
SOL (1)	503 (1,4)	-0.60	47 ± 15	2	**0.004-0.008**
49	346 (1,4)	-0.60	54 ± 3	2	**≤ 0.002**
53	267 (1,4)	-0.55	63 ± 5	2	**0.002- 0.008**
1,5-SOL	190 (1,5)	TBD	TBD	TBD	TBD
56	167 (mix)	-0.64	66 ± 1	2	**0.004**
50	137 (1,4)	-0.55	67 ± 4	4	0.008
48	114 (mix)	-0.54	57 ± 2	4	0.016
47	83 (mix)	-0.55	51 ± 12	2	0.032
52	75 (mix)	-0.58	54 ± 8	2	**≤ 0.002**
51	74 (mix)	-0.57	70	4	4
46	48 (1,4)	-0.59	16 ± 10	8	8
54	41	-0.54	64 ± 9	4	4
45	36 (1,4)	-0.66	68 ± 5	>32	>32

Figure 13

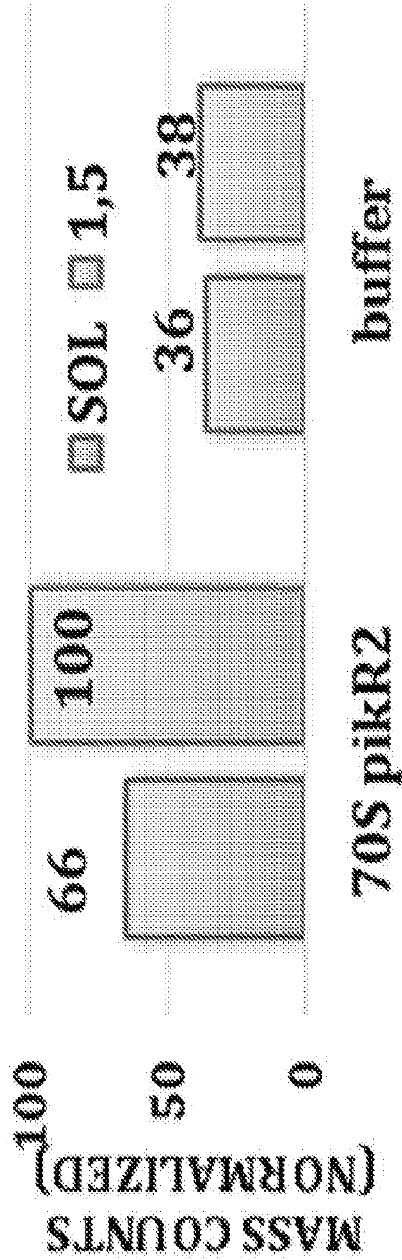
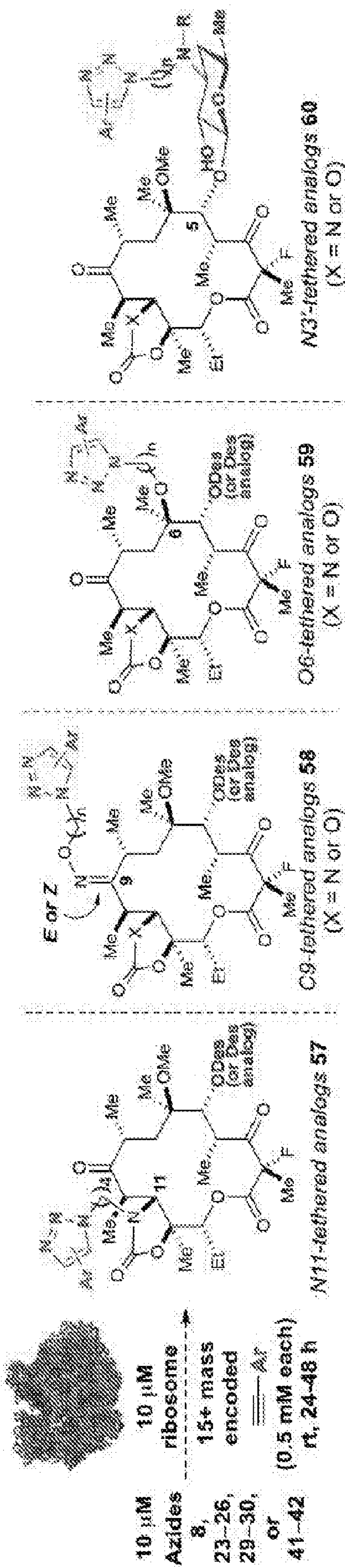


Figure 14



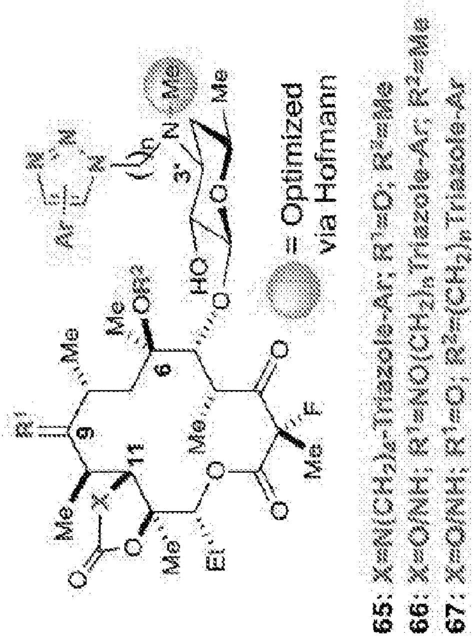
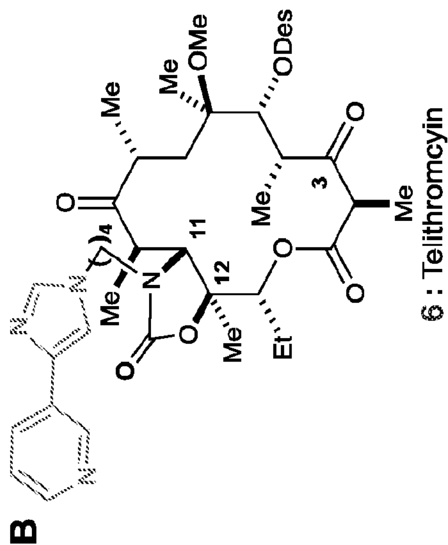


Figure 15

MIC	*Escherichia coli*				*Staphylococcus aureus*					*Streptomyces pneumoniae*					
Cmpd	DK pkk3 535	DK 2058 G	SQ 171	SQ171 A2058 G	ATCC 29213 (WT)	ATCC 33591 MRSA	UCN14 A2058 U	UCN17 A2058 G	UCN1 8 A2059	ATCC 49619 (WT)	*2196 erm B*	*655 mef A*	*1369 ermB mef A*	*319 A2059 G*	*2634 mef A 2059G*
SOL (1)	2	2	32	>64	<0.0625	32-64	8	4	4	0.004-0.008	0.5-1	0.25-0.5	0.5	0.008	0.125-0.25
43	1	1	32	>64	<0.0625	>128	32	8	8	0.004	2	0.5	0.5	0.008	0.125
44	4	4	>64	>64	0.5	>128	>128	128	128	0.016	>4	1	>4	0.25	0.5
45	>32	>32	>64	>64	128	>128	>128	>128	>128	4	>4	>4	>4	>4	>4
46	8	8	>64	>64	0.25	>128	128	>128	>128	0.0625	>4	4	>4	2	4
47	2	2	64	>64	0.25	>128	>128	128	128	0.031	>4	4	>4	0.25	1
48	4	4	>64	>64	0.125	>128	>128	128	>128	0.016	>4	2	>4	1	2
49	2	1	32	>64	<0.0625	>128	16	8	8	≤ 0.002	2	0.5	4	0.016	0.25
50	4	4	64	>64	<0.0625	128	128	32	32	0.008	4	1	>4	0.063	0.5
51	4	4	>64	>64	0.25	>128	>128	>128	>128	0.031	4	2	>4	0.5	2
52	2	2	32	>64	<0.0625	128	32	8	8	≤ 0.002	2	0.5	4	0.031	0.25
53	2	2	64	>64	<0.0625	>128	>128	32	32	0.002-0.008	4	1	>4	0.063	0.25
54	4	4	64	>64	<0.0625	32	32	32	32	0.016	>4	2	>4	0.5	1
55	2	2	32	>64	<0.0625	>128	32-64	16	16	0.016	1	0.5	>4	0.031	0.25
56	2	4	64	>64	<0.0625	128	32-64	16	8	0.004	4	1	>4	0.063	0.5

