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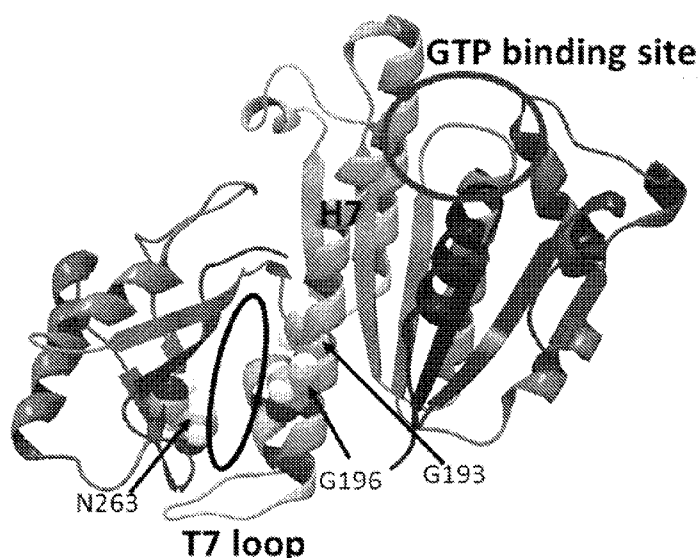
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[Continued on next page]

(54) Title: PYRIMIDINES FOR TREATMENT OF BACTERIAL INFECTIONS

Figure 1



(57) Abstract: Filamenting temperature-sensitive mutant Z (FtsZ) protein plays a crucial role in the bacterial cell division machinery and is a validated drug target for antibacterial agents. The present invention relates to FtsZ-interacting compounds that possess a 2,4,6-trisubstituted pyrimidine scaffold. Some of these compounds possess potent anti-staphylococcal properties and potent antibacterial activities against clinically isolated MRSA strains. Compounds have been identified to exhibit low spontaneous frequency of resistance, low toxicity as well as the ability to rescue *G. mellonella* larvae infected with lethal dose of the MRSA ATCC 43300 strain. Characterization by saturation transfer difference NMR, light scattering assay and GTPase hydrolysis assay with purified *S. aureus* FtsZ protein verified the interaction of 2,4,6-trisubstituted pyrimidine with the FtsZ protein, further confirmed by observations of iconic filamentous cell phenotype and mislocalization of the Z-ring formation. Taken together, these pyrimidine derivatives have the potential as effective treatment of staphylococcal infections.



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PYRIMIDINES FOR TREATMENT OF BACTERIAL INFECTIONS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application No. 62/319,894, filed April 8, 2016. The entire contents and disclosures of the preceding application are incorporated by reference into this application.

[0002] Throughout this application, various publications are cited. The disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

FIELD OF THE INVENTION

[0001] This invention relates to the synthesis of a novel series of amine linked 2,4,6-trisubstituted pyrimidines, which exhibited potent anti-staphylococcal activities by suppressing self-polymerization of cell division protein FtsZ.

BACKGROUND OF THE INVENTION

[0002] The inexorable rise in the incidence of serious bacterial infections caused by multiple antibiotic-resistant bacteria in healthcare and community associated settings has become a pressing threat of public health worldwide..¹ Of particular concern is the rise in incidence of methicillin resistant *Staphylococcus aureus* (MRSA) infections. MRSA is a human pathogen that can cause a wide range of illnesses, from mild skin and wound infections to pneumonia and bloodstream infections that cause sepsis and death. Centers for Disease Control and Prevention of United State estimates that over eighty thousand severe MRSA infections occur annually, resulting in eleven thousand deaths..² This scenario has driven the search for novel classes of anti-staphylococcal agents which act on novel bacterial drug targets.

[0003] The bacterial cell division machinery has been considered as an important field for exploring potential novel drug targets of antibacterial agents..³ The filamenting temperature-sensitive mutant Z (FtsZ) protein undoubtedly represents one of the well-characterized and exploitable antibacterial drug targets..⁴ FtsZ is a cytoplasmic protein and highly conserved tubulin-like guanosine triphosphatase (GTPase), playing an important role in bacterial cell division. In order for bacteria to carry out cell division, FtsZ monomers are required to localize mid-cell through the precise positioning of cell division site positioning protein and self-polymerize into single stranded straight protofilaments by means of head-to-tail association that curve upon hydrolysis of guanosine triphosphate (GTP) molecules..⁵ Consecutive lateral contacts between FtsZ protofilaments produce FtsZ bundles, which eventually lead to formation of a contractile ring called Z-ring at the mid-cell. Following subsequent involvement of other downstream cell division proteins, Z-ring contraction and depolymerisation completes the cell division process to furnish identical daughter cells. Small molecules interfering initial stage of FtsZ polymerization are capable of blocking bacterial cell division, causing abrogation

of bacterial cell viability eventually. These types of compounds have great potential to be developed as efficacious antimicrobial agents with a novel mode of action for clinical application. Several high resolution X-ray crystal structures of FtsZ homologs have been reported.⁶ These results contributed to the knowledge regarding the general organization of FtsZ protein structure, which is known to comprise two independent folding domains (Figure 1). The *N*-terminal domain forms a nucleotide binding site (GTP binding site, upper red circle of Figure 1), while the *C*-terminal domain contains a flexible loop (T7 loop). Both domains were interconnected via a long central helix 7 (H7) of high rigidity.

[0004] Many FtsZ-interacting compounds have been discovered and reported to bind either the GTP binding site or a cleft formed by the H7 helix, T7 loop and *C*-terminal β sheet (Figure 1, lower circle). Some exhibit potent antibacterial activity with minimum inhibitory concentration (MIC) at micromolar range. PC190723 (Figure 2)⁷ and its pro-drugs **1a**⁸ and **1b**⁹, and benzamide **2**¹⁰ have been demonstrated to exhibit *in vitro* and *in vivo* efficacy in a murine infection model. Moreover, X-ray crystallographic analysis revealed that PC190723 binds to a narrow cleft formed by the H7 helix, T7 loop and *C*-terminal β sheet (Figure 1, lower circle).¹¹ However, analysis of PC190723 drug resistant mutants across various MRSA strains revealed that all PC190723 drug resistant isolates had multiple mutations, resulting in amino acid substitutions that mapped to the FtsZ protein.¹² These mutations mainly occurred at amino acid positions 193, 196 and 263 (Figure 1), which accounted for over 90% of PC190723 drug resistant mutants. These results suggested that amino acid substitutions can alter slightly the overall shape of the binding pocket without interfering normal function of FtsZ. Nevertheless, this change resulted in PC190723 no longer binding to this pocket, therefore causing drug resistance. Such findings may hinder the potential of PC190723 and other related compounds¹³ from being developed into agents that exhibit the potential to target the same binding pocket for further clinical development. On the other hand, several compounds targeting the GTP binding site of FtsZ have also been demonstrated to exhibit potent antibacterial activity, including natural product chrysopaentin A **3**,¹⁴ C8-substituted GTP analog **4**¹⁵, berberine analog **5**¹⁶ and naphthol derivative **6**¹⁷ (Figure 2). Surprisingly, among these inhibitors, no drug resistant mutants have been reported in the literature so far presumably due to the fact that the GTP binding site is very important for recognizing the GTP molecule. Amino acid substitutions at this binding pocket may cause improper recognition of GTP molecule and thus hinder normal GTP hydrolysis process; therefore losing energy source to drive polymerization of FtsZ monomers.

[0005] Chan *et al.* identified a new class of FtsZ inhibitors exemplified by structure **7** bearing a 2,4,6-trisubstituted pyrimidine-and a chiral aminoquinuclidine moiety (Figure 2).¹⁸ Molecular docking study of compound **7** and the GTP molecule using *S. aureus* FtsZ revealed that the 2,4,6-trisubstituted pyrimidine moiety of **7** occupied exactly the same binding pocket as the guanosine moiety of GTP molecule through an extensive network of hydrogen bonds with FtsZ protein, suggesting that the pyrimidine moiety is crucial for binding.¹⁸ This class of

compounds has been demonstrated to inhibit GTPase hydrolysis activity of *S. aureus* FtsZ at low micromolar IC₅₀ value with moderate antimicrobial activity against *S. aureus* and *E. coli*. Interestingly, compound **7a** also exhibited strong synergistic effect against various MRSA and vancomycin-resistant *Enterococcus faecium* (VREF) strains when combined with clinically used β -lactam antibiotics.¹⁹ However, structural complexity of the chiral quinuclidine scaffold and limited availability of compound have prevented further development. Accordingly, specially designed small molecules able to mimic and compete with GTP molecules to bind the GTP binding site of FtsZ may have greater potential to be developed as antimicrobial agents without acquiring drug resistance.

SUMMARY OF THE INVENTION

[0006] The present invention discloses an efficient synthesis of novel amine-linked 2,4,6-trisubstituted pyrimidine compounds with the aims of replacing the complex quinuclidine scaffold of **7** with simple amines and improving the antimicrobial activity and selectivity. Compounds exhibiting potent antimicrobial activity and low toxicity can be further investigated regarding its interaction with FtsZ protein. Because of their structural novelty, potent antimicrobial activity and high selectivity against *S. aureus*, a new series of such compounds may represent a promising therapy.

[0007] The present invention relates to a novel class of pyrimidine. A composition comprising the novel pyrimidine or its pharmaceutically acceptable salt, and a pharmaceutically acceptable carrier can be used to treat bacterial infection in a subject by administering to the subject an effective amount of the composition.

DEFINITIONS & ABBREVIATIONS

[0008] The following terms are used to describe the present invention. In the absence of a specific definition set forth herein, the terms used to describe the present invention shall be given their common meaning as understood by those of ordinary skill in the art.

[0009] As used herein, the expression "FtsZ" refers to Filamenting temperature-sensitive mutant Z.

[0010] As used herein, the term "GTP" refers to guanosine triphosphate.

[0011] As used herein, the term "GTPase" refers to guanosine triphosphatase.

[0012] As used herein, the term "MIC" refers to minimum inhibition concentration.

[0013] As used herein, the term "MRSA" refers to methicillin resistant *Staphylococcus aureus*.

[0014] As used herein, the term "VREF" refers to vancomycin-resistant *Enterococcus faecium*.

[0015] As used herein, the term "ACN" refers to acetonitrile.

[0016] As used herein, the term "Boc" refers to tert-butyloxycarbonyl.

[0017] As used herein, the term "ACN" refers to acetonitrile.

- [0018] As used herein, the term “CBr₄” refers to carbon tetrabromide.
- [0019] As used herein, the term “CuI” refers to copper (I) iodide.
- [0020] As used herein, the term “DCM” refers to dichloromethane.
- [0021] As used herein, the term “DMSO” refers to dimethyl sulfoxide.
- 5 [0022] As used herein, the term “EA” refers to ethyl acetate.
- [0023] As used herein, the term “EDTA” refers to ethylenediaminetetraacetic acid.
- [0024] As used herein, the term “HCl” refers to hydrochloric acid.
- [0025] As used herein, the term “Hex” refers to hexane.
- [0026] As used herein, the term “MeOH” refers to methanol.
- 10 [0027] As used herein, the term “MgSO₄” refers to magnesium sulphate.
- [0028] As used herein, the term “Na₂CO₃” refers to sodium carbonate.
- [0029] As used herein, the term “NaCl” refers to sodium chloride.
- [0030] As used herein, the term “NaOH” refers to sodium hydroxide.
- [0031] As used herein, the term “NEt₃” refers to triethylamine.
- 15 [0032] As used herein, the term “NH₄HCO₃” refers to ammonium bicarbonate.
- [0033] As used herein, the term “Pd(PPh₃)₂Cl₂” refers to bis(triphenylphosphine)palladium(II) dichloride.
- [0034] As used herein, the term “PMSF” refers to phenylmethylsulfonyl fluoride.
- [0035] As used herein, the term “PPh₃” refers to triphenylphosphine.
- 20 [0036] As used herein, the term “TFA” refers to trifluoroacetic acid.
- [0037] As used herein, the term “THF” refers to tetrahydrofuran.
- [0038] As used herein, the term “Tris-HCl” refers to tris(hydroxymethyl)aminomethane hydrochloride.
- [0039] As used herein, the term “NMR” refers to nuclear magnetic resonance.
- 25 [0040] As used herein, the term “CDCl₃” refers to deuterated chloroform.
- [0041] As used herein, the term “ESI” refers to electron spray ionization.
- [0042] As used herein, the term “TLC” refers to thin-layer chromatography.
- [0043] As used herein, the term “aryl” refers to a phenyl or naphthyl group optionally substituted with an appropriate substituent.
- 30 [0044] As used herein, the term “heteroaryl” refers to a six-membered aromatic ring or two fused 6-membered aromatic rings containing at least one nitrogen (N) as the heteroatom, and optionally substituted with an appropriate substituent.
- [0045] As used herein, the term “optionally substituted phenyl” means that the phenyl group is unsubstituted or substituted with, but not limited to, halogen (F, Cl, Br), methyl, trifluoromethyl, methoxy, pyridyl, butyl, ethyl and propyl.
- 35 [0046] As used herein, the term “optionally substituted benzyl” means that the aromatic ring of the benzyl group is unsubstituted or substituted with, but not limited to, halogen (F, Cl, Br), methyl, trifluoromethyl, methoxy, pyridyl, butyl, ethyl and propyl.
- [0047] As used herein, the term “optionally substituted benzoyl” means that the aromatic

ring of the benzoyl group is unsubstituted or substituted with, but not limited to, halogen (F, Cl, Br), methyl, trifluoromethyl, methoxy, pyridyl, butyl, ethyl and propyl.

[0048] As used herein, the term “optionally substituted phenethyl” means that the aromatic ring of the phenethyl group is unsubstituted or substituted with, but not limited to, halogen (F, Cl, Br), methyl, trifluoromethyl, methoxy, pyridyl, butyl, ethyl and propyl.

[0049] As used herein, the term “optionally substituted cinnamoyl” means that the aromatic ring of the cinnamoyl group is unsubstituted or substituted with, but not limited to, halogen (F, Cl, Br), methyl, trifluoromethyl, methoxy, pyridyl, butyl, ethyl and propyl.

BRIEF DESCRIPTION OF THE DRAWINGS

10 [0050] Figure 1 shows a crystal structure of *S. aureus* FtsZ (PDB ID: 4DXD) with labeled GTP binding site, T7 loop, H7 helix and amino acids at positions 193, 196 and 263.

[0051] Figure 2 shows the chemical structures of FtsZ inhibitors **1-7**.

[0052] Figure 3 shows the synthesis of 2,4,6-trisubstituted pyrimidine **14**. Step (a): (i) 2 mol% Pd(PPh₃)₂Cl₂, 4 mol% CuI, NEt₃, THF, r.t. 1-2 h; (ii) Na₂CO₃, reflux 14 h; Step (b): conc. HCl, MeOH, r.t. 2 h; Step (c): PPh₃, CBr₄, THF, r.t. 3-4 h; Step (d): **amine01-39** (please refer to Table 2 for chemical structures), ACN, r.t. 24 h.

[0053] Figure 4 shows the synthesis of pyrimidines with a sulfonamide group. Step (a): 1-Boc-homopiperazine, ACN, rt., 14 h; Step (b): TFA, DCM, 0 °C, 2 h; Step (c): various sulfonyl chloride, NEt₃, DCM, 0 °C, 3 h.

20 [0054] Figure 5 shows a summary of SAR study. Black arrow indicates unfavorable substituents and grey arrow indicates favorable substituents.

[0055] Figure 6 shows a 1D (STD-off) spectrum of compound **14av_amine16** binding to the *S. aureus* FtsZ protein, with signal assignment (upper panel); and a corresponding Saturation Transfer Difference (STD) spectrum (lower panel) showing the STD effect (I_{STD}/I₀) for each proton.

[0056] Figure 7 shows the effect of compound **14av_amine16** on polymerization of the *S. aureus* FtsZ protein at various concentrations.

[0057] Figure 8A is ¹H NMR of **14dv_amine16**

[0058] Figure 8B is ¹³C NMR of **14dv_amine16**.

30 [0059] Figure 9A is ¹H NMR of **14dv_amine06**.

[0060] Figure 9B is ¹³C NMR of **14dv_amine06**.

[0061] Figure 10A is ¹H NMR of **14dv_amine20**.

[0062] Figure 10B is ¹³C NMR of **14dv_amine20**.

[0063] Figure 11A is ¹H NMR of **14dv_amine07**.

35 [0064] Figure 11B is ¹³C NMR of **14dv_amine07**.

[0065] Figure 12A is ¹H NMR of **14dv_amine08**.

[0066] Figure 12B is ¹³C NMR of **14dv_amine08**.