Figure 16



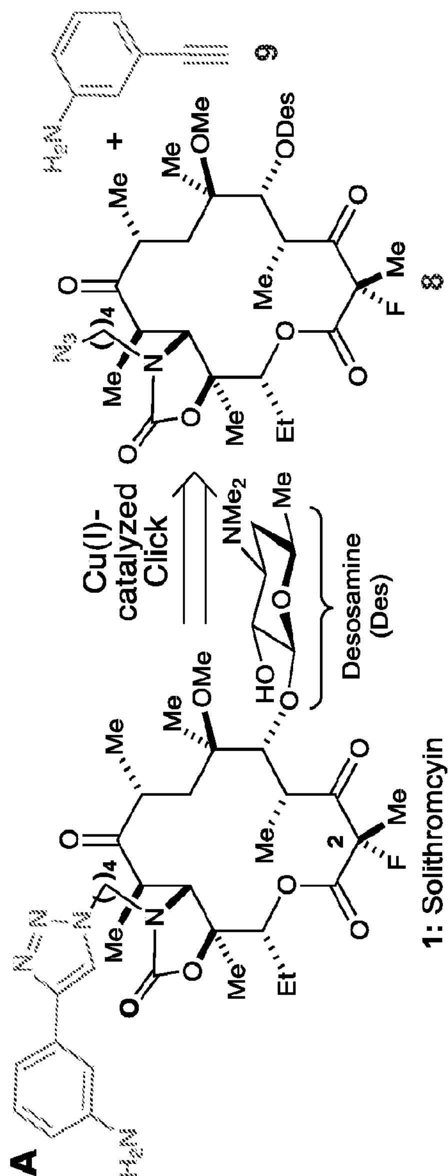


Figure 17A-17B

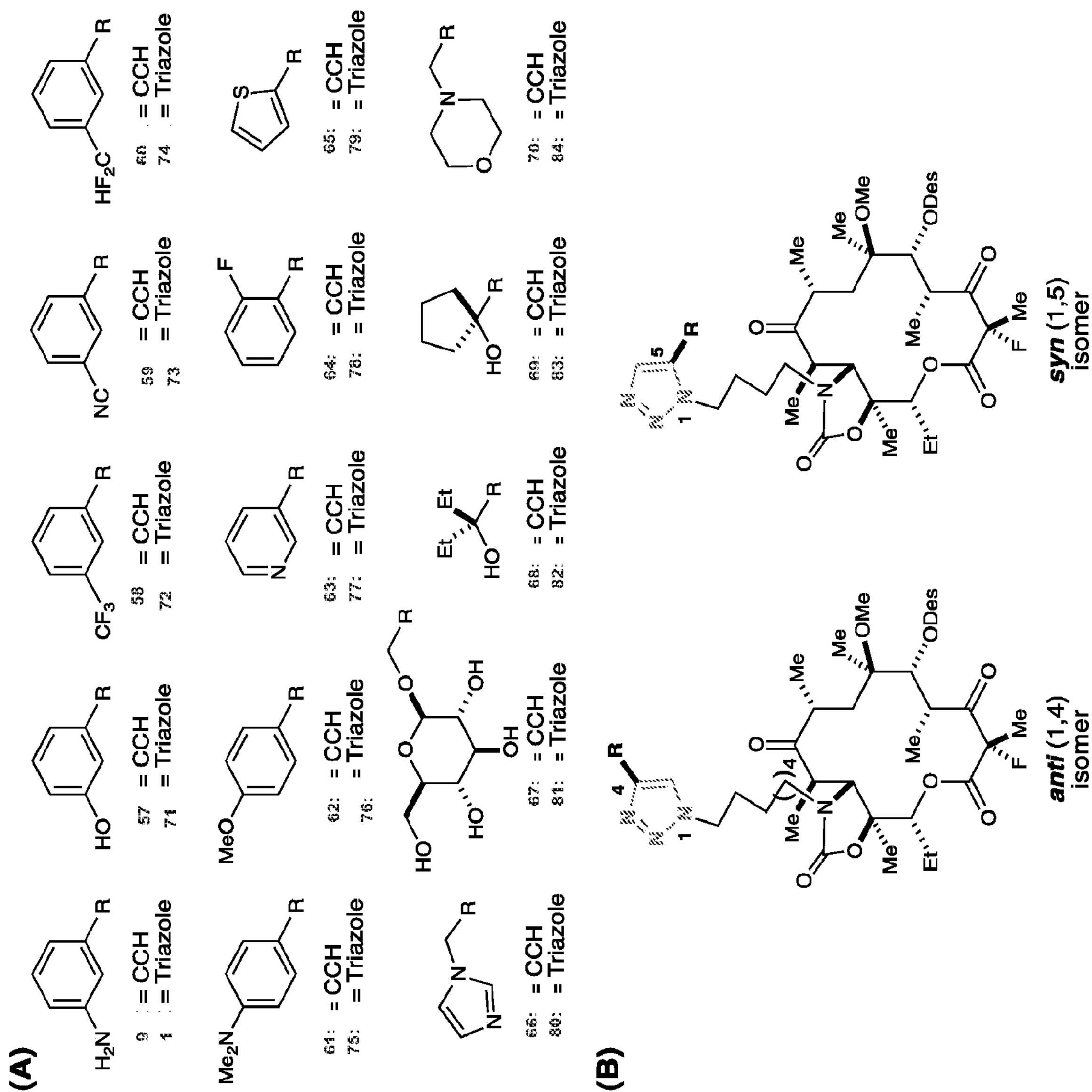


Figure 18A-18B

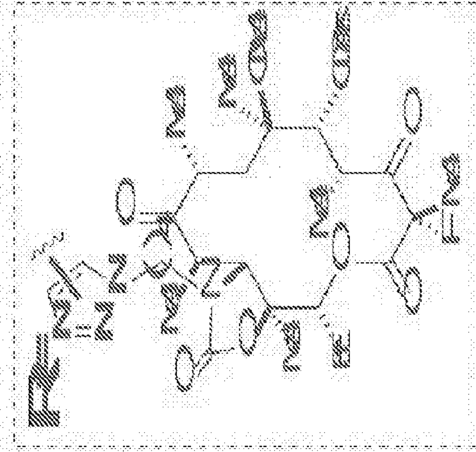


Figure 19

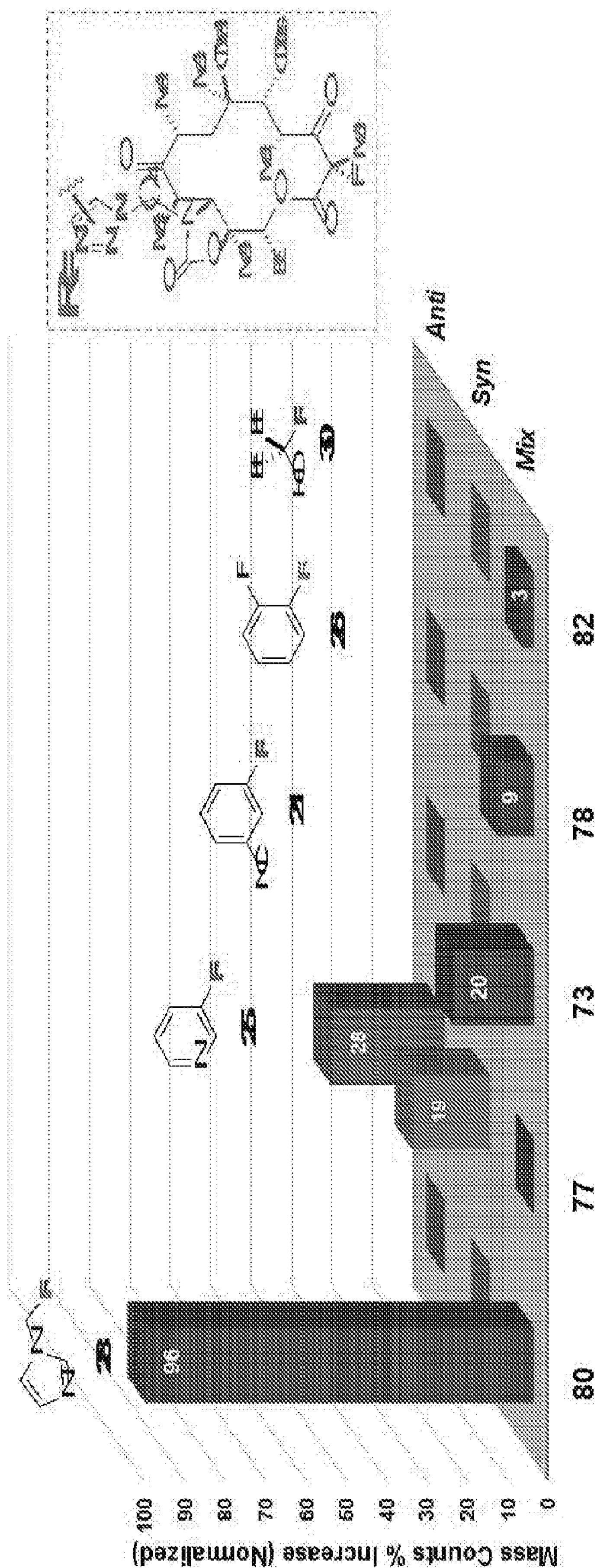


Figure 20

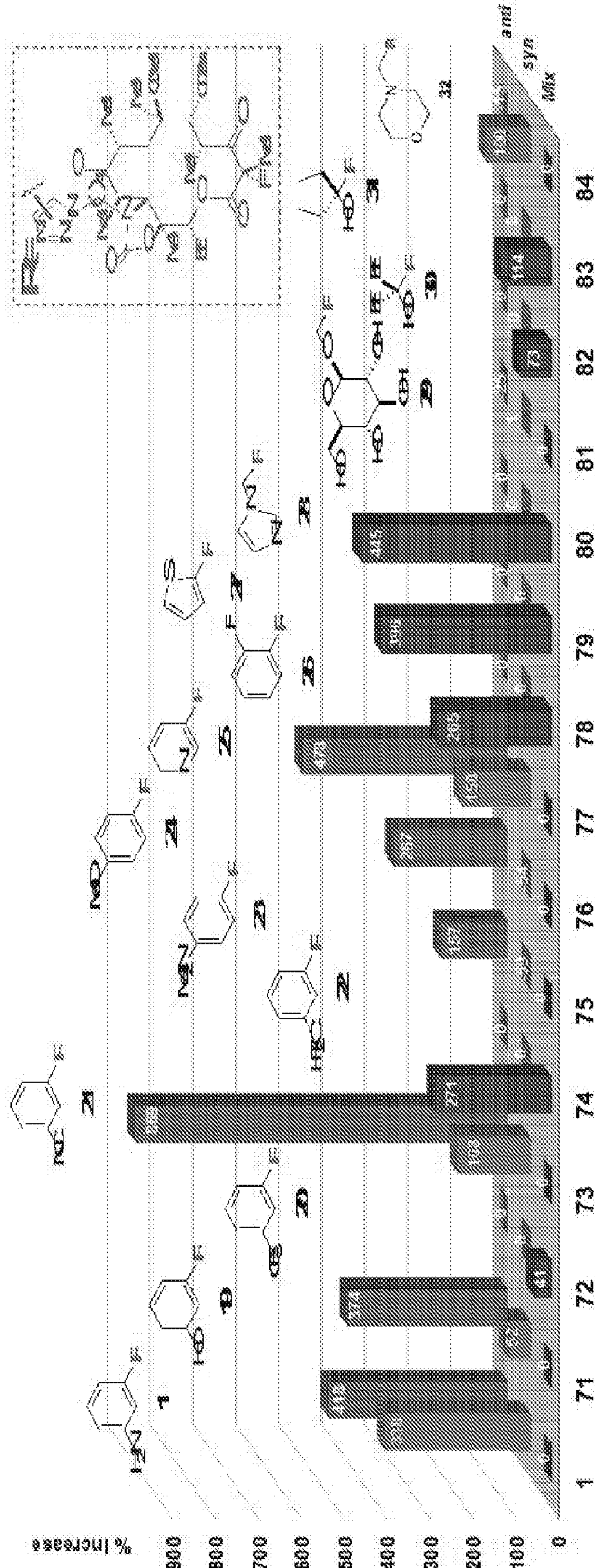


Figure 21

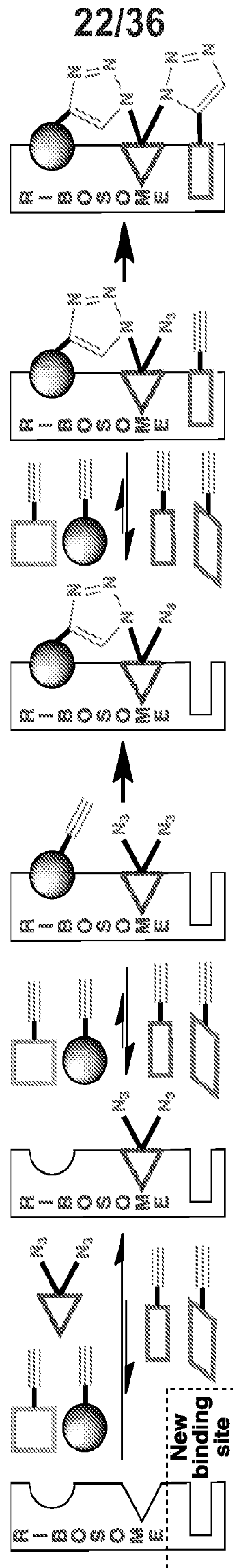