[0067] Figure 13A is ¹H NMR of **14av_amine16**.

[0068] Figure 13B is ¹³C NMR of **14av_amine16**.

- [0069] Figure 14A is ^1H NMR of **14av_amine20**.
 [0070] Figure 14B is ^{13}C NMR of **14av_amine20**.
 [0071] Figure 15A is ^1H NMR of **14az_amine40**.
 [0072] Figure 15B is ^{13}C NMR of **14az_amine40**.
 5 [0073] Figure 16A is ^1H NMR of **14az_amine41**.
 [0074] Figure 16B is ^{13}C NMR of **14az_amine41**.
 [0075] Figure 17A is ^1H NMR of **14az_amine42**.
 [0076] Figure 17B is ^{13}C NMR of **14az_amine42**.
 [0077] Figure 18A shows the frequency of resistance (FOR) study of bacterial growth in
 10 agar plates after treatment with DMSO (left) and **14av_amine16** (right).
 [0078] Figure 18B shows the Kaplan-Meier survival analysis of *G. mellonella* larvae
 following injection of various concentrations of compound **14av_amine16** without lethal dose
 of MRSA ATCC 43300 inoculation. Figure 18C Kaplan-Meier survival analysis of *G.*
mellonella larvae following injection of various concentrations of compound **14av_amine16**
 15 with lethal dose of MRSA ATCC 43300 inoculation. Larvae were considered dead if they did
 not respond to physical stimuli. Data presented are the mean of three independent assays.
 [0079] Figures 19A – 19F are the microscopic observation of a rod-shaped *B. subtilis* 168
 cell morphology after treatment with DMSO or compound **14av_amine16**. Figure 19A
 shows the effect of DMSO treatment on the cell morphology of *B. subtilis* 168. Figure 19B
 20 shows the effect of compound **14av_amine16** treatment on the cell morphology of *B. subtilis*
 168. Figure 19C shows the DMSO treated *B. subtilis* 168 with a functional green fluorescent
 protein tagged FtsZ. Figure 19D shows the corresponding phase-contrast microscopic pictures
 of Figure 19C. Figure 19E shows compound **14av_amine16** treated *B. subtilis* 168 with a
 functional green fluorescent protein tagged FtsZ. Figure 19F shows the corresponding phase-
 25 contrast microscopic pictures of Figure 19E.

DETAILED DESCRIPTION OF THE INVENTION

- [0080] The present invention discloses an efficient synthesis of a novel amine-linked 2,4,6-
 trisubstituted pyrimidine compound library with the aims of replacing the complex
 quinuclidine scaffold of **7** and improving the antimicrobial activity and selectivity.
 30 Compound **7** was divided into two parts (pyrimidine head and amine tail) as outlined in Figure
 2. Based on this approach, different pyrimidine heads and amine tails were used to assemble
 compound **14** of the present invention. Some examples of **14** were illustrated by Tables 1 and
 2, but were not meant to limit the scope of the invention. The amine tails include
 homopiperazine (**amine01-23**), piperazine (**amine24-34**) and linear amines (**amine 35-39**).

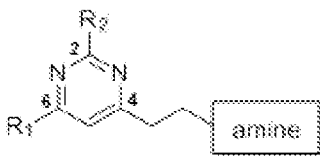
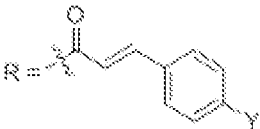
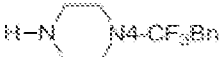
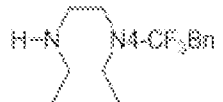
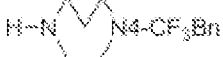
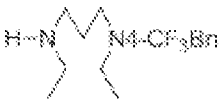
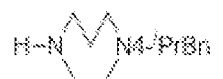


Table 1 Examples of 2,4,6-trisubstituted pyrimidine **14**

Entry	2,4,6-trisubstituted pyrimidine	Entry	2,4,6-trisubstituted pyrimidine	Entry	2,4,6-trisubstituted pyrimidine
1	14av_amine01	34	14av_amine34	67	14bv_amine08
2	14av_amine02	35	14av_amine35	68	14bv_amine10
3	14av_amine03	36	14av_amine36	69	14bv_amine11
4	14av_amine04	37	14av_amine37	70	14bv_amine12
5	14av_amine05	38	14av_amine38	71	14bv_amine13
6	14av_amine06	39	14av_amine39	72	14bv_amine14
7	14av_amine07	40	14aw_amine06	73	14bv_amine15
8	14av_amine08	41	14aw_amine07	74	14bv_amine16
9	14av_amine09	42	14aw_amine16	75	14bv_amine17
10	14av_amine10	43	14ax_amine06	76	14bv_amine18
11	14av_amine11	44	14ax_amine07	77	14cv_amine02
12	14av_amine12	45	14ax_amine16	78	14cv_amine06
13	14av_amine13	46	14ay_amine04	79	14cv_amine09
14	14av_amine14	47	14ay_amine05	80	14cv_amine16
15	14av_amine15	48	14ay_amine08	81	14dv_amine02
16	14av_amine16	49	14ay_amine09	82	14dv_amine06
17	14av_amine17	50	14ay_amine12	83	14dv_amine07
18	14av_amine18	51	14ay_amine13	84	14dv_amine08
19	14av_amine19	52	14ay_amine16	85	14dv_amine09
20	14av_amine20	53	14ay_amine17	86	14dv_amine10
21	14av_amine21	54	14ay_amine18	87	14dv_amine12
22	14av_amine22	55	14ay_amine20	88	14dv_amine16
23	14av_amine23	56	14az_amine07	89	14dv_amine20
24	14av_amine24	57	14az_amine08	90	14ay_amine40
25	14av_amine25	58	14az_amine09	91	14az_amine40
26	14av_amine26	59	14az_amine12	92	14ay_amine41
27	14av_amine27	60	14az_amine16	93	14az_amine41
28	14av_amine28	61	14az_amine17	94	14az_amine42
29	14av_amine29	62	14az_amine18	95	14az_amine43
30	14av_amine30	63	14az_amine20	96	14az_amine44
31	14av_amine31	64	14bv_amine02	97	14az_amine45
32	14av_amine32	65	14bv_amine04	98	14az_amine46
33	14av_amine33	66	14bv_amine05	99	14az_amine47

Table 2 Examples of amine tails

Cyclic amine tails containing 7-member homopiperazine ring	Cyclic amine tails containing 6-member homopiperazine ring
 amine01 R = Me amine02 R = Bn amine03 R = CH ₂ 4-Py amine04 R = 4-MeBn amine05 R = 4-MeOBn amine06 R = 4-iPrBn amine07 R = 4-tBuBn amine08 R = 4-BrBn amine09 R = 4-FBn amine10 R = 3-FBn amine11 R = 2-FBn amine12 R = 3,4-diFBn amine13 R = 4-ClBn amine14 R = 3-ClBn amine15 R = 2-ClBn amine16 R = 4-CF ₃ Bn amine17 R = 3-CF ₃ Bn amine18 R = 2-CF ₃ Bn amine19 R = C(=O)4-CF ₃ C ₆ H ₄ amine20 R = (CH ₂) ₂ 4-CF ₃ C ₆ H ₄  amine21 Y = Cl amine22 Y = H amine23 Y = OMe	amine24 R = 4-CF ₃ Bn amine25 R = 4-ClBn amine26 R = Bz amine27 R = C(=O)4-CF ₃ C ₆ H ₄ amine28 R = 3-CF ₃ Bn amine29 R = 4-FBn amine30 R = piperonyl amine31 R = 4-MeOBn amine32 R = 4-MeBn amine33 R = 4-CF ₃ C ₆ H ₄ amine34 R =  Linear amine tails amine35  amine36  amine37  amine38  amine39 

[0081] A general synthetic route to prepare target compound **14** is outlined in Figure 3. In step (a), the pyrimidine head (such as **11av**, **11aw**, **11ax**, **11ay**, **11az**, **11bv**, **11cv**, **11dv**) was obtained by coupling acid chloride **8** with protected terminal alkyne **9** in the presence of an amine under Sonogashira conditions followed by addition of excess amidinium salt (such as **10v-z**) in one-pot, under mild conditions and in good yields.¹⁹ R¹ of acid chloride **8** is selected from the group consisting of alkyl having 1-10 carbon atoms, aryl having one or two 6-

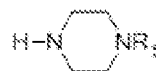
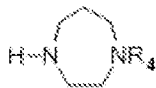
membered aromatic rings, or heteroaryl having one or two 6-membered aromatic rings with at least one nitrogen (N) as the heteroatom. The amidinium salt is not limited to those illustrated. One of ordinary skill in the art would readily appreciate that other amidinium salts would also be suitable for this reaction. For example, R² may be selected from an alkyl having 1-10 carbon atoms, an aryl having one or two 6-membered aromatic rings, or a heteroaryl having one or two 6-membered aromatic rings with at least one nitrogen (N) as the heteroatom in addition to those listed in Figure 3. The amidinium can be a hydrochloride or hydrobromide salt. The amine is selected from the group consisting of trimethylamine, *N,N*-diisopropylethylamine, trimethylamine, and *N,N*-dimethylbenzylamine.

5
10 [0082] In step (b) of Figure 3, deprotection of the silyl group affords an alcohol (such as **12av**, **12aw**, **12ax**, **12ay**, **12az**, **12bv**, **12cv**, **12dv**), typically in quantitative yield. In one embodiment, the reagent used for deprotection of the silyl group is selected from the group consisting of concentrated hydrochloride acid in methanol, tetra-*n*-butylammonium fluoride (TBAF), tetra-*n*-butylammonium fluoride (TBAF) with acetic acid (AcOH), and potassium fluoride (KF) in tetraethylene glycol.

15
20 [0083] In step (c) of Figure 3, the alcohol is converted into a bromide (such as **13av**, **13aw**, **13ax**, **13ay**, **13az**, **13bv**, **13cv**, **13dv**) in high yield. In one embodiment, the bromination reagent is selected from the group consisting of triphenylphosphine with carbon tetrabromide, and phosphorous tribromide with trimethylamine or pyridine. In another embodiment, the bromide is synthesized at a temperature below 50 °C. In an embodiment, the bromide is freshly prepared and used immediately for the next step.

25
30 [0084] In step (d) of Figure 3, amine-linked 2,4,6-trisubstituted pyrimidine **14** is constructed by mixing the bromide obtained in step (c) with an amine in an organic solvent. In one embodiment, the bromide is freshly prepared. In some embodiments, the amine is selected from the group consisting of homopiperazine ring **15** and R₄ is selected from alkyl having 1-10 carbon atoms, optionally substituted benzyl, optionally substituted phenyl, pyridylmethyl, optionally substituted benzoyl, optionally substituted phenethyl, piperonyl, 3-phenyl-2-propen-1-yl, optionally substituted cinnamoyl, alkyl sulphonyl, aryl sulphonyl and heteroaryl sulphonyl. In another embodiment, the amine is selected from the group consisting of 6-member homopiperazine ring **16** and R₅ is selected from alkyl having 1-10 carbon atoms, optionally substituted benzyl, optionally substituted phenyl, pyridylmethyl, optionally substituted benzoyl, optionally substituted phenethyl, piperonyl, 3-phenyl-2-propen-1-yl, optionally substituted cinnamoyl, alkyl sulphonyl, aryl sulphonyl and heteroaryl sulphonyl. In one embodiment, the amine is selected from the group consisting of H-N(R₆)-[CH₂]_m-N(R₇)(R₈), m is an integer selected from 2, 3, 4 and 5, and each of said R₆, R₇ and R₈ is independently selected from alkyl having 1-5 carbon atoms, and optionally substituted benzyl. In another embodiment, the amine is selected from the group consisting of amines listed in Table 2. One of ordinary skill in the art would readily appreciate that other amines would also undergo this reaction to give additional target compounds. In an embodiment, the organic

solvent is selected from the group consisting of acetonitrile (ACN), tetrahydrofuran (THF) and diethyl ether. In another embodiment, amine-linked 2,4,6-trisubstituted pyrimidine **14** is synthesized at room temperature.



5 7-member homopiperazine ring **15**

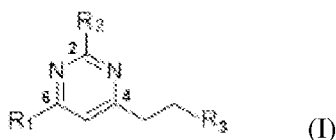
6-member homopiperazine ring **16**

[0085] Pyrimidine **14** with a sulfonamide group is synthesized as depicted in **Figure 4**. In step (a), treatment of a bromide (such as **13ay**, **13az**) with an amine affords a protected pyrimidine amine (such as **14ay_amine40**, **14az_amine 40**). In one embodiment, the amine is selected from the group consisting of 7-member homopiperazine ring **15** and 6-member
10 homopiperazine ring **16**, wherein R₄ and R₅ are Boc protecting group.

[0086] In step (b) of Figure 4, the protected pyrimidine amine is deprotected to furnish a pyrimidine amine (such as **14ay_amine41**, **14az_amine41**). In one embodiment, the Boc-protected pyrimidine amine is acidified with trifluoroacetic acid (TFA) in dichloromethane (DCM).

15 [0087] In step (c) of Figure 4, pyrimidine **14** with a sulfonamide group is synthesized by treating the product from step (b) with a sulfonyl chloride. In addition to those listed, the sulfonyl chloride is selected from the group consisting of methanesulfonyl chloride, *p*-toluenesulfonyl chloride, 4-fluorobenzenesulfonyl chloride, 1-naphthalenesulfonyl chloride, 8-quinolinesulfonyl chloride and 2-naphthalenesulfonyl chloride.

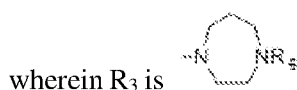
20 [0088] The present invention relates to a novel class of pyrimidine having a general structure (I) below:

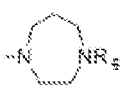


wherein R₁ and R₂ each independently represents alkyl having 1-10 carbon atoms, aryl having one or two 6-membered aromatic rings, or heteroaryl having one or two 6-membered aromatic
25 rings with at least one nitrogen (N) as the heteroatom; and R₃ represents a tertiary amine – NR₄R₅;

wherein R₁ is selected from the group consisting of alkyl having 1-8 carbon atoms, phenyl and substituted phenyl;

wherein R₂ is selected from the group consisting of alkyl having 1-8 carbon atoms, phenyl,
30 substituted phenyl, pyridin-2-yl, pyridin-3-yl and pyridin-4-yl;



wherein R₃ is  and R₆ is selected from alkyl having 1-10 carbon atoms, optionally substituted benzyl, optionally substituted phenyl, pyridylmethyl, optionally substituted

benzoyl, optionally substituted phenethyl, piperonyl, 3-phenyl-2-propen-1-yl, optionally substituted cinnamoyl, alkyl sulphonyl, aryl sulphonyl and heteroaryl sulphonyl;



substituted benzyl, optionally substituted phenyl, pyridylmethyl, optionally substituted benzoyl, optionally substituted phenethyl, piperonyl, 3-phenyl-2-propen-1-yl, optionally substituted cinnamoyl, alkyl sulphonyl, aryl sulphonyl and heteroaryl sulphonyl;

wherein R_3 is $-N(R_8)-[CH_2]_m-N(R_9)(R_{10})$, m is an integer selected from 2, 3, 4 and 5, and each of said R_8 , R_9 and R_{10} is independently selected from alkyl having 1-5 carbon atoms, and optionally substituted benzyl.

[0089] In certain embodiments, the pyrimidine (I) is selected from the group consisting of: 1-(2-(6-phenyl-2-(pyridin-4-yl)pyrimidin-4-yl)ethyl)-4-(4-(trifluoromethyl)benzyl)-1,4-diazepane;

1-(4-isopropylbenzyl)-4-(2-(6-phenyl-2-(pyridin-4-yl)pyrimidin-4-yl)ethyl)-1,4-diazepane;

1-(2-(6-phenyl-2-(pyridin-4-yl)pyrimidin-4-yl)ethyl)-4-(4-(trifluoromethyl)phenethyl)-1,4-diazepane;

1-(4-(*tert*-butyl)benzyl)-4-(2-(6-phenyl-2-(pyridin-4-yl)pyrimidin-4-yl)ethyl)-1,4-diazepane;

1-(4-bromobenzyl)-4-(2-(6-phenyl-2-(pyridin-4-yl)pyrimidin-4-yl)ethyl)-1,4-diazepane;

1-(2-(6-(pentan-3-yl)-2-(pyridin-4-yl)pyrimidin-4-yl)ethyl)-4-(4-(trifluoromethyl)benzyl)-1,4-diazepane; and

1-(2-(6-(pentan-3-yl)-2-(pyridin-4-yl)pyrimidin-4-yl)ethyl)-4-(4-(trifluoromethyl)phenethyl)-1,4-diazepane.