Figure 22

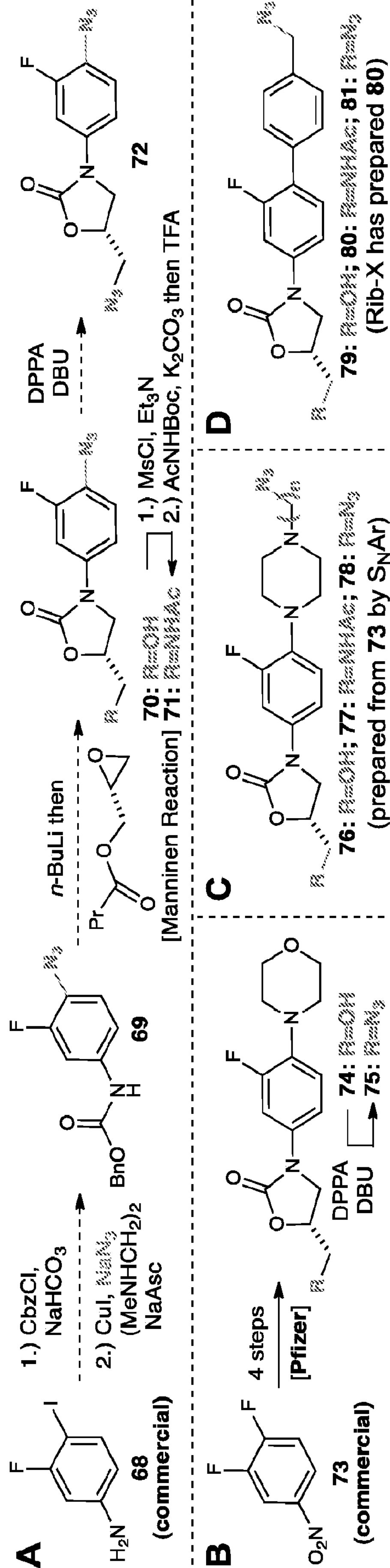


Figure 23A-23D

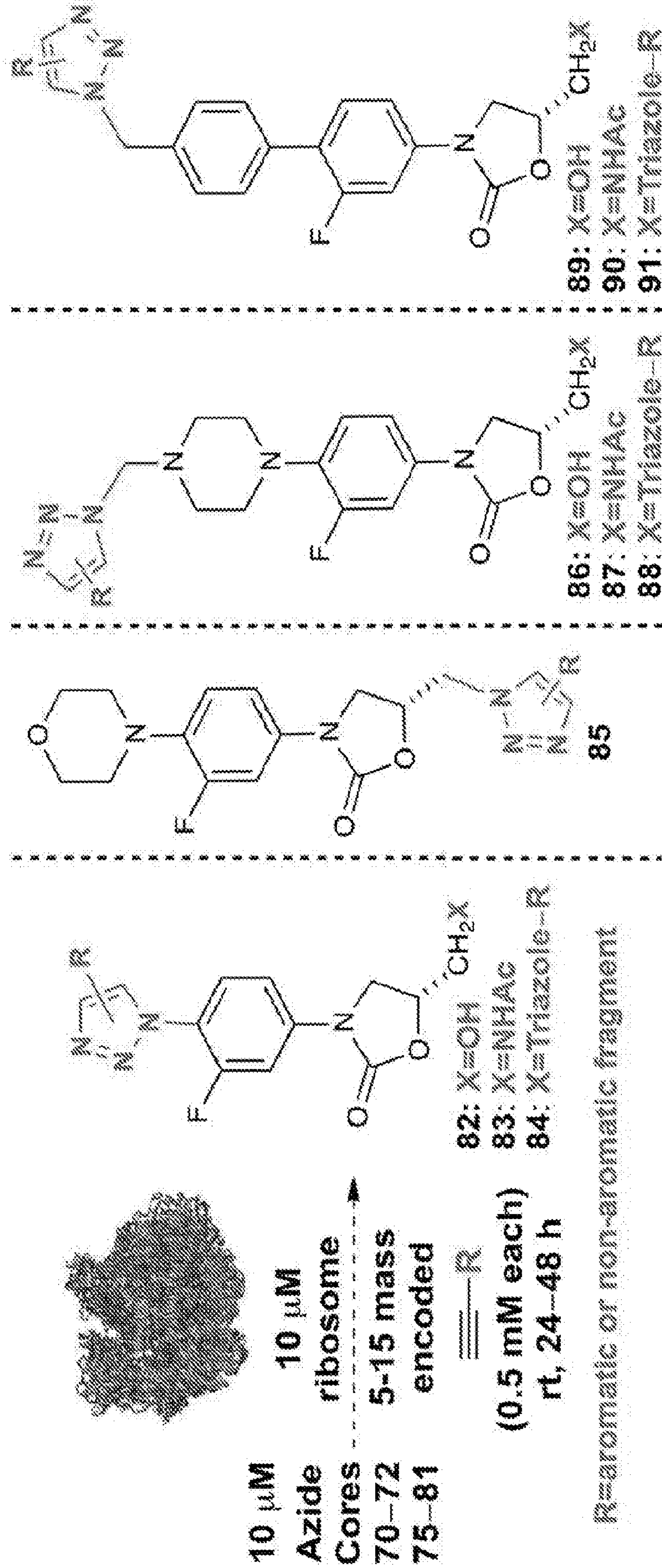


Figure 24

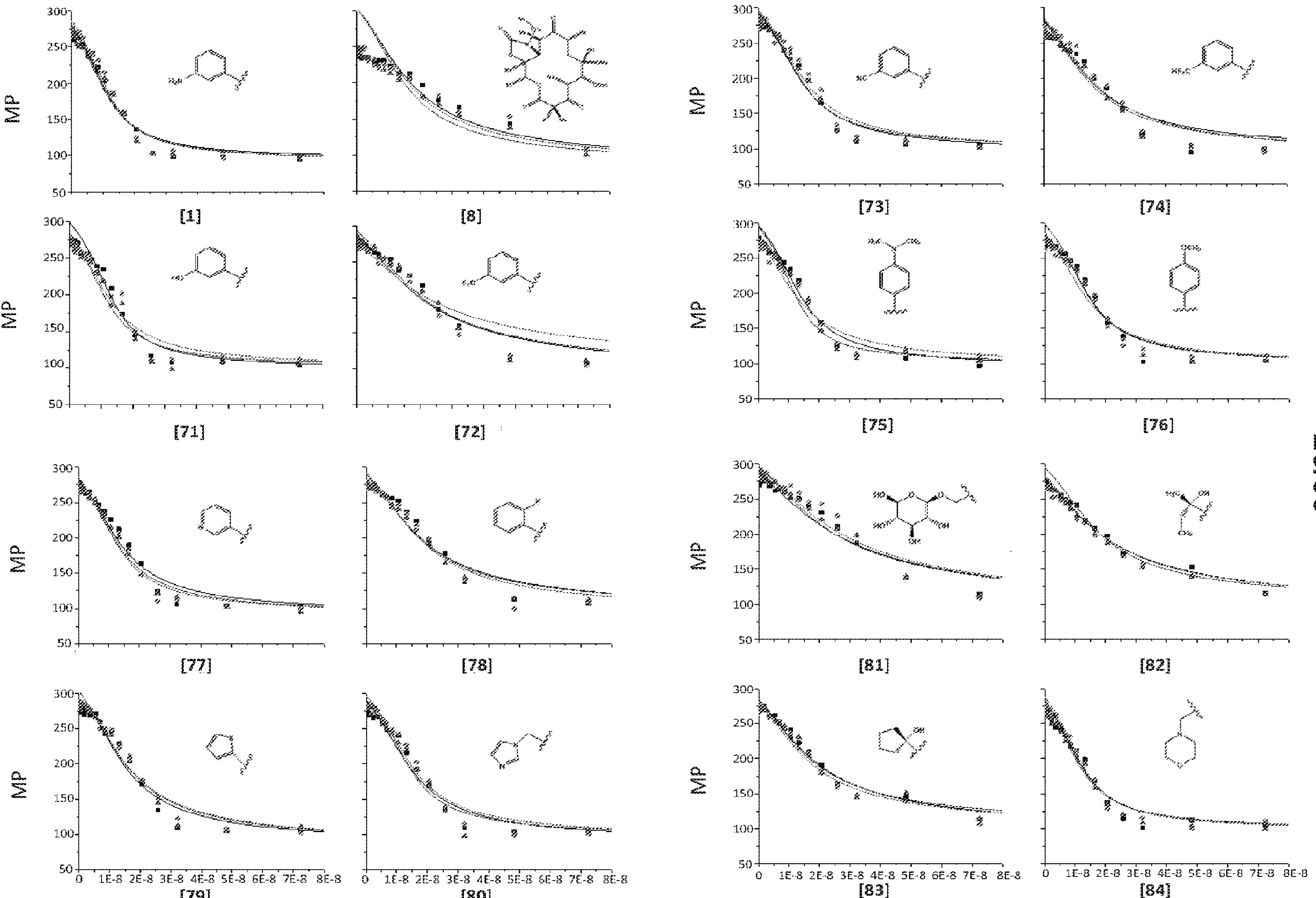


Figure 25

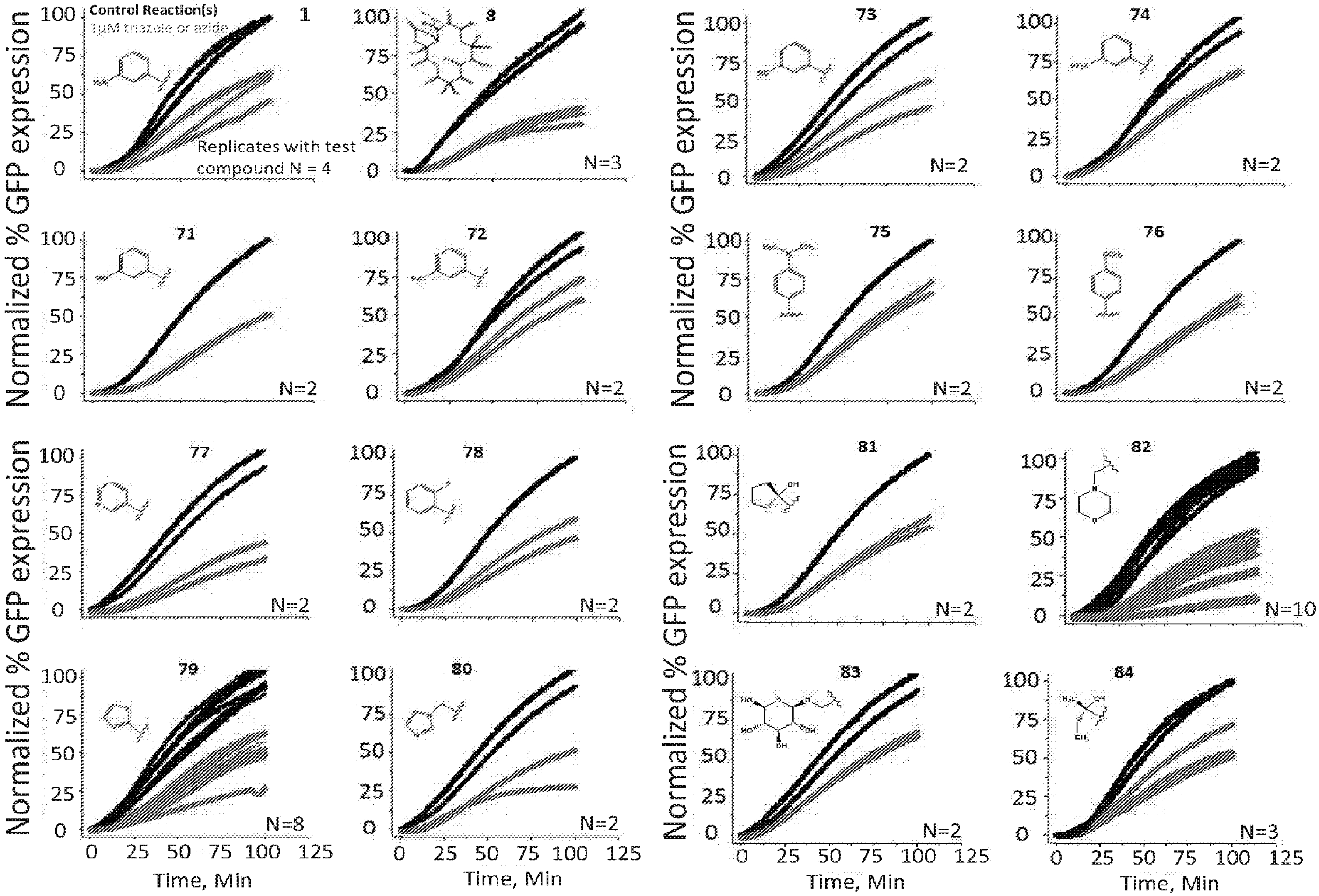


Figure 26

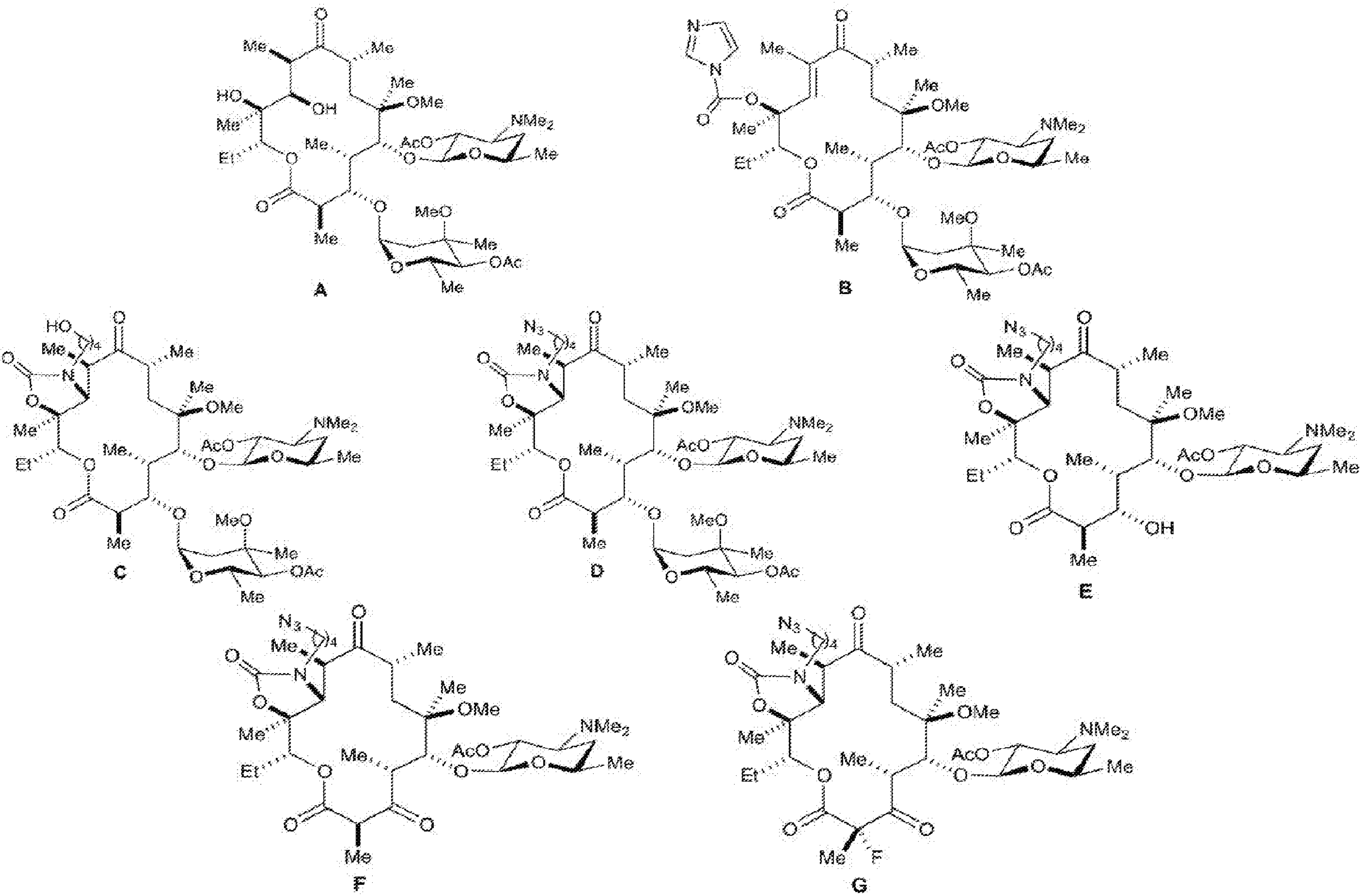


Figure 27