[0090] In one embodiment, a composition comprises the novel pyrimidine (I) or its pharmaceutically acceptable salt, and a pharmaceutically acceptable carrier can be used to treat bacterial infection in a subject by administering to the subject an effective amount of the composition. In another embodiment, the infection is caused by bacteria selected from the group consisting of *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecalis*, *Bacillus subtilis*, *Propionibacterium acnes*, and *Clostridium difficile*. In some embodiments, the infection is caused by Gram-negative bacteria, Gram-positive bacteria or multiple drug-resistant bacteria. In certain embodiments, the infection is a skin infection, a gastrointestinal infection, a respiratory infection, a urinary tract infection, or a reproductive tract infection. In one embodiment, the pyrimidine (I) is used to inhibit bacterial cell division. In another embodiment, the pyrimidine (I) inhibits Z-ring formation by filamenting temperature-sensitive mutant Z (FtsZ) protein within a bacterial cell. In one embodiment, the pyrimidine (I) binds to the guanosine triphosphate (GTP) binding site of FtsZ within a bacterial cell. In another embodiment, the composition is administered in combination with other compounds to achieve synergistic treatment of the bacterial infection. In some embodiments, the subject is a vertebrate, a mammal or human. In certain embodiments, the composition is administered orally, nasally, aurally, ocularly, sublingually, buccally,

systemically, transdermally, mucosally, via cerebral spinal fluid injection, vein injection, muscle injection, peritoneal injection, subcutaneous injection, nasal or mucosal administration, or by inhalation. In a further embodiment, the composition is formulated in the form of cream, gel, ointment, suppository, tablet, granule, injection, powder, solution, suspension, spray, patch or capsule.

[0091] Minimum inhibition concentration (MIC) evaluation of the 2,4,6-trisubstituted pyrimidine **14** derivatives against *S. aureus* and *E. coli* bacterial strains identifies potent and selective anti-staphylococcal activity. Antibacterial activities of the 2,4,6-trisubstituted pyrimidine **14** derivatives are studied against clinically isolated MRSA strains. The potential for development of resistance against the compounds of the present invention is investigated, which is then followed by the evaluation of in vivo toxicity and efficacy. Binding between the compounds of the present invention and *S. aureus* FtsZ protein can be characterized by saturation transfer difference NMR. The effect of suppression on FtsZ self-polymerization can be verified by light scattering assay with purified *S. aureus* FtsZ protein. The mode of action of the antibacterial activity can be investigated by microscopic observation of a rod-shaped *B. subtilis* 168 cell morphology. Taken together, these pyrimidine derivatives represent a novel scaffold for further optimization and may have potential to be developed into a therapy for treating staphylococcal infection in the future.

[0092] The invention will be better understood by reference to the Experimental Details which follow, but those skilled in the art will readily appreciate that the specific experiments are provided only for illustrative purpose, and are not meant to limit the invention scope as described herein, which is defined by the claims following thereafter.

[0093] Throughout this application, various references or publications are cited. Disclosures of these references or publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains. It is to be noted that the transitional term “comprising”, which is synonymous with “including”, “containing” or “characterized by”, is inclusive or open-ended, and does not exclude additional, un-recited elements or method steps.

EXAMPLE 1

General procedure for Synthesis of Pyrimidine 14

(1) Characterization of compounds

[0094] All NMR spectra were recorded on a Bruker Advance-III spectrometer at 400.13 MHz for ^1H and 100.62 MHz for ^{13}C . All NMR measurements were carried out at room temperature, and the chemical shifts are reported as parts per million (ppm) in unit relative to the resonance of CDCl_3 (7.26 ppm in the ^1H , 77.0 ppm for the central line of the triplet in the ^{13}C modes, respectively). Low-resolution and high-resolution mass spectra were obtained on a Micromass Q-TOF-2 by electron spray ionization (ESI) mode. All reagents and solvents were reagent grade and were used without further purification unless otherwise stated. The plates used for thin-layer chromatography (TLC) were E. Merck Silica Gel 60F₂₅₄ (0.25 mm

thickness), and they were visualized under short UV light (254 nm). Chromatographic purifications were carried out using MN silica gel 60 (230–400 mesh). Compound purity was determined by an Agilent 1100 series HPLC installed with a Prep-Sil Scalar column (4.6 mm × 250 mm, 5 µm) at UV detection of 254 nm (reference at 450 nm). All tested compounds were shown to have >95% purity according to HPLC. **Amine20** was prepared from homopiperazine and 1-(2-bromoethyl)-4-trifluoromethylbenzene. **Amine38** was prepared from *N,N*-diethyl-1,3-diaminopropane and 4-(trifluoromethyl)benzyl bromide. Other amines used in this study are commercially available.

(2) General procedure for synthesis of pyrimidine **11**

[0095] Step (a) of Figure 3: To a well-stirred solution of Pd(PPh₃)₂Cl₂ (0.4 mmol) and CuI (0.8 mmol) in degassed THF (150 mL) under nitrogen atmosphere, was added NEt₃ (13 mmol), acid chloride **8** (10 mmol) and alkyne **9** (10 mmol) successively. The reaction mixture was stirred for 3 h until complete conversion as monitored by TLC. Then, Na₂CO₃ (34 mmol) and amidinium hydrochloride salt **10** (12 mmol) were added to the reaction mixture. The resulting suspension was heated at reflux for 14 h. After cooling to room temperature, the reaction mixture was filtered through a short pad of silica gel to remove excess Na₂CO₃. The obtained pale brown filtrate was evaporated under reduced pressure to give a crude oil which was subjected to flash column chromatography on silica gel with gradient elution (5% EA in Hex to 30% EA in Hex) to furnish the desired product **11**.

(3) General procedure for synthesis of alcohol **12**

[0096] Step (b) of Figure 3: To a well-stirred solution of pyrimidine **11** (5 mmol) in methanol (20 mL) at room temperature, was added excess conc. hydrochloric acid (5 mL) dropwise. The reaction mixture was stirred for 2 h until complete conversion as monitored by TLC. Then, a 2 M NaOH solution was added to neutralize the reaction mixture (pH 7) followed by extraction with DCM. The combined organic layers were dried over MgSO₄, filtered and evaporated to afford the desired product **12**, which is pure enough for next step.

(4) General procedure for synthesis of bromide **13**

[0097] Step (c) of Figure 3: To a well-stirred solution of alcohol **12** (3 mmol) and PPh₃ (3.6 mmol) in THF (50 mL) at room temperature, was added carbon tetrabromide (3.6 mmol) once. The reaction mixture was stirred for 3 h until the complete conversion as monitored by TLC. The reaction mixture was filtered through a short pad of silica gel. The obtained pale brown filtrate was evaporated under reduced pressure to give a crude oil which was subjected to flash column chromatography on silica gel with gradient elution (5% EA in Hex to 20% EA in Hex) to furnish the desired product **13**.

(5) General procedure for synthesis of pyrimidine **14**

[0098] Step (d) of Figure 3: To a well-stirred solution of slightly excess amine (1.1 mmol) in ACN (10 mL) at room temperature, was added freshly prepared bromide **13** (1 mmol) solution in ACN (10 mL) once. The reaction mixture was stirred for 14 h until the complete conversion as monitored by TLC. The reaction mixture was evaporated under reduced pressure

to give a crude oil which was subjected to flash column chromatography on silica gel with gradient elution (1% MeOH in DCM to 5% MeOH in DCM) to furnish the desired product **14**.

[0099] Based on this approach, eight pyrimidine heads (Figure 3) and thirty-nine secondary amine tails (Table 2) were designed and synthesized. As shown in Table 2, cyclic amine tails (for 7-membered homopiperazine ring, **amines 01-23**, and for 6-membered piperazine ring, **amines 24-34**) and linear amine tails (**amines 35-39**) were compared.

EXAMPLE 2

Synthesis of 14dv_amine16

[0100] 1-(2-(6-phenyl-2-(pyridin-4-yl)pyrimidin-4-yl)ethyl)-4-(4-(trifluoromethyl)benzyl)-1,4-diazepane (**14dv_amine16**) was prepared according to Example 1 and was obtained as a colorless oil (0.17 g, 33% yield); ¹H NMR (400 MHz, CDCl₃) δ 8.77 - 8.82 (m, 2H), 8.40 - 8.44 (m, 2H), 8.20 - 8.24 (m, 2H), 7.64 (s, 1H), 7.52 - 7.58 (m, 5H), 7.45 (d, *J* = 7.82 Hz, 2H), 3.68 (s, 2H), 3.19 (s, 4H), 2.89 - 3.04 (m, 4H), 2.69 - 2.80 (m, 4H), 1.89 - 1.96 (m, 2H) (Figure 8A); ¹³C NMR (101 MHz, CDCl₃) δ 169.7, 164.1, 162.2, 150.4, 145.3, 143.4, 136.6, 131.1, 129.0, 128.9, 127.2, 125.2, 125.2, 125.1, 122.2, 115.4, 62.0, 56.9, 55.3, 54.3, 54.2, 53.8, 35.5, 27.1 (Figure 8B); LRMS (ESI) *m/z* 518 (M⁺ + H, 100); HRMS (ESI) calcd for C₃₀H₃₁N₅F₃ (M⁺ + H) 518.2532, found 518.2528.

EXAMPLE 3

Synthesis of 14dv_amine06

[0101] 1-(4-isopropylbenzyl)-4-(2-(6-phenyl-2-(pyridin-4-yl)pyrimidin-4-yl)ethyl)-1,4-diazepane (**14dv_amine06**) was prepared according to Example 1 and was obtained as a colorless oil (0.12 g, 24% yield); ¹H NMR (400 MHz, CDCl₃) δ 8.75 - 8.81 (m, 2H), 8.37 - 8.41 (m, 2H), 8.18 - 8.24 (m, 2H), 7.65 (s, 1H), 7.50 - 7.55 (m, 3H), 7.27 - 7.32 (m, *J* = 7.82 Hz, 2H), 7.14 - 7.18 (m, *J* = 7.83 Hz, 2H), 3.76 (s, 2H), 3.21 - 3.31 (m, 4H), 3.05 - 3.12 (m, 4H), 2.83 - 2.99 (m, 5H), 2.07 - 2.14 (m, 2H), 1.22 (d, *J* = 6.85 Hz, 6H) (Figure 9A); ¹³C NMR (101 MHz, CDCl₃) δ 168.8, 164.3, 162.2, 150.4, 148.9, 145.1, 136.4, 131.3, 129.6, 129.0, 127.3, 126.7, 122.1, 115.4, 61.8, 56.4, 53.4, 53.3, 53.0, 34.8, 33.8, 25.0, 23.9 (Figure 9B); LRMS (ESI) *m/z* 492 (M⁺ + H, 100); HRMS (ESI) calcd for C₃₂H₃₈N₅ (M⁺ + H) 492.3127, found 492.3149.

EXAMPLE 4

Synthesis of 14dv_amine20

[0102] 1-(2-(6-phenyl-2-(pyridin-4-yl)pyrimidin-4-yl)ethyl)-4-(4-(trifluoromethyl)phenethyl)-1,4-diazepane (**14dv_amine20**) was prepared according to Example 1 and was obtained as a pale brown oil (0.14 g, 26% yield); ¹H NMR (400 MHz, CDCl₃) δ 8.75 (d, *J* = 5.87 Hz, 2H), 8.35 (d, *J* = 5.87 Hz, 2H), 8.15 - 8.22 (m, 2H), 7.65 (s, 1H), 7.45 - 7.53 (m, 5H), 7.25 (d, *J* = 8.31 Hz, 2H), 3.23 - 3.39 (m, 4H), 3.08 - 3.22 (m, 6H), 3.01 - 3.07 (m, 2H), 2.92 - 3.00 (m, 4H), 2.12 - 2.24 (m, 2H) (Figure 10A); ¹³C NMR (101 MHz, CDCl₃) δ 168.2, 164.4, 162.2, 150.4, 145.0, 142.2, 136.2, 131.3, 129.0, 127.2, 125.5, 125.5, 125.5, 122.1, 115.4, 58.7, 56.1, 53.0, 53.0, 52.8, 34.3, 32.2, 24.2 (Figure 10B); LRMS (ESI) *m/z* 532 (M⁺ + H, 100); HRMS (ESI) calcd for C₃₁H₃₃N₅F₃ (M⁺ + H) 532.2688, found 532.2676.

EXAMPLE 5

Synthesis of 14dv_amine07

[0103] 1-(4-(*tert*-butyl)benzyl)-4-(2-(6-phenyl-2-(pyridin-4-yl)pyrimidin-4-yl)ethyl)-1,4-diazepane (**14dv_amine07**) was prepared according to Example 1 and was obtained as a colorless oil (0.18 g, 36% yield); ¹H NMR (400 MHz, CDCl₃) δ 8.75 - 8.79 (m, 2H), 8.35 - 8.39 (m, 2H), 8.18 - 8.23 (m, 2H), 7.66 (s, 1H), 7.50 - 7.55 (m, 3H), 7.32 (s, 4H), 3.83 (s, 2H), 3.27 - 3.40 (m, 4H), 3.12 - 3.22 (m, 4H), 2.95 - 3.09 (m, 4H), 2.20 (br. s., 2H), 1.28 (s, 9H) (Figure 11A); ¹³C NMR (101 MHz, CDCl₃) δ 168.2, 164.4, 162.2, 151.7, 150.5, 145.0, 136.2, 131.3, 129.7, 129.1, 127.3, 125.8, 122.1, 115.4, 61.4, 56.1, 53.1, 53.0, 52.4, 34.6, 34.3, 31.3, 24.0 (Figure 11B); LRMS (ESI) *m/z* 506 (M⁺ + H, 100); HRMS (ESI) calcd for C₃₃H₄₀N₅ (M⁺ + H) 506.3284, found 506.3281.

EXAMPLE 6

Synthesis of 14dv_amine08

[0104] 1-(4-bromobenzyl)-4-(2-(6-phenyl-2-(pyridin-4-yl)pyrimidin-4-yl)ethyl)-1,4-diazepane (**14dv_amine08**) was prepared according to Example 1 and was obtained as a pale brown oil (0.14 g, 27% yield); ¹H NMR (400 MHz, CDCl₃) δ 8.80 (d, *J* = 5.87 Hz, 2H), 8.44 (d, *J* = 6.36 Hz, 2H), 8.20 - 8.24 (m, 2H), 7.62 (s, 1H), 7.53 - 7.58 (m, 3H), 7.39 - 7.44 (m, *J* = 8.31 Hz, 2H), 7.17 - 7.23 (m, *J* = 8.31 Hz, 2H), 3.57 (s, 2H), 3.09 (s, 4H), 2.89 (t, *J* = 5.87 Hz, 2H), 2.79 - 2.85 (m, 2H), 2.63 - 2.73 (m, 4H), 1.84 (quin, *J* = 5.87 Hz, 2H) (Figure 12A); ¹³C NMR (101 MHz, CDCl₃) δ 170.4, 164.0, 162.2, 150.4, 145.4, 138.6, 136.8, 131.3, 131.1, 130.4, 129.0, 127.2, 122.2, 120.6, 115.4, 87.4, 61.9, 57.2, 55.2, 55.0, 54.3, 53.9, 36.1, 27.8 (Figure 12B); LRMS (ESI) *m/z* 529 (M⁺ + H, 100); HRMS (ESI) calcd for C₂₉H₃₁N₅Br (M⁺ + H) 528.1763, found 528.1762.

EXAMPLE 7

Synthesis of 14av_amine16

[0105] 1-(2-(6-(pentan-3-yl)-2-(pyridin-4-yl)pyrimidin-4-yl)ethyl)-4-(4-(trifluoromethyl)benzyl)-1,4-diazepane (**14av_amine16**) was prepared according to Example 1 and was obtained as a colorless oil (0.27 g, 53% yield); ¹H NMR (400 MHz, CDCl₃) δ 8.72 - 8.80 (m, 2H), 8.31 - 8.38 (m, 2H), 7.52 - 7.60 (m, *J* = 8.31 Hz, 2H), 7.40 - 7.49 (m, *J* = 8.31 Hz, 2H), 6.99 (s, 1H), 3.67 (s, 2H), 2.96 - 3.08 (m, 4H), 2.83 - 2.90 (m, 2H), 2.77 - 2.83 (m, 2H), 2.66 - 2.72 (m, 4H), 2.57 (tt, *J* = 5.81, 8.38 Hz, 1H), 1.73 - 1.87 (m, 6H), 0.83 (t, *J* = 7.58 Hz, 6H) (Figure 13A); ¹³C NMR (101 MHz, CDCl₃) δ 173.9, 169.0, 161.9, 150.3, 145.7, 143.9, 128.8, 125.6, 125.2, 125.1, 125.1, 125.1, 122.9, 122.2, 119.1, 62.2, 57.2, 55.3, 55.2, 54.3, 53.9, 51.0, 35.9, 27.8, 27.5, 12.0 (Figure 13B); LRMS (ESI) *m/z* 512 (M⁺ + H, 100), 534 (M⁺ + Na, 13); HRMS (ESI) calcd for C₂₉H₃₇N₅F₃ (M⁺ + H) 512.3001, found 512.2986.