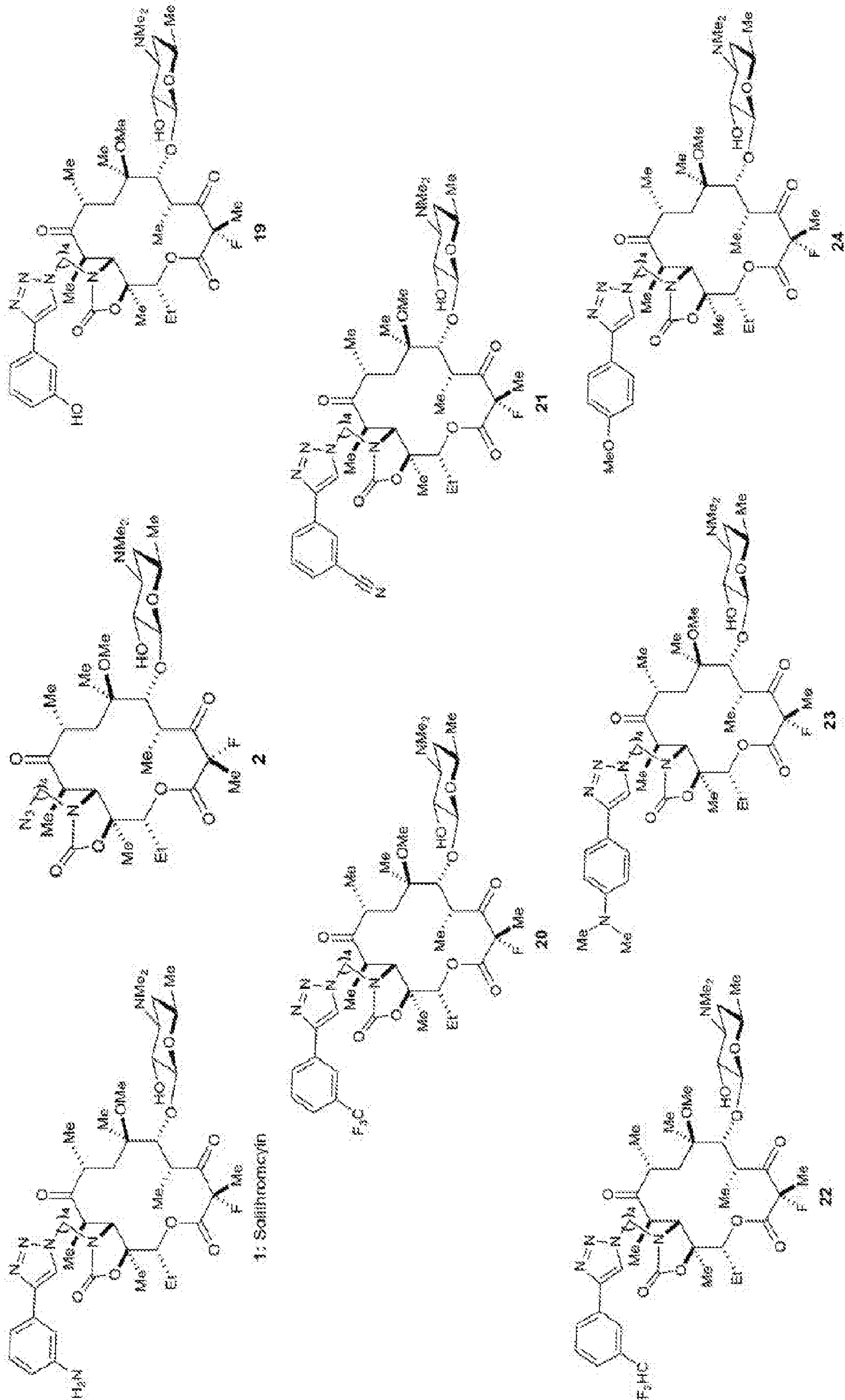


Figure 28

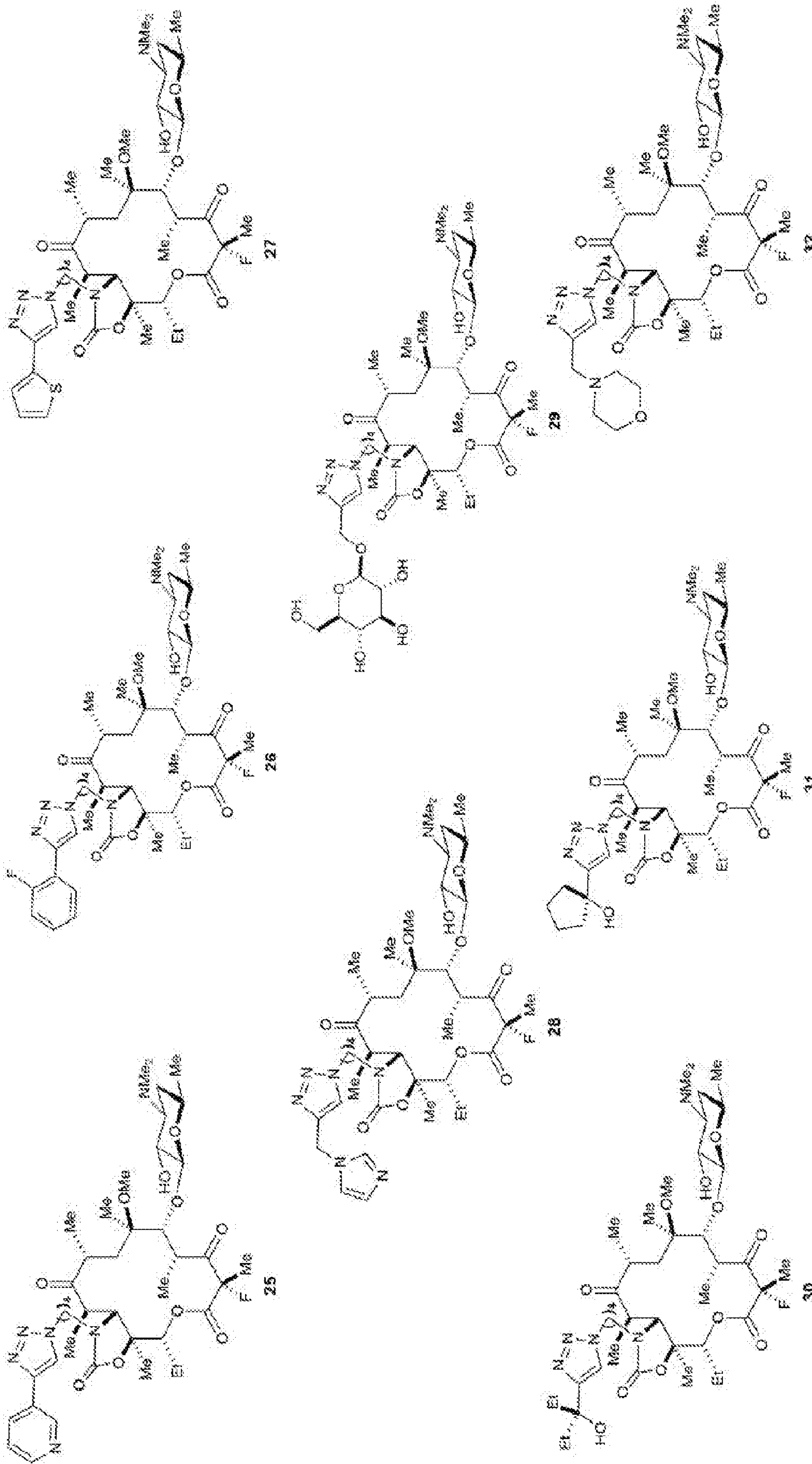
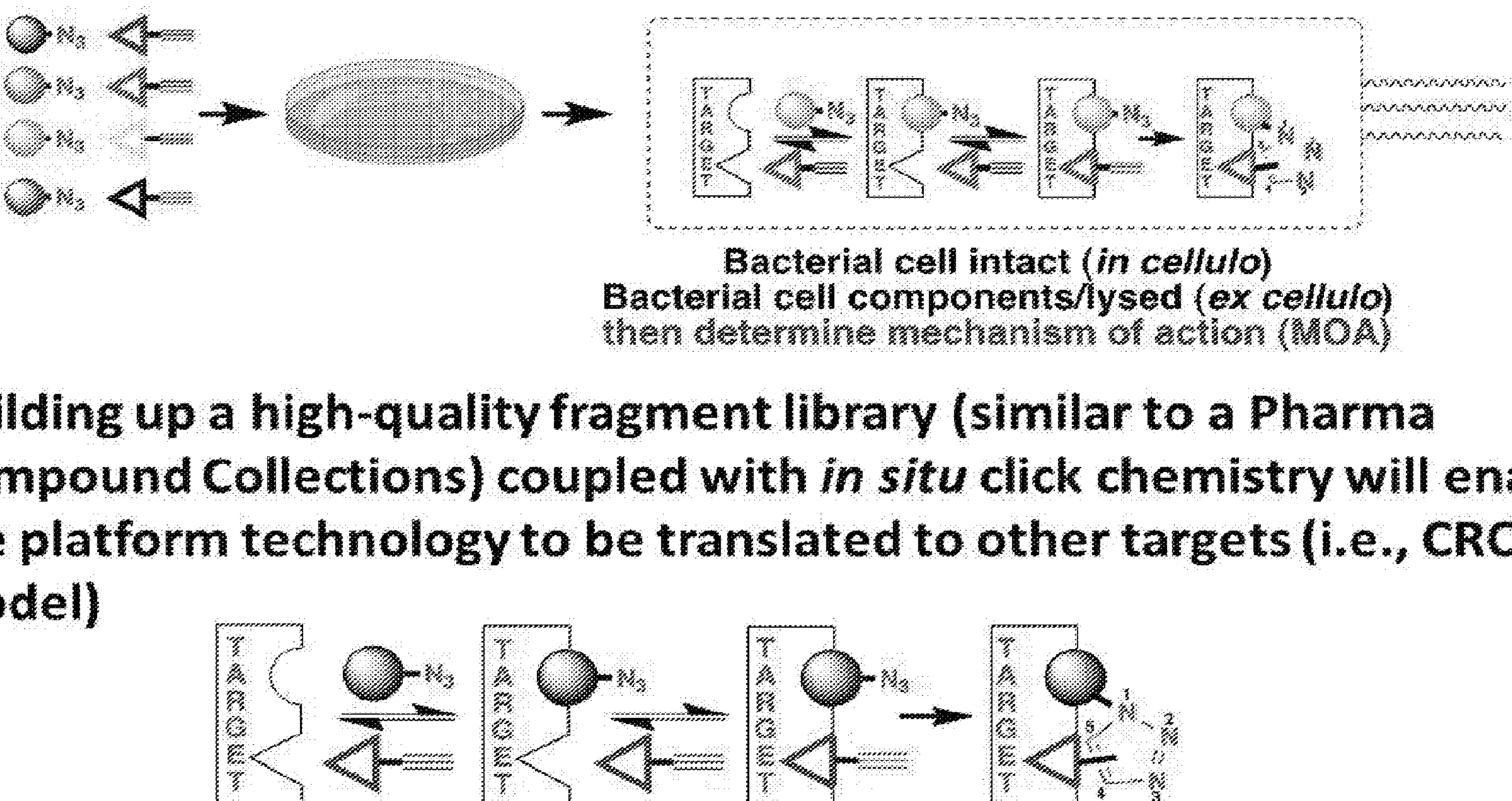


Figure 29

- **Translate method into a species- or resistance-selective *in cellulo* to enable rapid phenotypic screening (kill vs no kill) by a synthetic lethal mechanism to discover new antibiotics. *Ex cellulo* is also viable. The target and mode-of-action need not be restricted to the ribosome.**



- **Building up a high-quality fragment library (similar to a Pharma Compound Collections) coupled with *in situ* click chemistry will enable the platform technology to be translated to other targets (i.e., CRO Model)**