EXAMPLE 8

Synthesis of 14av_amine20

[0106] 1-(2-(6-(pentan-3-yl)-2-(pyridin-4-yl)pyrimidin-4-yl)ethyl)-4-(4-(trifluoromethyl)phenethyl)-1,4-diazepane (**14av_amine20**) was prepared according to

Example 1 and was obtained as a pale brown oil (0.16 g, 30% yield); ^1H NMR (400 MHz, CDCl_3) δ 8.75 (d, $J = 5.87$ Hz, 2H), 8.30 (d, $J = 6.36$ Hz, 2H), 7.54 (d, $J = 7.83$ Hz, 2H), 7.31 (d, $J = 7.82$ Hz, 2H), 7.02 (s, 1H), 3.34 - 3.44 (m, 2H), 3.24 - 3.32 (m, 2H), 3.18 - 2.96 (m, 10H), 2.77 - 2.96 (m, 2H), 2.53 - 2.61 (m, 1H), 2.29 (br. s., 2H), 1.72 - 1.83 (m, 4H), 0.81 (t, $J = 7.34$ Hz, 6H) (Figure 14A); ^{13}C NMR (101 MHz, CDCl_3) δ 174.9, 166.3, 162.0, 150.4, 145.1, 142.0, 129.0, 125.7, 125.6, 122.1, 119.3, 58.7, 56.0, 52.9, 52.8, 52.3, 51.0, 33.6, 32.2, 27.4, 23.7, 12.0 (Figure 14B); LRMS (ESI) m/z 526 ($\text{M}^+ + \text{H}$, 100); HRMS (ESI) calcd for $\text{C}_{30}\text{H}_{39}\text{N}_5\text{F}_3$ ($\text{M}^+ + \text{H}$) 526.3158, found 526.3177.

EXAMPLE 9

Synthesis of Pyrimidine 14 with a Sulfonamide Group

(1) Synthesis of Boc-protected pyrimidine **14az_amine40**

[0107] *tert*-butyl-4-(2-(6-(pentan-3-yl)-2-(pyridin-2-yl)pyrimidin-4-yl)ethyl)-1,4-diazepane-1-carboxylate (**14az_amine40**) was prepared according to Example 1 and was obtained as a colorless oil (0.13 g, 26% yield); ^1H NMR (400 MHz, CDCl_3) δ 8.78 (br. s., 1H), 8.34 - 8.54 (m, 1H), 7.64 - 7.85 (m, 1H), 7.29 (s, 3H), 6.82 - 7.07 (m, 1H), 3.42 (br. s., 2H), 3.35 (br. s., 2H), 2.99 (br. s., 2H), 2.87 - 2.95 (m, 2H), 2.57 - 2.72 (m, 5H), 1.62 - 1.83 (m, 6H), 1.38 (br. s., 9H), 0.69 - 0.84 (m, 6H) (Figure 15A); ^{13}C NMR (101 MHz, CDCl_3) δ 173.8, 169.3, 163.2, 155.7, 149.9, 136.6, 124.3, 123.9, 118.4, 79.1, 57.0, 55.9, 55.8, 54.7, 54.4, 50.8, 47.0, 46.5, 45.9, 45.0, 36.1, 28.4, 27.9, 27.8, 27.4, 11.9 (Figure 15B); LRMS (ESI) m/z 454 ($\text{M}^+ + \text{H}$, 100); HRMS (ESI) calcd for $\text{C}_{26}\text{H}_{40}\text{N}_5\text{O}_2$ ($\text{M}^+ + \text{H}$) 454.3182, found 454.3164.

(2) Synthesis of pyrimidine **14az_amine41**

[0108] 1-(2-(6-(pentan-3-yl)-2-(pyridin-2-yl)pyrimidin-4-yl)ethyl)-1,4-diazepane (**14az_amine41**): A round-bottom flask was charged with **14az_amine40** (80 mg, 0.18 mmol) and DCM (5 mL). The solution was cooled to 0 °C with an ice bath. An equal volume of TFA (5 mL) was then added dropwise, and the reaction mixture was stirred vigorously at 0 °C for 2 h. After the mixture was stirred, the reaction was quenched by pouring the mixture into a conical flask containing water. The resultant mixture was basified to pH 10 by using potassium hydroxide solution. The mixture was continuously extracted with DCM. The combined organic layers were dried over MgSO_4 , filtered, and evaporated to give **14az_amine41** (55 mg, 88% yield) as a pale brown oil; ^1H NMR (400 MHz, CDCl_3) δ 8.84 (d, $J = 4.89$ Hz, 1H), 8.51 (d, $J = 7.83$ Hz, 1H), 7.74 - 7.88 (m, 1H), 7.32 - 7.40 (m, 1H), 7.02 (s, 1H), 2.95 - 3.10 (m, 4H), 2.83 - 2.95 (m, 4H), 2.69 - 2.81 (m, 4H), 2.64 (t, $J = 7.09$ Hz, 1H), 1.69 - 1.82 (m, 6H), 0.82 (t, $J = 7.34$ Hz, 6H) (Figure 16A); ^{13}C NMR (101 MHz, CDCl_3) δ 173.8, 169.6, 163.2, 155.8, 150.0, 136.7, 124.3, 123.9, 118.4, 58.2, 57.7, 54.4, 53.4, 50.8, 49.2, 47.4, 36.2, 30.6, 27.4, 11.9 (Figure 16B); LRMS (ESI) m/z 354 ($\text{M}^+ + \text{H}$, 100), 376 ($\text{M}^+ + \text{Na}$, 14); HRMS (ESI) calcd for $\text{C}_{21}\text{H}_{32}\text{N}_5$ ($\text{M}^+ + \text{H}$) 354.2658, found 354.2657.

(3) Synthesis of pyrimidine **14az_amine42**

[0109] 1-(methylsulfonyl)-4-(2-(6-(pentan-3-yl)-2-(pyridin-2-yl)pyrimidin-4-yl)ethyl)-1,4-diazepane (**14az_amine42**): A round-bottom flask was charged with **14az_amine41** (33 mg,

0.09 mmol), methanesulfonyl chloride (30 mg, 0.26 mmol), triethylamine (5 mL) and DCM (5 mL). The reaction mixture was stirred at 0°C for 2 h. When TLC indicated complete consumption of starting material, the reaction mixture was poured into a separating funnel containing 1 M hydrochloric acid solution. The mixture was continuously extracted with DCM.

- 5 The combined organic layers were dried over MgSO₄, filtered, and evaporated to give a crude reaction mixture, which was subjected to purification by flash column chromatography on silica gel with 10–40% EA in Hex as eluent to furnish **14az_amine42** as a pale brown oil (24 mg, 60%): ¹H NMR (400 MHz, CDCl₃) δ 8.84 (d, *J* = 3.91 Hz, 1H), 8.52 (d, *J* = 8.31 Hz, 1H), 7.83 (dt, *J* = 1.71, 7.70 Hz, 1H), 7.36 (dd, *J* = 5.14, 7.09 Hz, 1H), 6.99 (s, 1H), 3.31 - 3.46 (m, 4H), 2.98 - 3.07 (m, 4H), 2.76 - 2.87 (m, 7H), 2.55 - 2.69 (m, 1H), 1.86 (td, *J* = 5.87, 11.74 Hz, 2H), 1.70 - 1.82 (m, 4H), 0.83 (t, *J* = 7.34 Hz, 6H) (Figure 17A); ¹³C NMR (101 MHz, CDCl₃) δ 174.0, 169.1, 163.3, 155.7, 150.0, 136.7, 124.4, 124.0, 118.4, 57.2, 56.2, 54.1, 50.8, 48.5, 46.9, 37.2, 36.1, 28.5, 27.4, 11.9 (Figure 17B); LRMS (ESI) *m/z* 432 (M⁺ + H, 100); HRMS (ESI) calcd for C₂₂H₃₄N₅O₂S (M⁺ + H) 432.2433, found 432.2446.

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EXAMPLE 10

Evaluation of antimicrobial activities and SAR study

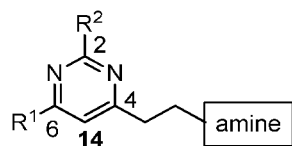
- [0110] This example illustrates the evaluation of 2,4,6-trisubstituted pyrimidines **14** for their antimicrobial activities against the Gram positive *S. aureus* strain ATCC29213 and the Gram negative *E. coli* strain ATCC25922 by measuring their MIC, which is the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism in a broth dilution susceptibility test according to the standard method approved by the Clinical and Laboratory Standards Institute (CLSI) guidelines.²¹

- [0111] Procedure of Antimicrobial susceptibility test: The MIC value of compounds was measured in the antimicrobial susceptibility test using the broth microdilution procedure in accordance with the CLSI guidelines.²¹ Cation-adjusted Mueller Hinton broth (CA-MHB) was used for all the *S. aureus* strains in the assay. Cells in exponential phase of growth were diluted to approximately 5 × 10⁵ cfu/mL. Stock solutions of each compound were freshly dissolved in dimethyl sulfoxide (DMSO). Then, serial dilutions of each compound in CA-MHB medium were added into 96-well microplate. The final percentage of DMSO in the assay was 1%. Control experiments were performed with 1% DMSO instead of the compound solution. After being incubated at 37 °C for 18 h, OD₆₀₀ value of cells was measured on a microplate reader and the percentage of bacterial cell inhibition with respect to controls was calculated. The MIC value was defined as the lowest compound concentration at which the growth of bacteria was inhibited by ≥90%. Three independent assays were performed for each compound.

- [0112] Results: Parent compound **7b** (Figure 1, bottom right) was selected as positive control for comparison purpose. Compound **7b** inhibited the growth of both bacterial strains with MIC at 125 μM, demonstrating moderate antibacterial activity and no selectivity of killing between two bacterial strains (Table 3, Entry 1). In contrast, some compounds of the present invention display superior antimicrobial activity and selectivity. The MIC screening results are

summarized in Table 3, in which compounds with MIC values against *S. aureus* less than 20 μ M are shown in Entries 1-23. In general, among the newly synthesized compounds, most of them displayed potent inhibitory activities against the growth of *S. aureus* but not *E. coli* at a concentration of 125 μ M. The weak activity against *E. coli* may be attributed to their poor penetration ability into the cytoplasm of Gram negative bacteria, which have an outer membrane of low permeability. For compounds listed in Table 3, seven compounds displaying superior potency (MIC at 5 – 8 μ M) and selectivity (>15) were identified, namely **14dv_amine16**, **14dv_amine06**, **14dv_amine20**, **14dv_amine07**, **14dv_amine08**, **14av_amine16** and **14av_amine20** (Table 3, Entry 2 – 8). Compared with compound **7b** (Entry 1), their potency was improved by 15 to 25-fold and their selectivity by >15 -fold. Interestingly, these compounds possess the common structural features of a 4-pyridyl (4-Py) group at position 2 and a cyclic 7-membered homopiperazine ring substituted with a benzyl group at position 4. Detailed SAR analysis of 2,4,6-trisubstituted pyrimidines **14** derivatives are depicted in Figure 5. For the pyrimidine head, 4-pyridyl group of R^2 of **14av_amine16** was very important for potent antibacterial activity. Replacing this function group with others such as the 3-pyridyl of **14ay_amine16**, 2-pyridyl of **14az_amine16**, phenyl of **14ax_amine16** and methyl groups of **14aw_amine16** resulted in weak antibacterial activity, implying that nitrogen atom of 4-Py group is crucial for maintaining potent antibacterial activity. For R^1 group of pyrimidine head, both phenyl of **14dv_amine16** and 3-pentyl group of **14av_amine16** were favorable substituents. Less bulky *iso*-propyl group of **14bv_amine16** and linear *n*-pentyl group of **14cv_amine16** both caused poor antibacterial activity. In general, pyrimidine head bearing 4-pyridyl group of R^2 and phenyl or 3-pentyl groups of R^1 exhibited the most potent antibacterial activity. For SAR analysis of the amine tail (**14av_amine01** – **14av_amine39**), there were two important structural features that gave rise to potent antibacterial activity: (1) cyclic 7-member homopiperazine ring with substituted benzyl group; (2) the benzyl group substituted with a bulky group (*tert*-butyl, trifluoromethyl, *iso*-propyl, bromo) at *para*-position. Replacing the amine tail with cyclic 6-member piperazine ring of **14av_amine24**, linear 1,2-diamine of **14av_amine35** – **36** or 1,3-diamine of **14av_amine37** - **38** resulted in poor antibacterial activity. It was also worthy to note that replacing the benzylic carbon with a rigid functional group, such as carbonyl group of **14av_amine19**, sulfonyl group of **14az_amine43** – **44** or α , β -unsaturated ketone group of **14av_amine21** – **23**, caused poor antibacterial activity. These results suggested that a more flexible and freely rotatable functional group should be installed at this position. This observation was further supported by the low MIC value of compound **14av_amine20**, in which the benzylic carbon was replaced with a freely rotatable CH_2CH_2 group.

Table 3. MIC values of 2,4,6-trisubstituted pyrimidines against *S. aureus* 29213 and *E. coli* ATCC 25922 as well as calculated selectivity index (MIC^c / MIC^b).



Entry	Compound No.	R ¹	R ²	Amine No.	MIC ^b	MIC ^c	MIC ^c / MIC ^b
1	7	N.A.	N.A.	N.A.	125	125	1
2	14dv_amine16	phenyl	4-Py	16	5	>125	>25
3	14dv_amine06	phenyl	4-Py	06	5	>125	>25
4	14dv_amine20	phenyl	4-Py	20	5	>125	>25
5	14dv_amine07	phenyl	4-Py	07	5	>125	>25
6	14dv_amine08	phenyl	4-Py	08	5	>125	>25
7	14av_amine16	3-pentyl	4-Py	16	8	>125	>15
8	14av_amine20	3-pentyl	4-Py	20	8	>125	>15
9	14cv_amine06	<i>n</i> -pentyl	4-Py	06	10	>125	>12
10	14cv_amine16	<i>n</i> -pentyl	4-Py	16	10	>125	>12
11	14dv_amine12	phenyl	4-Py	12	10	>125	>12
12	14dv_amine10	phenyl	4-Py	10	10	>125	>12
13	14av_amine28	3-pentyl	4-Py	28	10	>125	>12
14	14av_amine38	3-pentyl	4-Py	38	10	>125	>12
15	14av_amine39	3-pentyl	4-Py	39	10	>125	>12
16	14av_amine24	3-pentyl	4-Py	24	12	>125	>10
17	14av_amine35	3-pentyl	4-Py	35	12	>125	>10
18	14av_amine13	3-pentyl	4-Py	13	13	>125	>9
19	14av_amine06	3-pentyl	4-Py	06	16	>125	>7
20	14av_amine07	3-pentyl	4-Py	07	16	>125	>7
21	14av_amine08	3-pentyl	4-Py	08	16	>125	>7
22	14av_amine17	3-pentyl	4-Py	17	16	>125	>7
23	14av_amine18	3-pentyl	4-Py	18	16	>125	>7

N.A.: not applicable.

MIC^b : Minimum inhibitory concentration in uM against *S. aureus*

MIC^c : Minimum inhibitory concentration in uM against *E. coli*

MIC^c / MIC^b : Selectivity index

EXAMPLE 11

Antibacterial activity against drug-resistant *S. aureus* strains

- 5 [0113] Procedure for expression and purification of *S. aureus* FtsZ protein: *S. aureus* FtsZ protein was prepared as previous reported.^{16a} In brief, *E. coli* BL21(DE3) cells, transformed with a pRSET-A-S vector carrying *S. aureus* FtsZ with a 6-histidine tag attached, were streaked

on a nutrient agar plate containing 50 µg/mL ampicillin. After overnight incubation at 37 °C, a single colony was inoculated into 5 mL of Luria–Bertani (LB) medium in the presence of 50 µg/mL ampicillin, which was then incubated at 37 °C, with shaking at 250 rpm for 16 h. The overnight culture was transferred into a fresh 2×TY medium in a dilution ratio of 1:100 and 50 µg/mL ampicillin was then added, followed by incubation at 37 °C with shaking at 250 rpm. When OD₆₀₀ reached 0.8, protein expression was induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h. Cells were harvested by centrifugation at 9000 rpm for 20 min at 4 °C. The cell pellet was resuspended in 20 mL of solubilization buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM PMSF and 1 mM EDTA, pH 7.4) and then lysed with 1 mg/mL of lysozyme. The mixture was incubated for 1 h on ice. The cells were disrupted by sonication and the crude lysate obtained was centrifuged at 13,000 rpm for 1 h at 4 °C. The supernatant containing 6-histidine tagged *S. aureus* FtsZ was collected and loaded onto a nickel charged HiTrap chelating column pre-equilibrated with starting buffer (20 mM sodium phosphate buffer, 0.5 M NaCl, pH 7.4). The column was then washed with 8 column volumes of the starting buffer to remove the unbound proteins, and the histidine-tagged enzyme was eluted by a linear gradient of 0–0.2 M imidazole. Fractions containing *S. aureus* FtsZ were pooled, buffer-exchanged with 20 mM NH₄HCO₃ (pH 8.0) at 4°C, lyophilized, and stored at –20°C. A stock solution of *S. aureus* FtsZ for the subsequent Saturation Transfer Difference (STD) NMR study and light scattering assay and GTPase activity assay was prepared from the lyophilized powder.

[0114] Results: Three compounds, namely **14av_amine16**, **14dv_amine16** and **14dv_amine06**, were selected to be tested against nine in-house clinically isolated bacterial strains including *S. aureus* ATCC29247, which is an ampicillin resistant strain, *S. aureus* ATCC BAA-41, ATCC BAA-1717, ATCC BAA-1720, ATCC 43300 which are methicillin resistant strains and four USA300 strains, (#417, #757, #1799 and #2690), which are the predominant strain type of community-associated MRSA strains in the United States. MIC screening results are summarized in Table 4. All compounds were found to retain potent antibacterial activities against these antibiotic-resistant strains with MIC values ranging from 5 to 12 µM. Compound **14dv_amine16** and **14dv_amine06** displayed the most potent antibacterial activity against *S. aureus* ATCC 29247 and ATCC BAA-1717, with MIC at 5 µM. Compared with methicillin, which is the clinically used antibiotic with MIC values higher than 30 µM against most of the MRSA, these pyrimidine derivatives exhibited significantly lower MIC values and thus the potential to be developed into new anti-staphylococcal agents in the future. It was worthy to mention that compounds **7** did not exhibit any antibacterial activity against these clinically isolated *S. aureus* strains (MIC > 100 µM).

Table 4. MIC values of selected pyrimidines against nine clinically isolated *S. aureus* strains.

Compound No.	MIC (µM)								
	Strain 1	Strain 2	Strain 3	Strain 4	Strain 5	Strain 6	Strain 7	Strain 8	Strain 9

14av_amine16	8	8	12	12	12	12	12	12	12
14dv_amine16	5	5	10	10	10	10	10	10	10
14dv_amine06	5	5	10	10	10	10	10	10	10
Methicillin	N.D.	>30	>30	>30	>30	>30	30	30	15

N.D.: not determined; Strain 1: ATCC 29247; Strain 2: ATCC BAA-1717; Strain 3: ATCC BAA-1720; Strain 4: ATCC BAA-41; Strain 5: ATCC 43300; Strain 6: USA300 #417; Strain 7: USA300 #757; Strain 8: USA300 #1799; Strain 9: USA300 #2690.

EXAMPLE 12

Investigation of potential for development of resistance and in vivo biological evaluation

[0115] This example evaluates the spontaneous resistance rate of compounds in MRSA and measures the *in vivo* toxicity and efficacy.

a) Frequency of resistance (FOR) determination

[0116] Procedure: MRSA ATCC 43300 cells were grown to late-exponential phase (1×10^9 CFU/mL) and spread on agar plates containing 1% DMSO or compound **14av_amine16** at 8 folds of MIC level. The plates were incubated for 48 hours to allow resistant mutants to grow. The spontaneous FOR was calculated as the number of resistance colonies divided by the number of CFUs originally plated. The assay was performed in triplicates.

[0117] Results: As shown in Figure 18A (left), almost confluent bacterial growth was observed for DMSO treated bacterial cells yet no colony was observed for plates treated with compound **14av_amine16** (Figure 18A, right) after 48 hours' incubation, implying that the rate of emergence of spontaneous resistant mutants was as low as $< 1 \times 10^9$. This finding confirmed that the chance of occurrence of mutational changes and confer resistance to inhibitors acting on GTP binding site of FtsZ protein is extremely low. This low frequency of spontaneous resistance implies a reduced probability of rapid development of resistance against compound **14av_amine16** in clinical practice.

b) Evaluation of the toxicity and antimicrobial efficacy using a *G. mellonella* model of infection

[0118] Procedure: Compound **14av_amine16** was tested in a *Galleria mellonella* model, which are an easy and inexpensive *in vivo* model with no ethical constraints for investigating antibacterial activity of a compound.²² The *G. mellonella* model of *S. aureus* infection was used as previously described.²⁴ Briefly, 1 mL aliquots of overnight cultures of *S. aureus* 43300 were pelleted by centrifugation and washed with sterile PBS before being resuspended in 100 μ L of PBS. *G. mellonella* larvae (N = 10) that weighed 200–300 mg were then inoculated with 10 μ L of *S. aureus* (2.5×10^6 CFU) into the last left proleg. Larvae were then treated with compounds at 1 h before bacterial inoculation. Treatments were performed in the same manner as infection, except that compounds were injected into the next left proleg moving toward the head of the

larvae. Larvae were then incubated at 37 °C and mortality rates were monitored at 12 h interval for 48 h; larvae were considered dead if they did not respond to physical stimuli. Data was analyzed for statistical significance using a log rank and χ square test with 1 degree of freedom. Various concentrations of compound **14av_amine16** dissolved in 50% PEG in saline (0 mg/Kg, 50 mg/Kg and 100 mg/Kg) were injected into the hemocoel of last-instar *G. mellonella* larvae and survival was scored over time.

[0119] To examine the feasibility of using *G. mellonella* for compound **14av_amine16** toxicity study, the ability of compound **14av_amine16** to kill *G. mellonella* larvae was examined.

[0120] The efficacy of compound **14av_amine16** against *G. mellonella* larvae infected with MRSA ATCC 43300 was investigated. Inoculation of lethal dose of 2.5×10^6 CFU/larva of MRSA ATCC 43300 led to a significant death rate. All larvae injected with 50% PEG in saline (0 mg/Kg) died within a 24 hours' infection period.

[0121] Results: All concentrations of compound **14av_amine16** tested did not kill *G. mellonella* larvae during a 48-hours period (Figure 18B). These data indicate that formulation of 50% PEG in saline and compound **14av_amine16** are nontoxic and safe against *G. mellonella* larvae.

[0122] Injection of compound **14av_amine16** at doses of 50 mg/Kg to infected larvae was found to prolong the survival time of infected larvae to 36 hours (Figure 18C). It is observed that 20% survival rate of infected larvae over the infection period with increased dosing with **14av_amine16** at 100 mg/Kg. Compared to the vehicle group, it was found to be highly significant ($p < 0.05$). Further increased dosing did not lead to improved efficacy (Data not shown). These data indicated that compound **14av_amine16** is capable of preventing or delaying the lethal effect of MRSA ATCC 43300 in *G. mellonella* larvae.

EXAMPLE 13

Saturation Transfer Difference (STD) NMR study

[0123] Procedure STD NMR study: STD NMR experiments were performed on a Bruker Avance III 600 NMR spectrometer equipped with a QCI cryoprobe at 298K. Spectra were recorded with a carrier set at 0.5 ppm for on-resonance irradiation and 30 ppm for off-resonance irradiation. Control spectra were recorded under identical conditions without compound to test for artifacts. Selective protein saturation was accomplished using a train of 40 Gauss-shaped pulses (50 ms with 1 ms delay between pulses) for a total saturation time of 2 s at an experimentally determined optimal power (7.7625 W on the probe); a T1 ρ spinlock filter (20 - 50 ms) was incorporated to suppress protein resonances. STD spectra were recorded using a minimum of 128 scans and 32k points covering a spectral width of 6600Hz. The free induced decay (FID) was multiplied by an exponential line-broadening function of 1 Hz and zero-filled by a factor of two prior to Fourier transformation. On- and off- resonance spectra were processed independently and subtracted to provide a difference spectrum. Spectra processing was performed using Topspin software (Bruker). Group epitope mapping were performed by

integrated the STD signal of the individual protons with respect to the strongest STD signal, which was assigned to a value of 100%.

[0124] Results: STD NMR spectroscopy is a powerful and unique tool that can detect the magnetization that was transferred from a protein to a bound ligand proton. It is commonly used to detect binding of low molecular weight compound to large biomolecules.²³ In order to get further insights of the interaction between compounds **14av_amine16** and *S. aureus* FtsZ protein, STD NMR spectroscopy is employed to characterize the binding properties and identify epitopes of small molecules that bind to a protein receptor. *S. aureus* FtsZ protein was expressed and purified as described in a previous reported.^{16a} STD NMR spectroscopy was performed and the relative degrees of saturation for individual protons of compound **14av_amine16** are displayed in Figure 6 with the integral value of the largest signal set to 100%. The line boarding observed is caused by compound **14av_amine16** being in close contact with the FtsZ protein, resulting in slow tumbling rate of the protein-ligand complex, which also confirmed that compound **14av_amine16** indeed bind to FtsZ protein. All of the protons of compound **14av_amine16** showed some degree of enhancement, demonstrating that the molecule, except the homopiperazine moiety, is bound in the active site of FtsZ protein. The largest amount of saturation transfer was observed for H1, indicating the pyrimidine ring of compound **14av_amine16** are making more intimate contacts with the active site residue of FtsZ protein.

EXAMPLE 14

S. aureus FtsZ polymerization and GTPase hydrolysis assay.

[0125] This example determines whether the binding of compound **14av_amine16** to *S. aureus* FtsZ protein led to a change in the polymerization activity and GTPase activity of the protein itself.

a) *S. aureus* FtsZ polymerization assay

[0126] Procedure: The polymerization of recombinant *S. aureus* FtsZ was measured using a 90° light scattering in a thermostatically (37 °C) controlled Fluorescence Spectrometer LS 50B according to previous reports.^{16a,19} Both excitation and emission wavelengths were fixed to 600 nm with a slit width of 2.5 nm. *S. aureus* FtsZ (12.5 μM) in 50 mM 4-morpholinepropanesulfonic acid (MOPS) buffer (pH 6.5) was incubated with DMSO or different concentrations of compound for 10 min at 25 °C. Then, 50 mM KCl and 10 mM MgCl₂ were added to establish a baseline. After 8 min, the final concentration of 1 mM GTP was added at the last fraction and the increase in light scattering measured for an additional 2000 seconds. The rate and extent of polymerization were measured. Appropriate blanks were subtracted from all experimental data. Results shown are the average of three independent experiments.

[0127] Results: To assay this function, an *in vitro* light scattering assay, in which FtsZ polymerization was detected in solution by a time-dependent increase in light scattering as reflected by an increase in solution absorbance at 600 nm, was employed. Figure 7 shows the

relative time-dependence absorbance at 600 nm of *S. aureus* FtsZ in the presence of **14av_amine16** at concentrations ranging from 0 to 30 μ M. It was clearly demonstrated that **14av_amine16** potently suppresses the self-polymerization of *S. aureus* FtsZ protein with the magnitude of these suppressing effects increasing with increasing compound concentration.

- 5 Complete inhibition of FtsZ polymerization was observed at 30 μ M of **14av_amine16**. These results suggested that **14av_amine16** inhibits the proliferation of *S. aureus* via suppressing the self-polymerization of FtsZ protein.

b) GTPase hydrolysis assay

- [0128] Procedure: The inhibitory effect of compound **14av_amine16** on GTPase hydrolysis activity was investigated according to the protocol described previously.¹⁸ The GTPase activity of *S. aureus* FtsZ was estimated in a 96-well microplate using a CytoPhos phosphate assay Biochem Kit based on the manufacturer's instructions. Purified *S. aureus* FtsZ (7.5 μ M) was incubated with serial dilutions of compound in 50 mM 4-morpholinepropanesulfonic acid (MOPS, pH 6.5) buffer at 25 °C for 10 minutes. 1% DMSO was used for control experiment.
- 15 Then 5 mM of $MgCl_2$ and 200 mM of KCl were added followed by addition of 500 μ M GTP and incubation at 37 °C. After half an hour, the reactions were quenched by adding 100 μ L of Cytophos reagent. The precipitated inorganic phosphate was quantified by measuring the absorbance at 650 nm in a microplate reader. Three independent assays were performed in triplicate.

- 20 [0129] Results: Compounds **7a** and **7b** inhibited the GTPase hydrolysis activity with IC_{50} at 73 μ M and 189 μ M respectively. However, compound **14av_amine16** at 50 μ M and 75 μ M displayed only moderate inhibition of the GTPase hydrolysis activity at about $20 \pm 3\%$ and $25 \pm 4\%$ respectively. GTPase hydrolysis activity at higher compound concentration ($>80 \mu$ M) cannot be measured due to poor compound solubility, which causes precipitation in aqueous
- 25 medium. Nonetheless, it seems very likely that compound **14av_amine16** suppresses the self-polymerization of FtsZ protein, probably via disrupting the GTPase hydrolysis activity of FtsZ protein.

EXAMPLE 15

Effects on the *B. subtilis* 168 cell morphology and localization of the Z-ring

- 30 [0130] This example investigates the underlying mode of action of the antibacterial activity of compound **14av_amine16** by microscopic observation of a rod-shaped *B. subtilis* 168 cell morphology.

a) Bacterial Morphology of *B. subtilis* 168

- [0131] Procedure: The *B. subtilis* 168 cells were grown in LB medium. The cultures at an A_{600} of 0.01 from an overnight culture were inoculated in the same medium containing different concentrations of the test compounds and grown at 37°C for 4 h. The cells for morphology studies were harvested and resuspended in 100 μ L of PBS buffer containing 0.25% agarose. 10 μ L of the suspension mixture were then placed on a microscopic slide pretreated with 0.1% (w/v) poly-L-lysine and the morphology of the bacterial cells was observed under a phase-
- 35

contrast optical microscope at 40 × magnification. The images were captured using an Olympus Bio Imaging Navigator FSX 100 microscope.

[0132] Results: Compound **14av_amine16** exhibited antibacterial activity against *B. subtilis* 168 with MIC value at 12 µM. Treatment of *B. subtilis* cell at a sub-lethal concentration of compound **14av_amine16** significantly increased the cell length with average cell length > 20 µm (Figure 19B) as compared with the DMSO treated cells (cell length < 5 µm, Figure 19A). Interestingly, such phenomenon of cell elongation was also observed for other FtsZ inhibitors reported, strongly suggesting that **14av_amine16** interacts with FtsZ protein *in vivo*. The iconic filamentous cell phenotype of FtsZ inhibitors was believed to be caused by the disruption and mislocalization of the Z-ring.

b) Z-ring Visualization of *B. subtilis* 168

[0133] Procedure: A single colony of FtsZ GFP fusion stain of *B. subtilis* 168 was grown in 5 ml 2×TY medium with 35 mg/L chloramphenicol for 14 hours at 30°C. An overnight culture was then diluted to an OD₆₀₀ of 0.01 by 2×TY medium containing the absence (DMSO) and presence of 6 µM test compounds and 40 µM of IPTG, it grew at 30°C after 4 hours of incubation; the cells were pelleted and re-suspended in 300 µL PBS buffer containing 1% agarose. 10 µL suspensions were then placed on the microscopic slide. The morphology of bacterial cell was observed under a fluorescent and phase-contrast microscope at 40 × magnification, using the software of Olympus FSX100® Bio Imaging Navigator to capture images.

[0134] Results: A fluorescence microscopic analysis of dynamic Z-ring formation in *B. subtilis* 168 was carried out by using a functional green fluorescent protein tagged FtsZ in *B. subtilis* 168. In the absence of compound (DMSO treated), fluorescent foci corresponding to Z-ring formation were observed at mid-cell. Each cell possesses only one fluorescent focus, indicating the proper Z-ring formation and localization (Figure 19C, white arrow). In contrast, upon exposure to compound **14av_amine16**, bacteria cell lacked mid-cell foci. Instead, FtsZ protein was randomly distributed as discrete multiple foci (Figure 19E, white arrows) throughout the whole elongated cell, demonstrating that compound **14av_amine16** caused obvious mislocalization of the Z-ring.