Figure 30

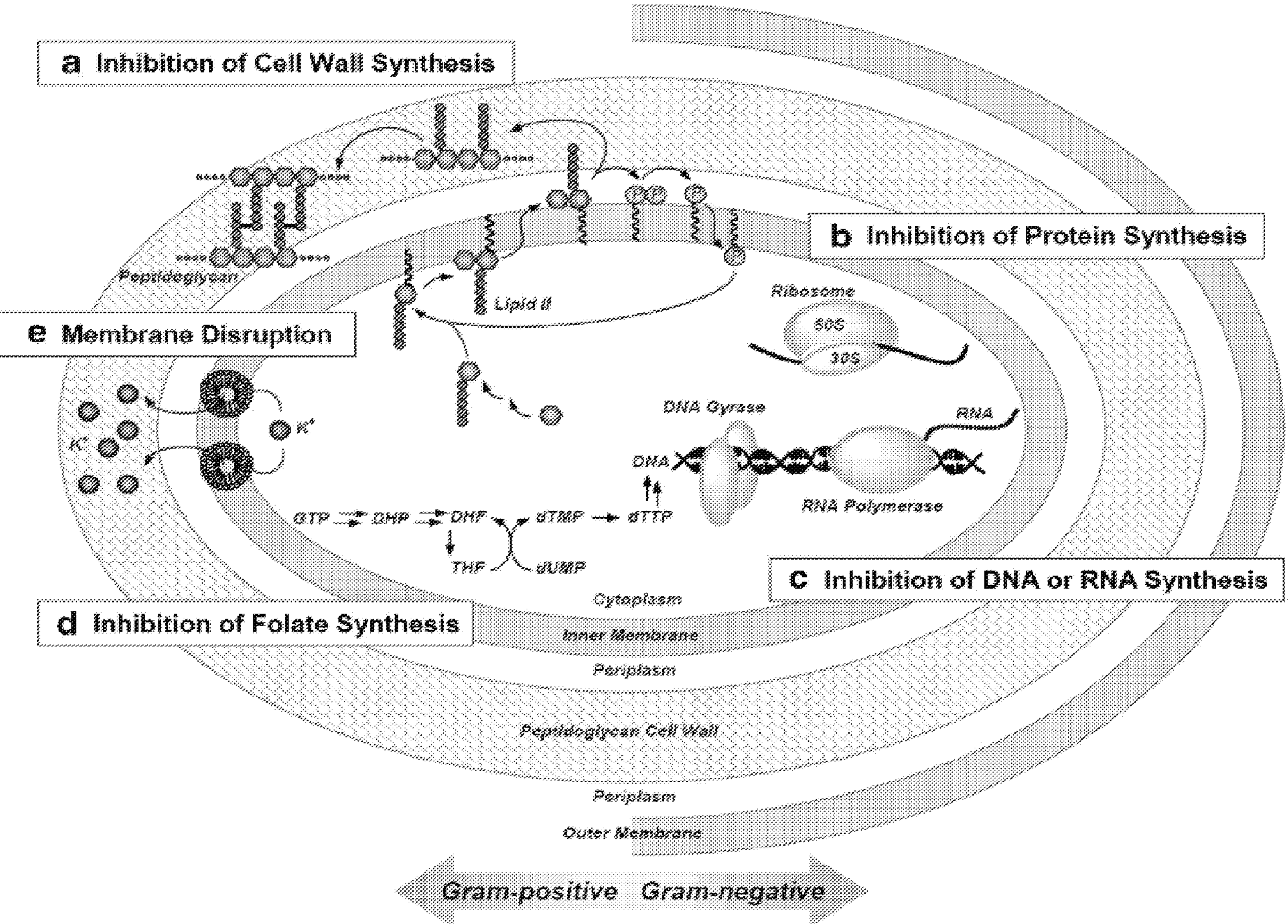


Figure 31

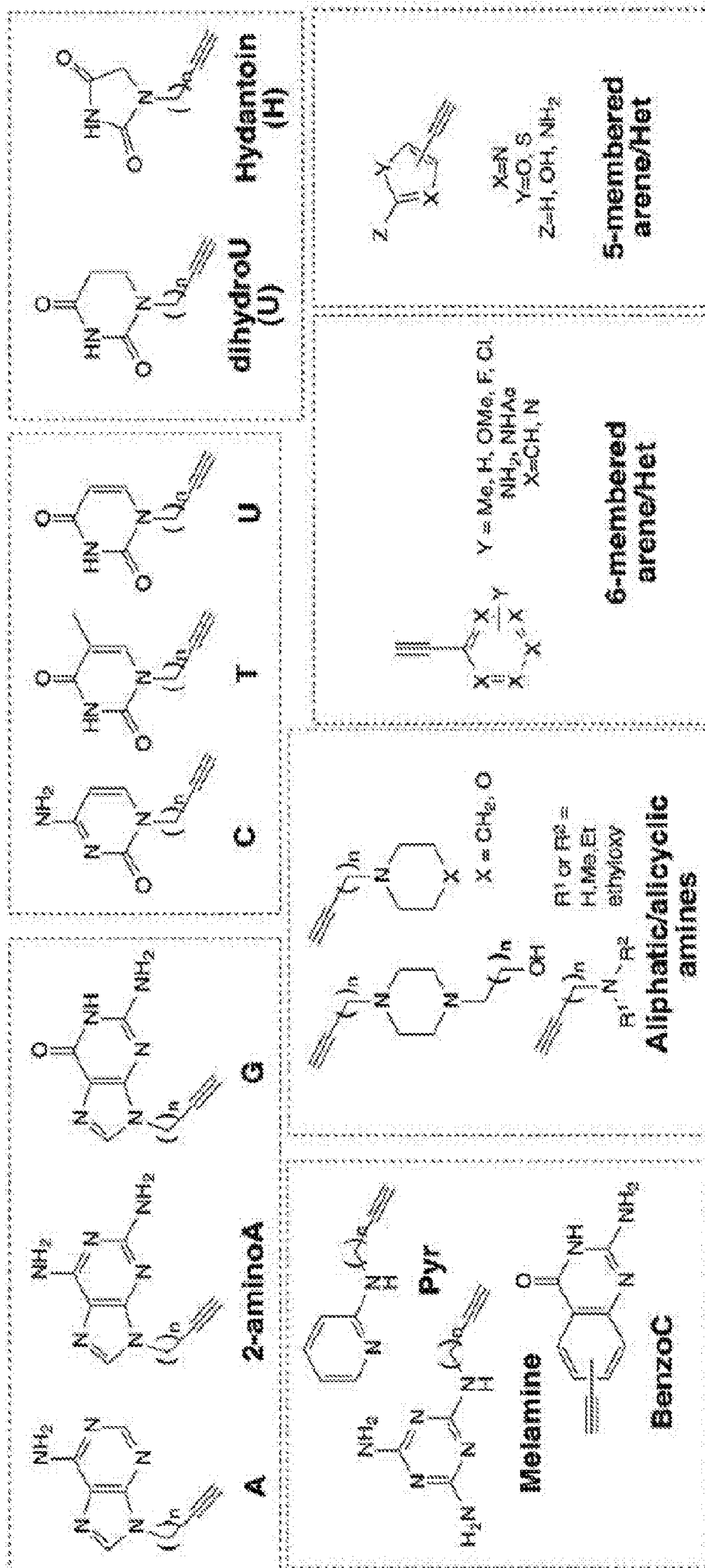


Figure 32

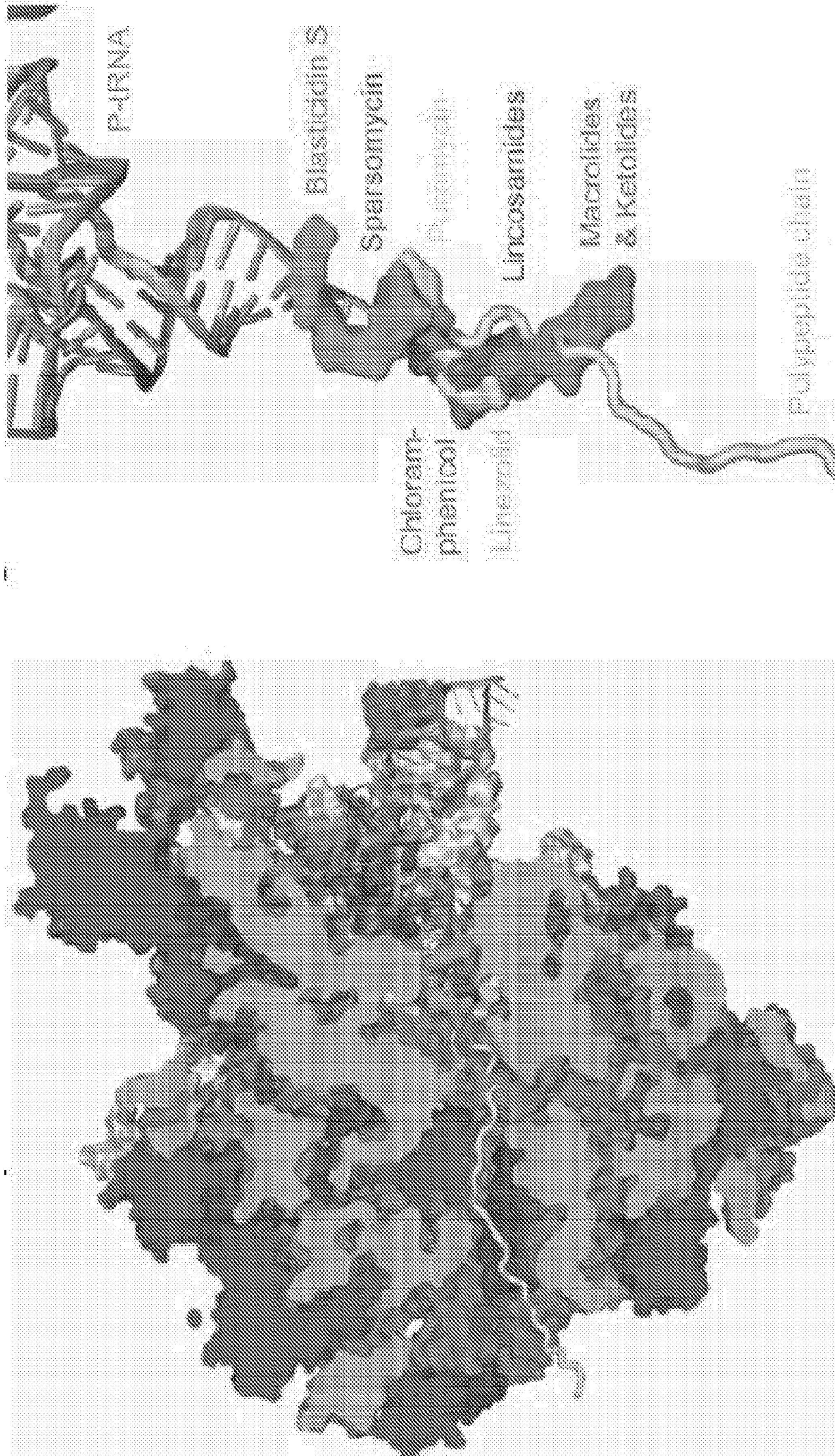


Figure 33

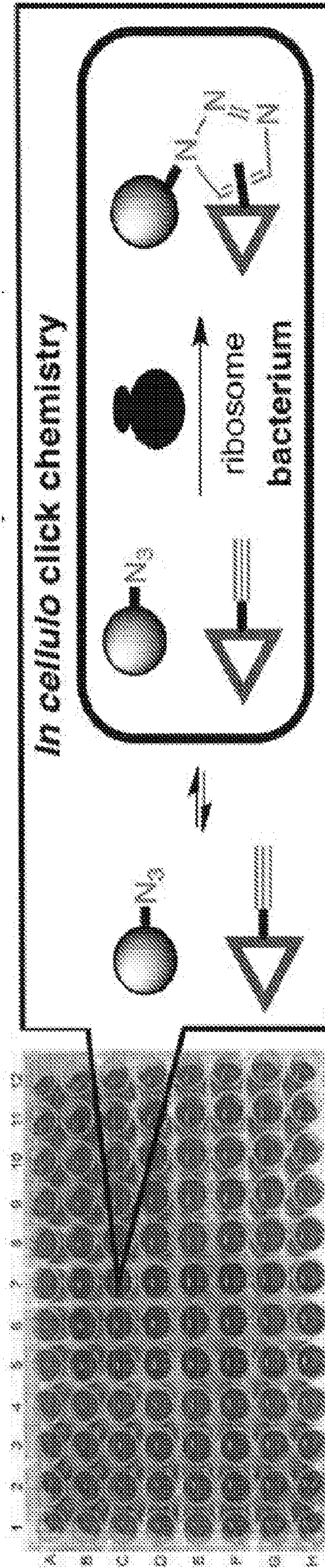
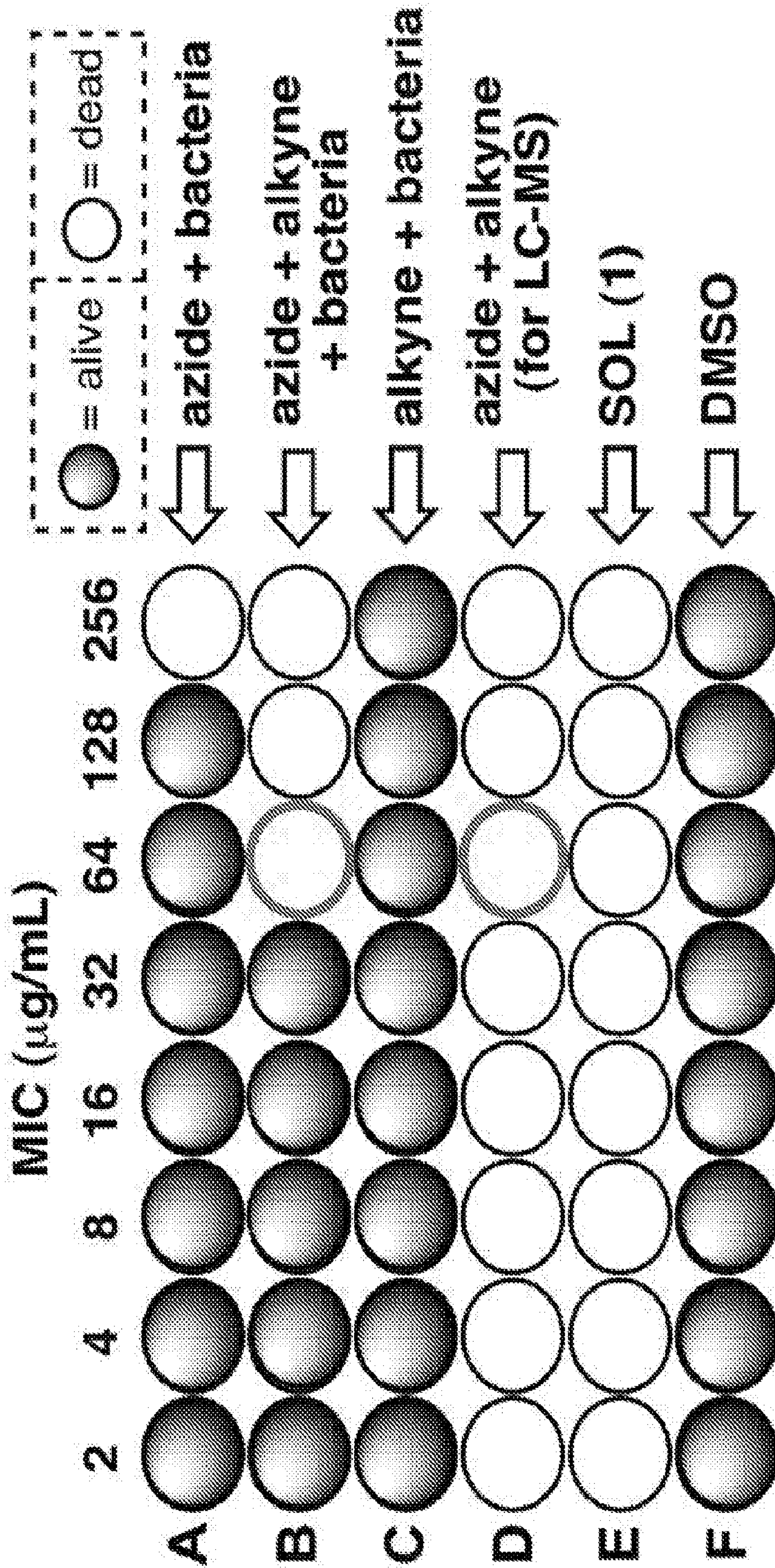