[0135] In summary, a new class of amine-linked 2,4,6-trisubstituted pyrimidines with potent anti-staphylococcal activity was discovered. SAR studies of these compounds identified seven compounds possessing potent and selective anti-staphylococcal activity with MIC values of 5 µM. These compounds exhibited potent antibacterial activities against clinically isolated MRSA strains. These promising results led to efficacy testing of **14av_amine16**, which revealed a very low spontaneous frequency of resistance and limited toxicity against *G. mellonella* larvae. Investigation of the mode of action suggests that compound **14av_amine16** exerted its antibacterial activity by suppressing FtsZ polymerization, resulting in obvious mislocalization of the Z-ring formation. The observed potency makes the 2,4,6-trisubstituted pyrimidines of the present invention excellent anti-staphylococcal agents.

References

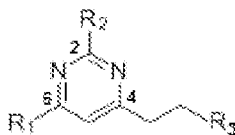
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What is claimed is:

1. A pyrimidine having the structure of formula (I):



(I)

- 5 wherein R_1 and R_2 each independently represents alkyl having 1-10 carbon atoms, aryl having one or two 6-membered aromatic rings, or heteroaryl having one or two 6-membered aromatic rings with at least one nitrogen (N) as the heteroatom; and R_3 represents a tertiary amine $-NR_4R_5$.
- 10 2. The pyrimidine of claim 1, wherein R_1 is selected from the group consisting of alkyl having 1-8 carbon atoms, phenyl and substituted phenyl.
3. The pyrimidine of claim 1, wherein R_2 is selected from the group consisting of alkyl having 1-8 carbon atoms, phenyl, substituted phenyl, pyridin-2-yl, pyridin-3-yl and pyridin-4-yl.

- 15 4. The pyrimidine of claim 1, wherein R_3 is  and R_6 is selected from alkyl having 1-10 carbon atoms, optionally substituted benzyl, optionally substituted phenyl, pyridylmethyl, optionally substituted benzoyl, optionally substituted phenethyl, piperonyl, 3-phenyl-2-propen-1-yl, optionally substituted cinnamoyl, alkyl sulphonyl, aryl sulphonyl and heteroaryl sulphonyl.

- 20 5. The pyrimidine of claim 1, wherein R_3 is  and R_7 is selected from alkyl having 1-10 carbon atoms, optionally substituted benzyl, optionally substituted phenyl, pyridylmethyl, optionally substituted benzoyl, optionally substituted phenethyl, piperonyl, 3-phenyl-2-propen-1-yl, optionally substituted cinnamoyl, alkyl sulphonyl, aryl sulphonyl and heteroaryl sulphonyl.

- 25 6. The pyrimidine of claim 1, wherein R_3 is $-N(R_8)-[CH_2]_m-N(R_9)(R_{10})$, m is an integer selected from 2, 3, 4 and 5, and each of said R_8 , R_9 and R_{10} is independently selected from alkyl having 1-5 carbon atoms, and optionally substituted benzyl.

7. The pyrimidine of claim 1, selected from the group consisting of:
 1-(2-(6-phenyl-2-(pyridin-4-yl)pyrimidin-4-yl)ethyl)-4-(4-(trifluoromethyl)benzyl)-1,4-diazepane;
 1-(4-isopropylbenzyl)-4-(2-(6-phenyl-2-(pyridin-4-yl)pyrimidin-4-yl)ethyl)-1,4-diazepane;
 1-(2-(6-phenyl-2-(pyridin-4-yl)pyrimidin-4-yl)ethyl)-4-(4-(trifluoromethyl)phenethyl)-1,4-diazepane; 1-(4-(*tert*-butyl)benzyl)-4-(2-(6-phenyl-2-
- 30

- (pyridin-4-yl)pyrimidin-4-yl)ethyl)-1,4-diazepane;
1-(4-bromobenzyl)-4-(2-(6-phenyl-2-(pyridin-4-yl)pyrimidin-4-yl)ethyl)-1,4-diazepane;
1-(2-(6-(pentan-3-yl)-2-(pyridin-4-yl)pyrimidin-4-yl)ethyl)-4-(4-(trifluoromethyl)benzyl)-1,4-diazepane; and
1-(2-(6-(pentan-3-yl)-2-(pyridin-4-yl)pyrimidin-4-yl)ethyl)-4-(4-(trifluoromethyl)phenethyl)-1,4-diazepane.
8. A composition comprising the pyrimidine of any of claims 1-7 or its pharmaceutically acceptable salt, and a pharmaceutically acceptable carrier.
9. Use of the composition of claim 8 for the preparation of a medicament for treating a bacterial infection in a subject, said medicament comprising a therapeutically effective amount of said pyrimidine.
10. The use of claim 9, wherein the infection is a skin infection, a gastrointestinal infection, a respiratory infection, a urinary tract infection, or a reproductive tract infection.
11. The use of claim 9, wherein said infection is caused by one or more of the bacteria selected from the group consisting of *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecalis*, *Bacillus subtilis*, *Propionibacterium acnes*, and *Clostridium difficile*.
12. The use of claim 9, wherein the infection is caused by one or more of the bacteria selected from the group consisting of Gram-negative bacteria and Gram-positive bacteria.
13. The use of claim 9, wherein the infection is caused by multiple drug-resistant bacteria.
14. The use of claim 9, wherein the pyrimidine inhibits bacterial cell division.
15. The use of claim 9, wherein the pyrimidine inhibits Z-ring formation by filamenting temperature-sensitive mutant Z (FtsZ) protein within a bacterial cell.
16. The use of claim 9, wherein the pyrimidine binds to the guanosine triphosphate (GTP) binding site of FtsZ within a bacterial cell.
17. The use of claim 9, wherein said medicament is administered in combination with other compounds to achieve synergistic treatment of the bacterial infection.
18. The use of claim 9, wherein the subject is a vertebrate, a mammal or human.
19. The use of claim 9, wherein the medicament is administered orally, nasally, aurally, ocularly, sublingually, buccally, systemically, transdermally, mucosally, via cerebral spinal fluid injection, vein injection, muscle injection, peritoneal injection, subcutaneous injection, or by inhalation.
20. The use of claim 9, wherein the medicament is formulated in the form of a cream, gel, ointment, suppository, tablet, granule, injection, powder, solution, suspension, spray, patch or capsule.