Figure 34



Experiments	1	2	3	4
Azide+alkyne+bacteria (Row B)	475567	346204	576483	488653
Azide+alkyne (Row D)	174540	155429	206624	267377
Azide+alkyne+bacteria+**AZY**	ND	ND	192555	271439

Figure 35

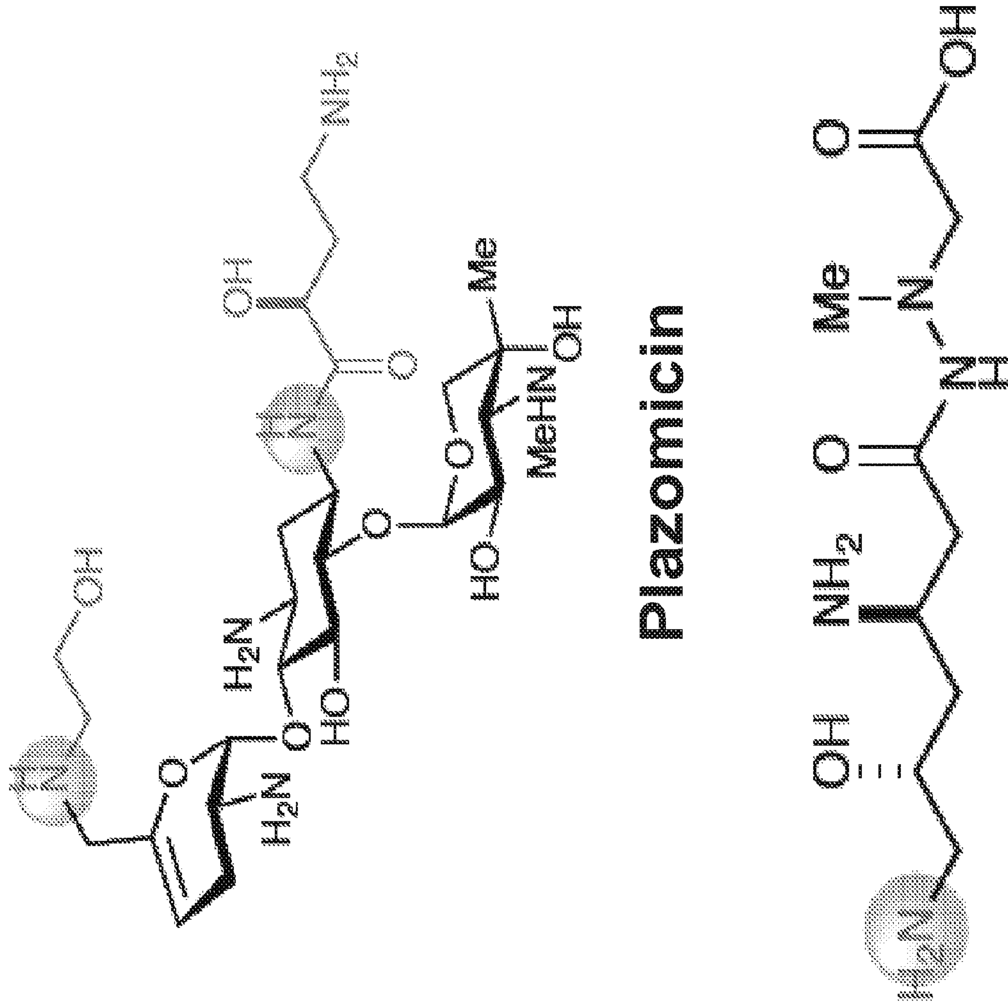
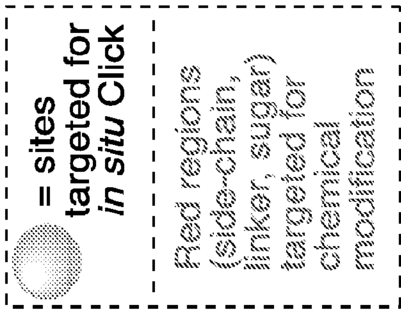


Figure 36



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US16/39196

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C40B 30/00; C12Q 1/04 (2016.01)
CPC - A61K 31/00; C40B 40/00; C12Q1/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) Classifications: C40B 30/00; C12Q 1/04 (2016.01)
CPC Classifications: A61K 31/00; C40B 40/00; C12Q1/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data); Google; Pubmed; EBSCO.
Search Terms Used: In Situ Click Chemistry, antibiotic, "In Situ Click Chemistry", coli, aureus, pneumonia, influenza, catarrhalis, pneumophila, gonorrhoeae, pyogenes, A2058G, UCN 14, chromolith chromatography, Q-TOF LC-MS, Macrolide, Oxazolidinone

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	(TIEU, W et al.) Optimising in situ Click Chemistry: the Screening and Identification of Biotin Protein Ligase Inhibitors. Chemical Science. 2013, Vol. 4; pages 3533-3537; page 3533, first column, first paragraph; DOI: 10.1039/C3SC51127H	1-5, 14-15, 17-18 -------------------- 6-13, 16, 19
Y	(GLASSFORD, I et al.) Desmethyl Macrolides: Synthesis and Evaluation of 4-Desmethyl Telithromycin. ACS Medical Chemical Letters. 16 July 2014, Vol. 5; pages 1021-1026; page 1021, first column, 2nd paragraph; DOI: 10.1021/ml5002097	6-11
Y Y	(MCILWRICK, R) Monolithic Silica Columns: Benefits and Applications of Chromolith (R) HPLC Columns. Presentation [online]. August 2006 [Retrieved on 14 September 2016]. Retrieved from the Internet: <URL: http://66.35.87.110/~kvcv/images/documenten/analytische/hplc/14h45%20High%20speed%20analysis%20with%203%20mm%20moinolithic%20hplc%20columns%20using%20standard%20hplc%20instruments%20and%20pressures.pdf>; page 18	12
Y	US 2010/0237235 A1 (OZBAL, CC et al.) 23 September 2010; paragraphs [0134], [0186]	13
Y	(KIRST, ha) 11th International Conference on the Chemistry of Antibiotics and other Bioactive Compounds (ICCA-11) Donostia—San Sebastian, Spain 29 September–2 October 2009. The Journal of Antibiotics. 2010, Vol. 63; pages 45-48; page 46, first column, third paragraph; DOI:10.1038/ja.2009.115	16
Y	(PHETSANG, W et al.) An Azido-oxazolidinone Antibiotic for Live Bacterial Cell Imaging and Generation of Antibiotic Variants. Bioorganic & Medicinal Chemistry. 15 August 2014, Vol. 22, No. 16; pages 4490–4498; abstract; page 4492, second column, first paragraph; DOI: 10.1016/j.bmc.2014.05.054	19

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 September 2016 (19.09.2016)	17 OCT 2016

Name and mailing address of the ISA/	Authorized officer
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774