Figure 1

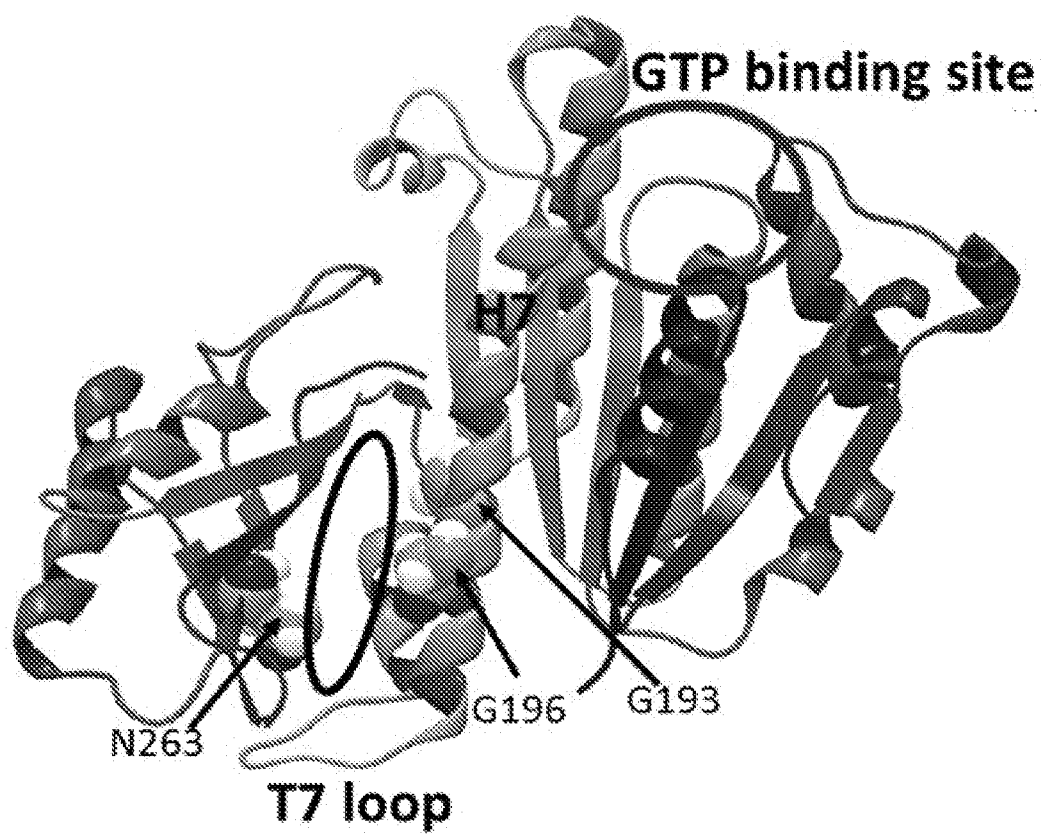


Figure 2

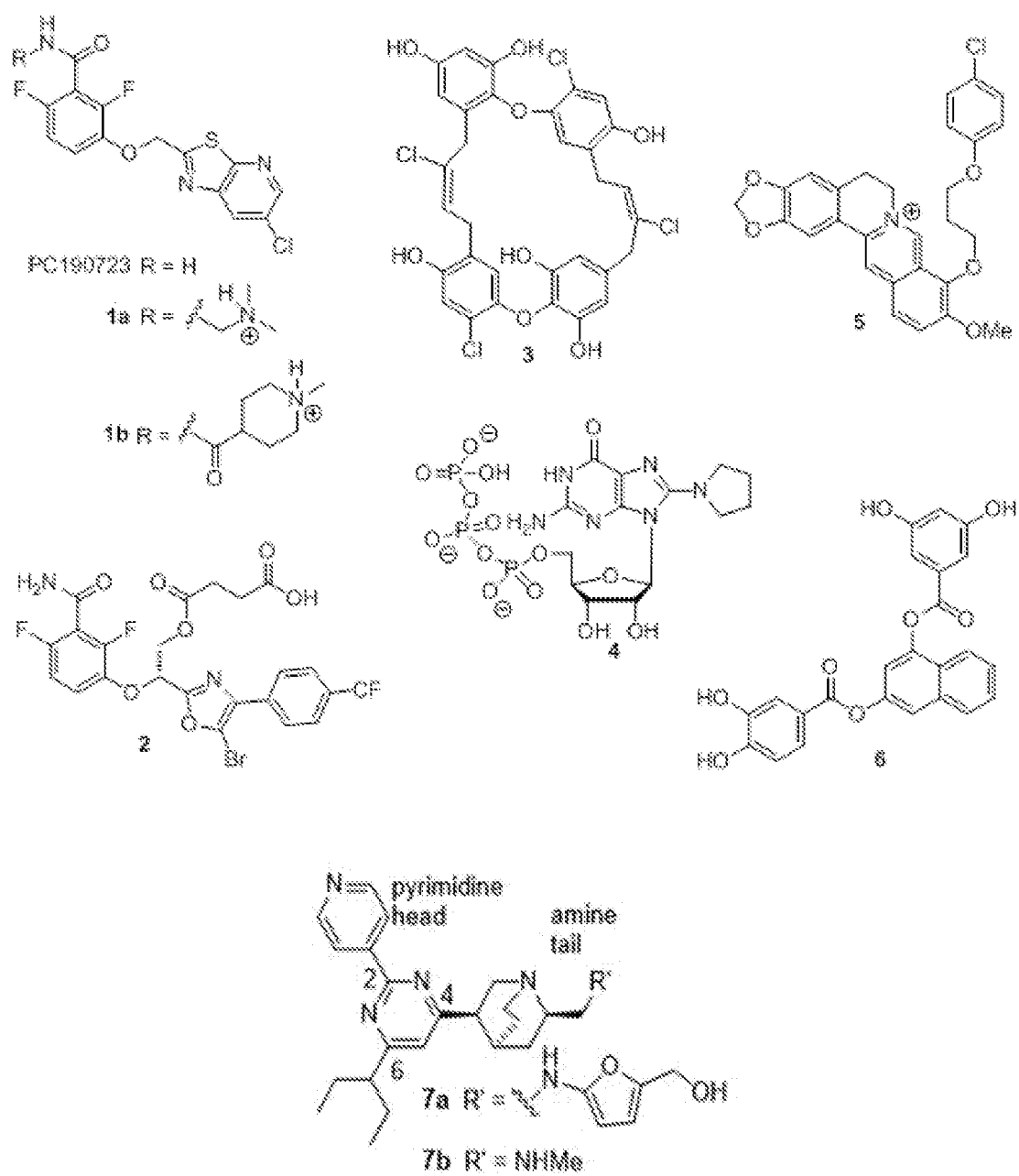


Figure 3

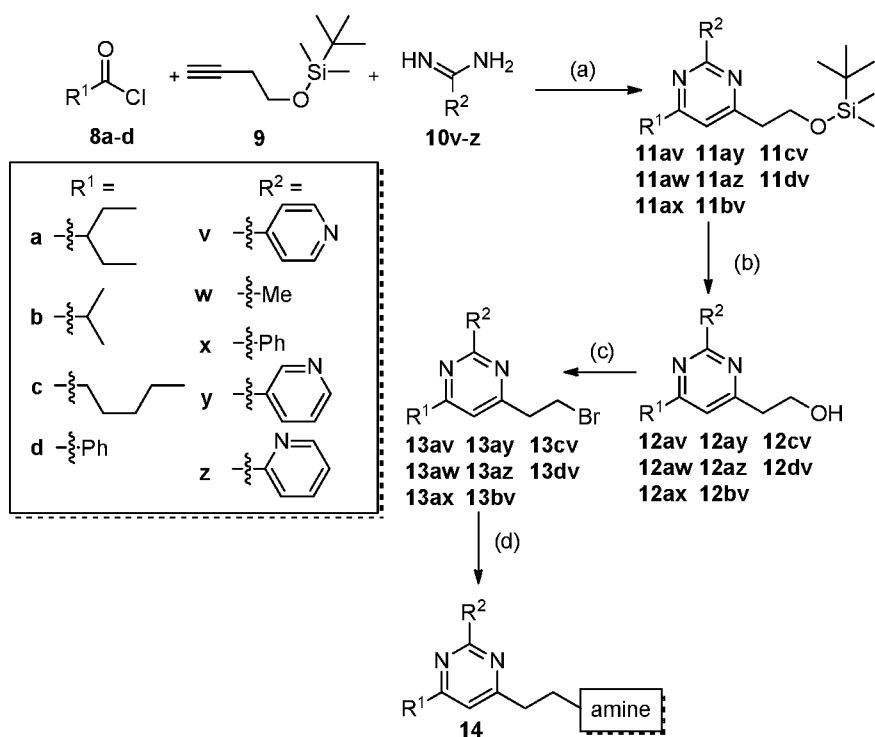


Figure 4

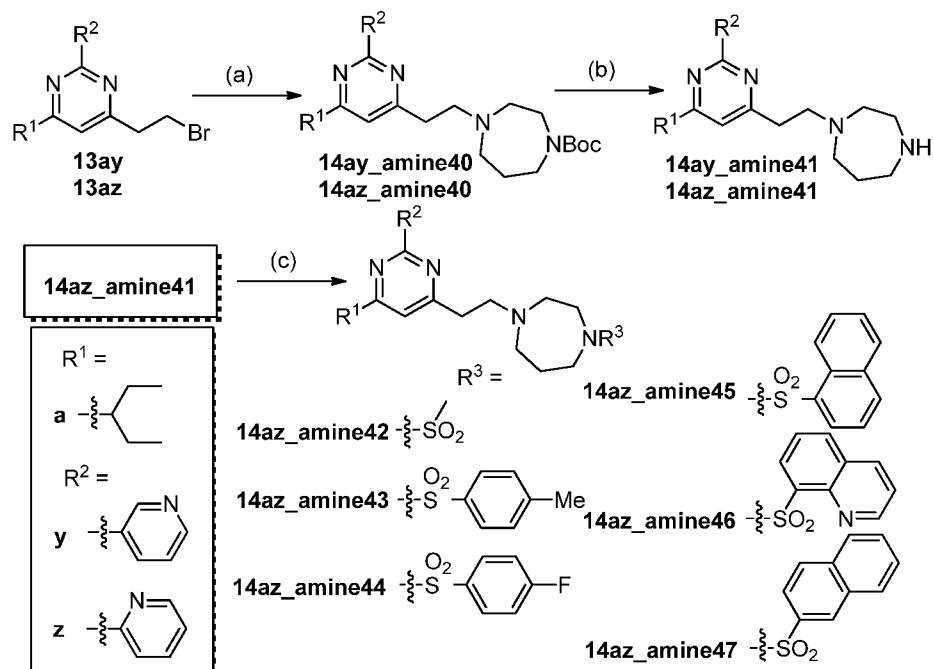


Figure 5

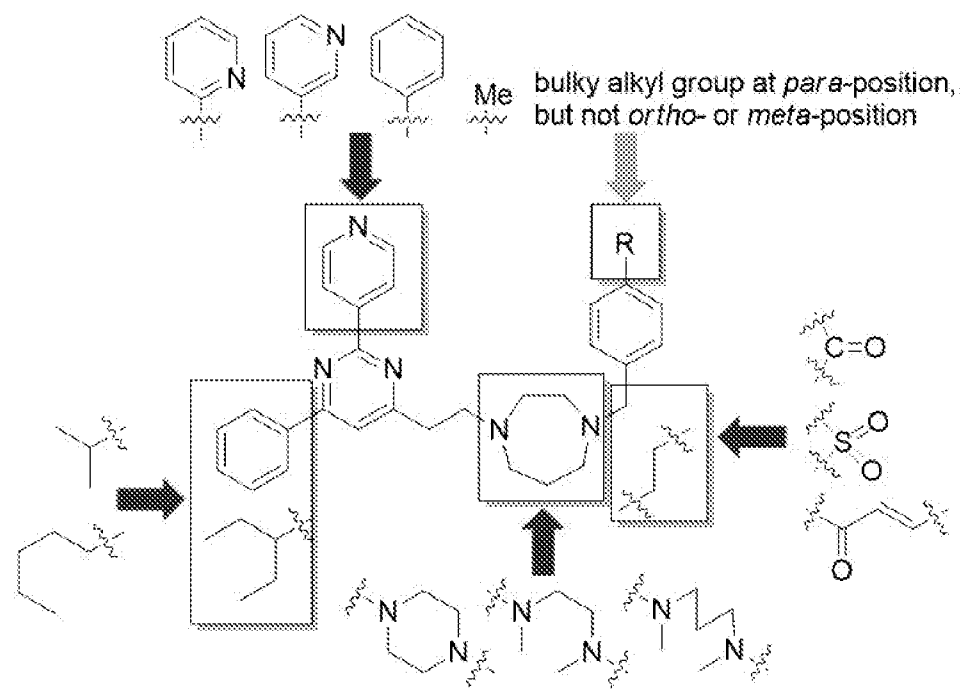


Figure 6

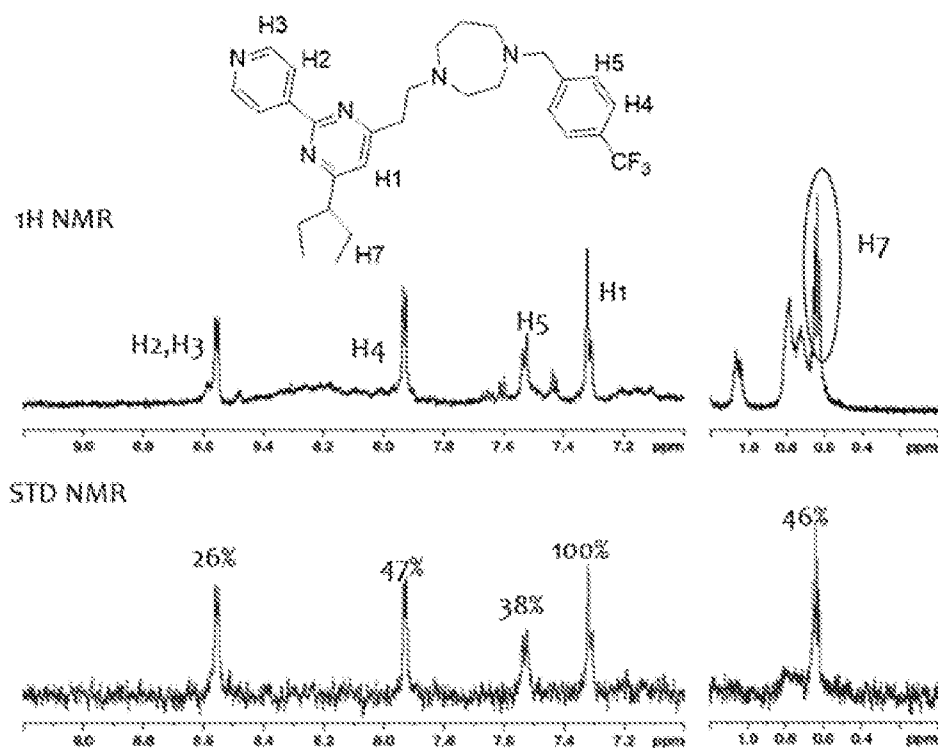


Figure 7

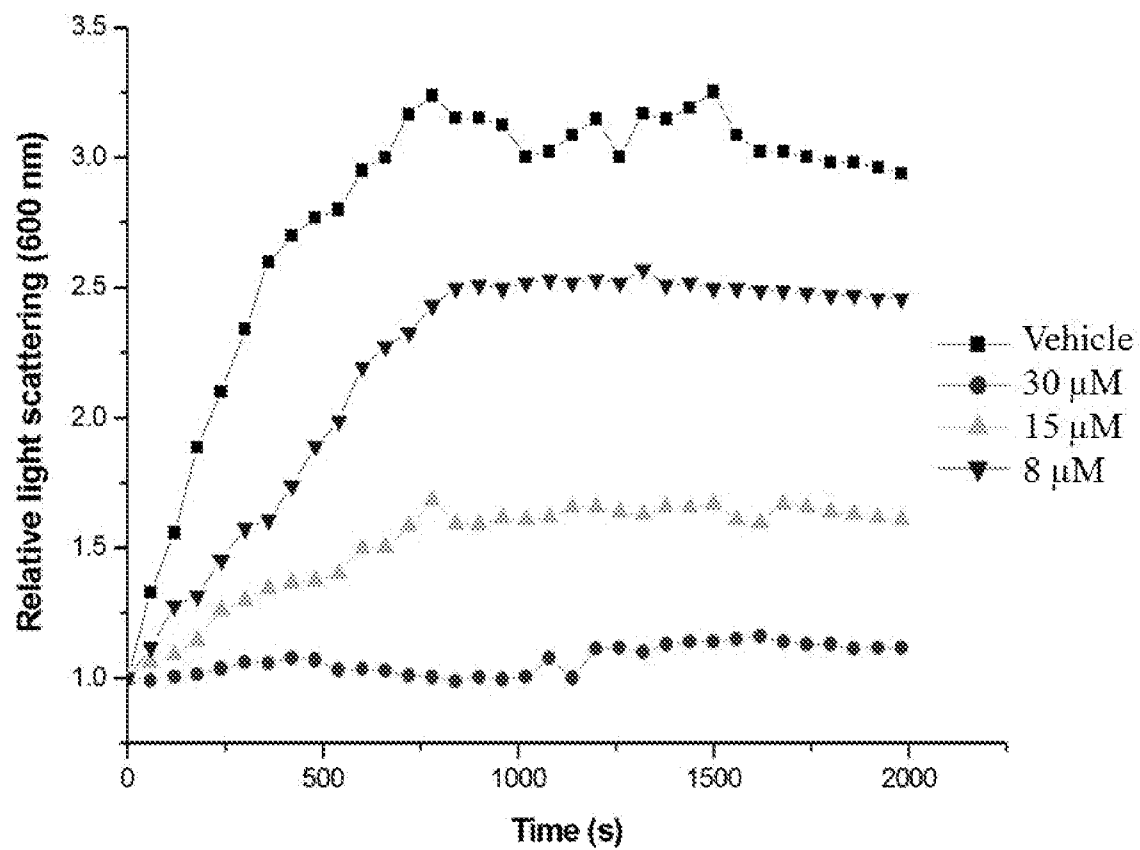


Figure 8A

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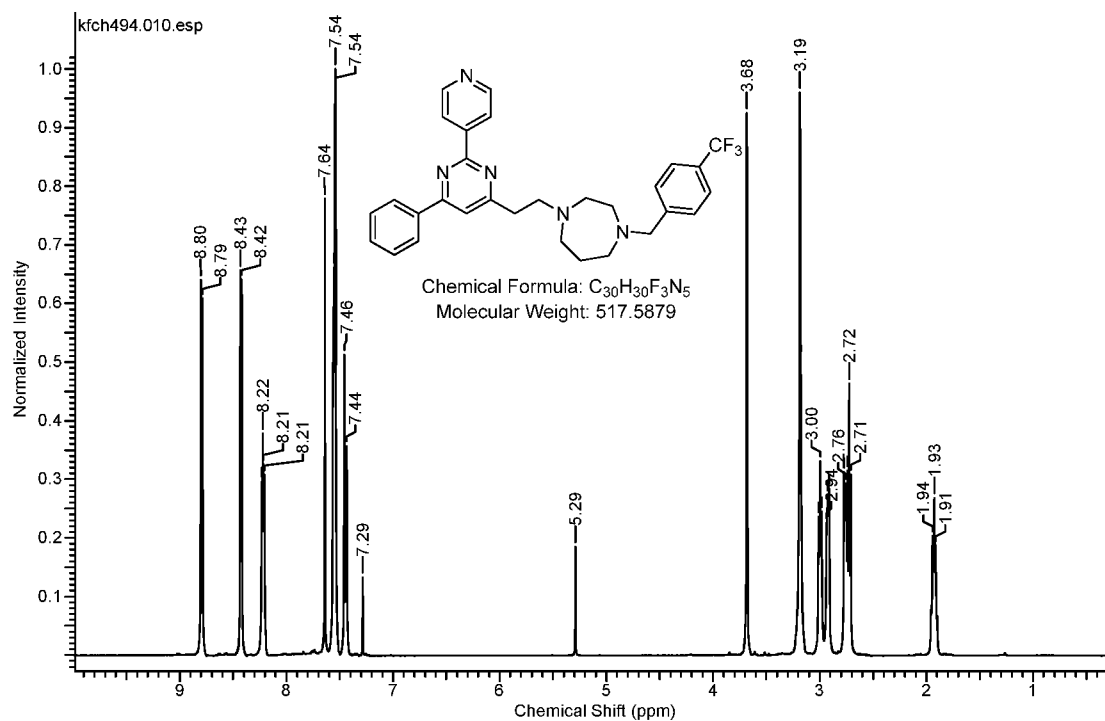


Figure 8B

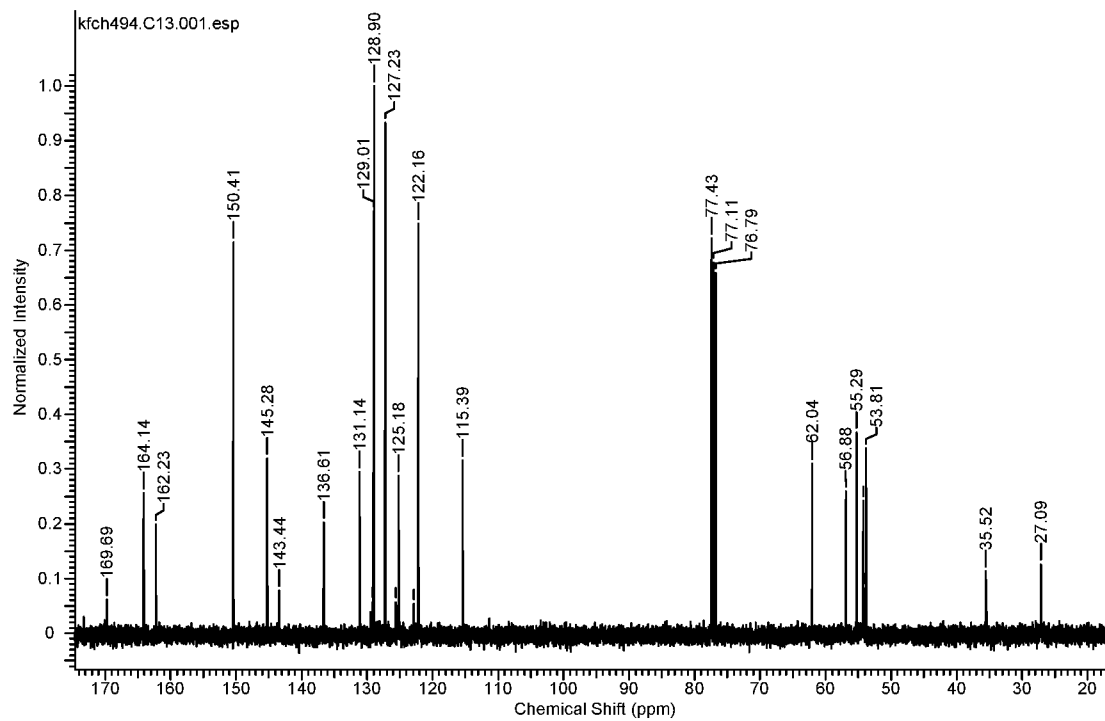


Figure 9A

14dv_amine06

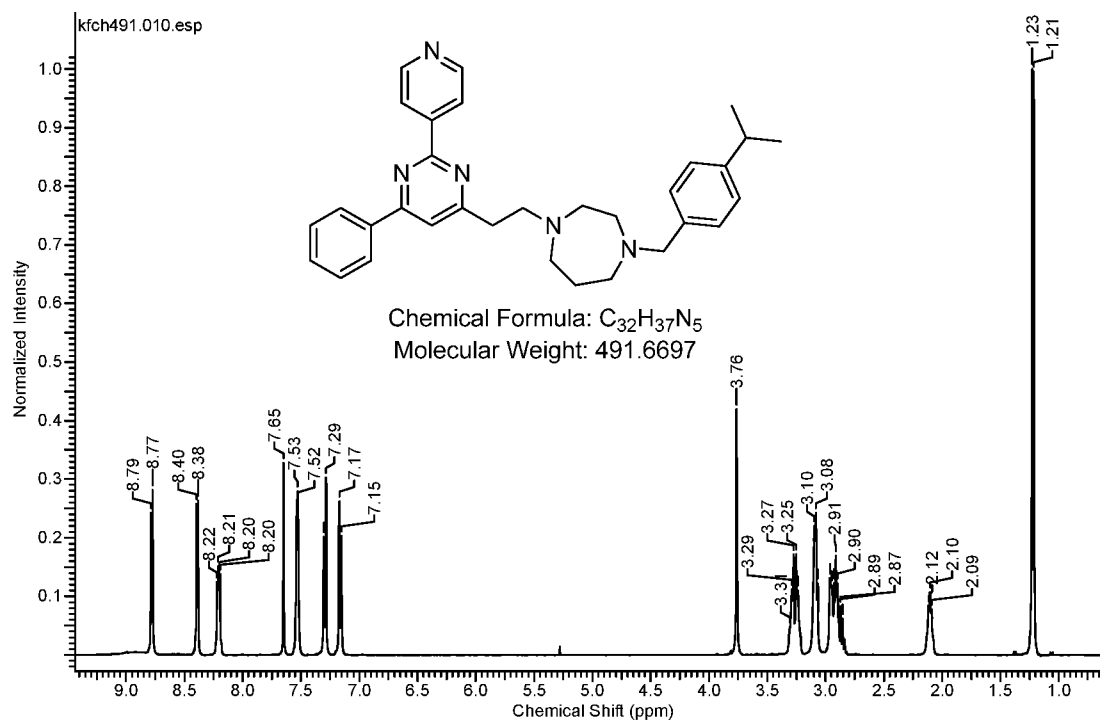


Figure 9B

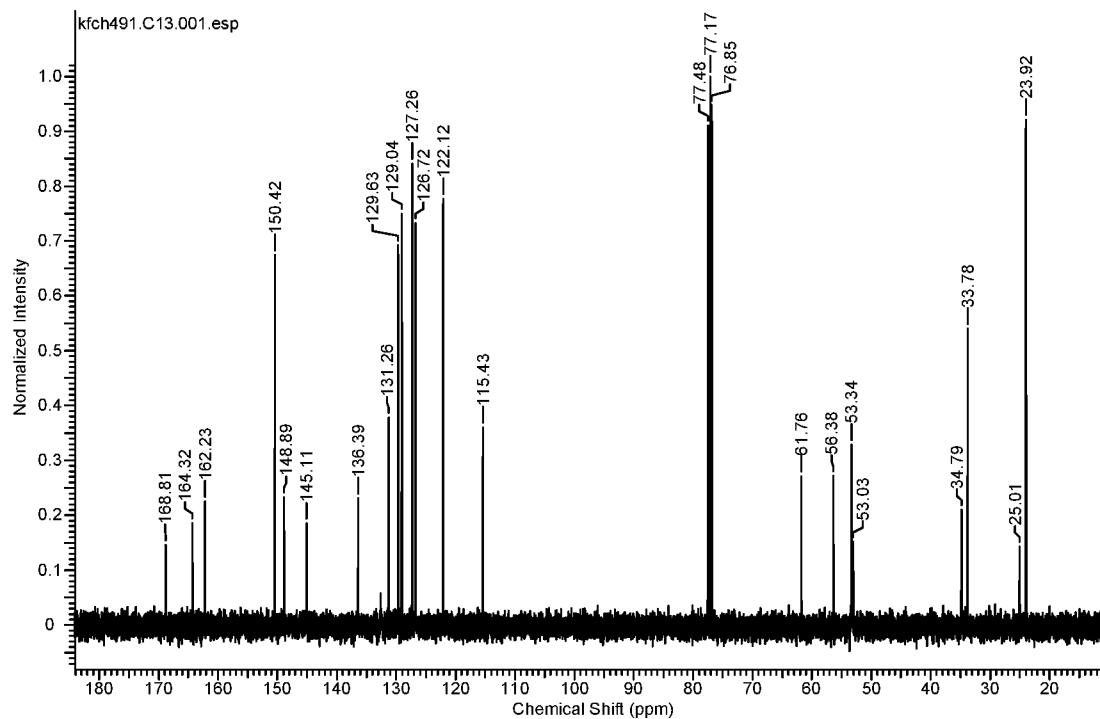


Figure 10A

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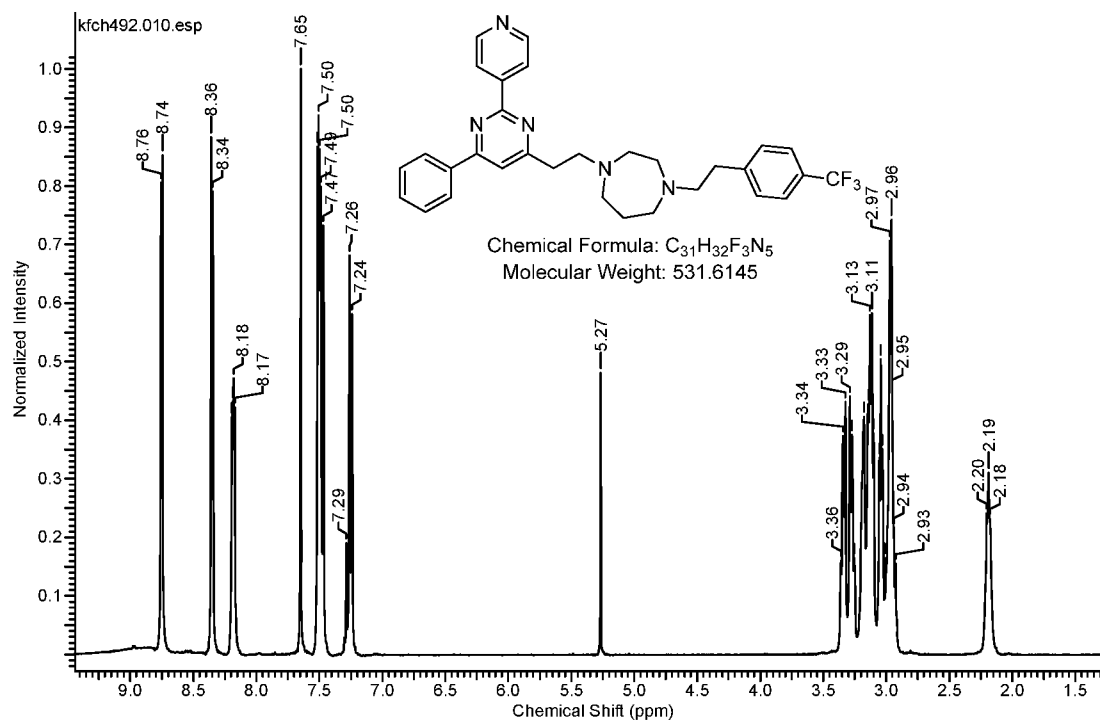


Figure 10B

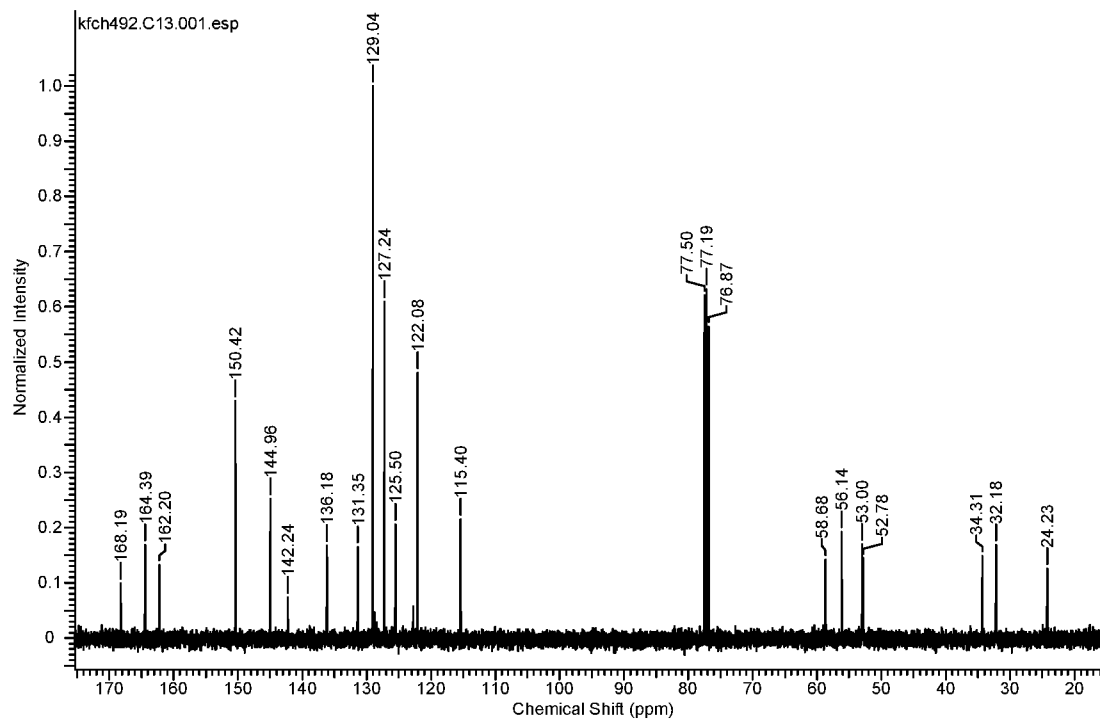


Figure 11A

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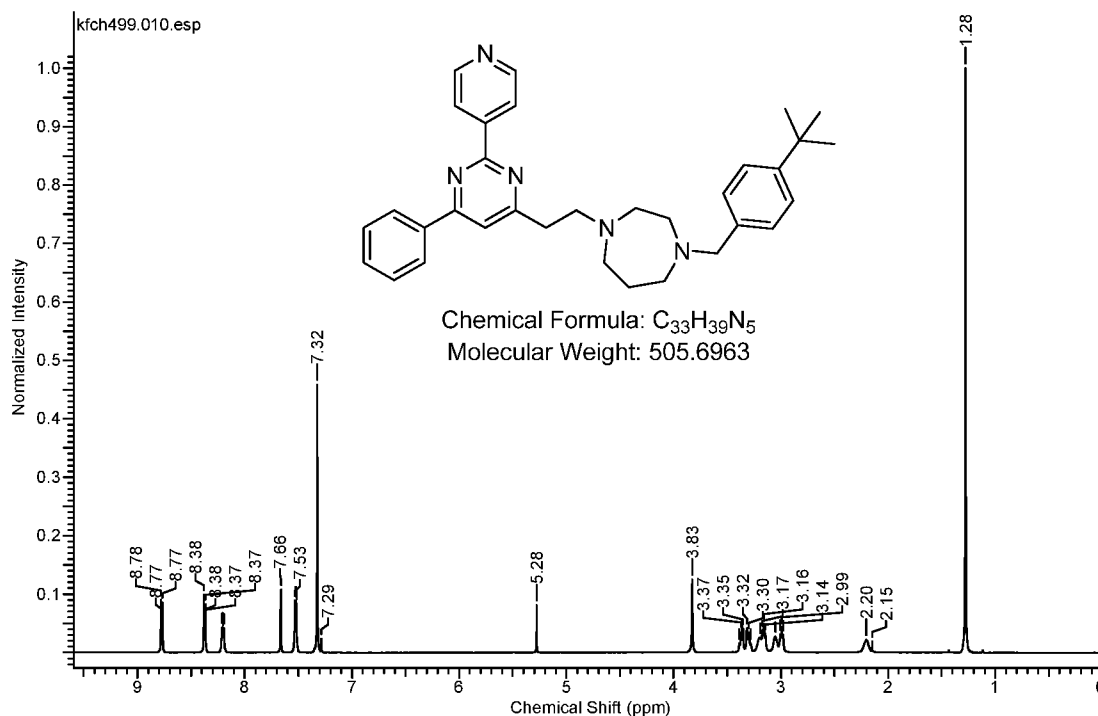


Figure 11B

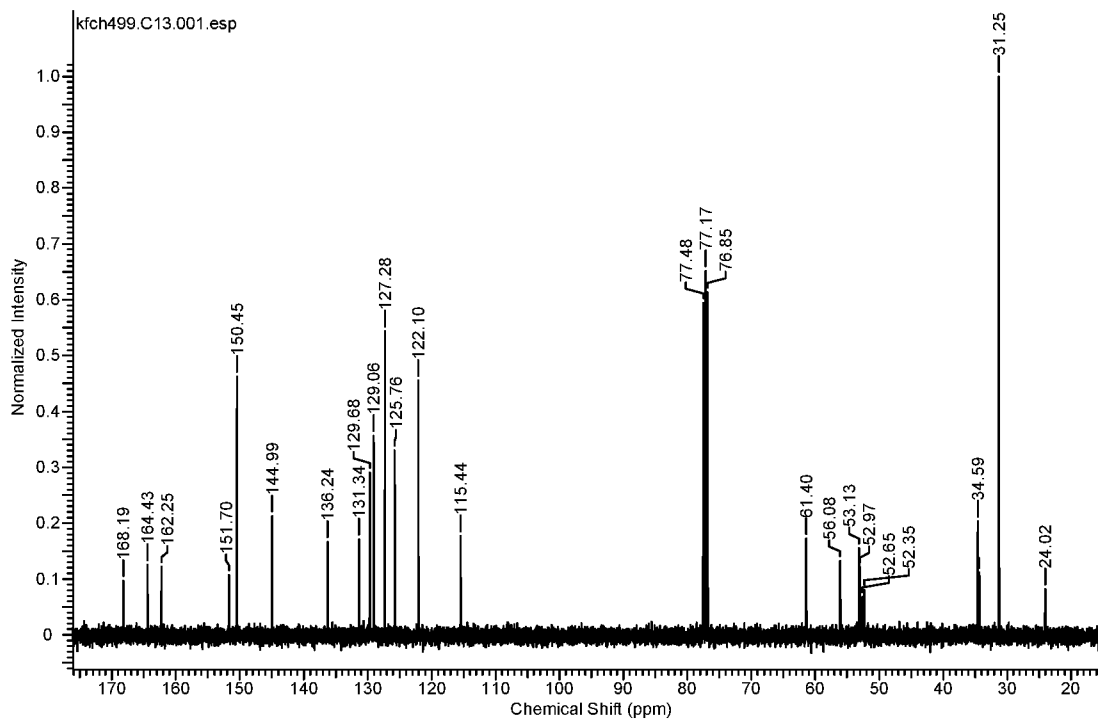


Figure 12A

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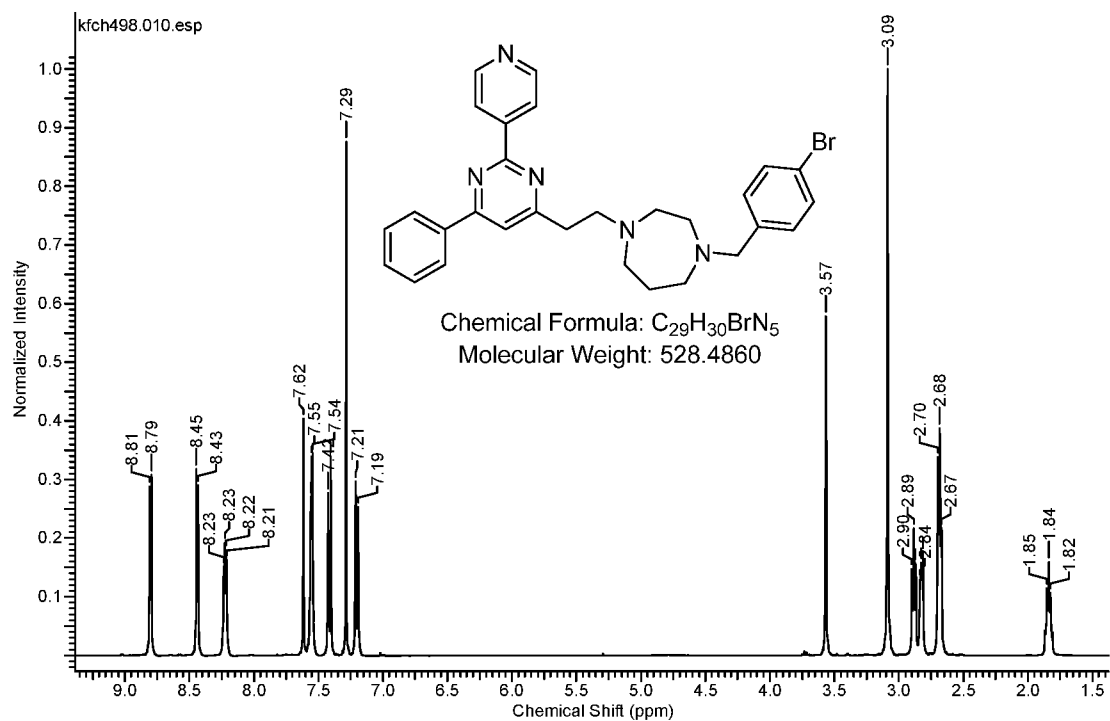


Figure 12B

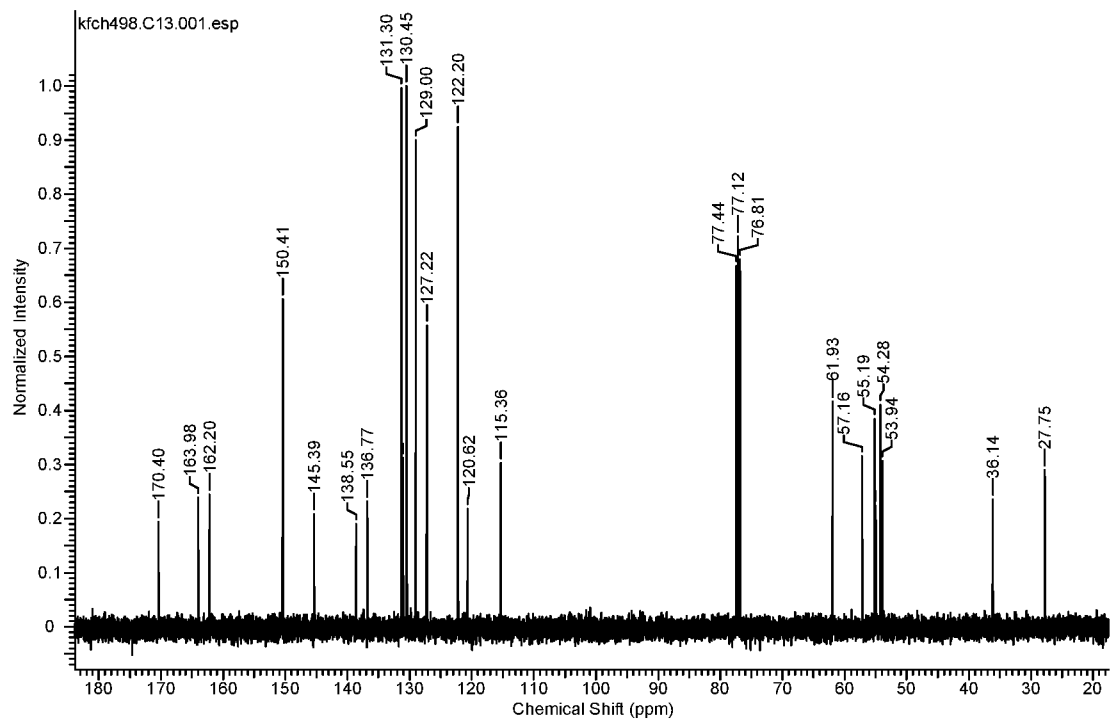


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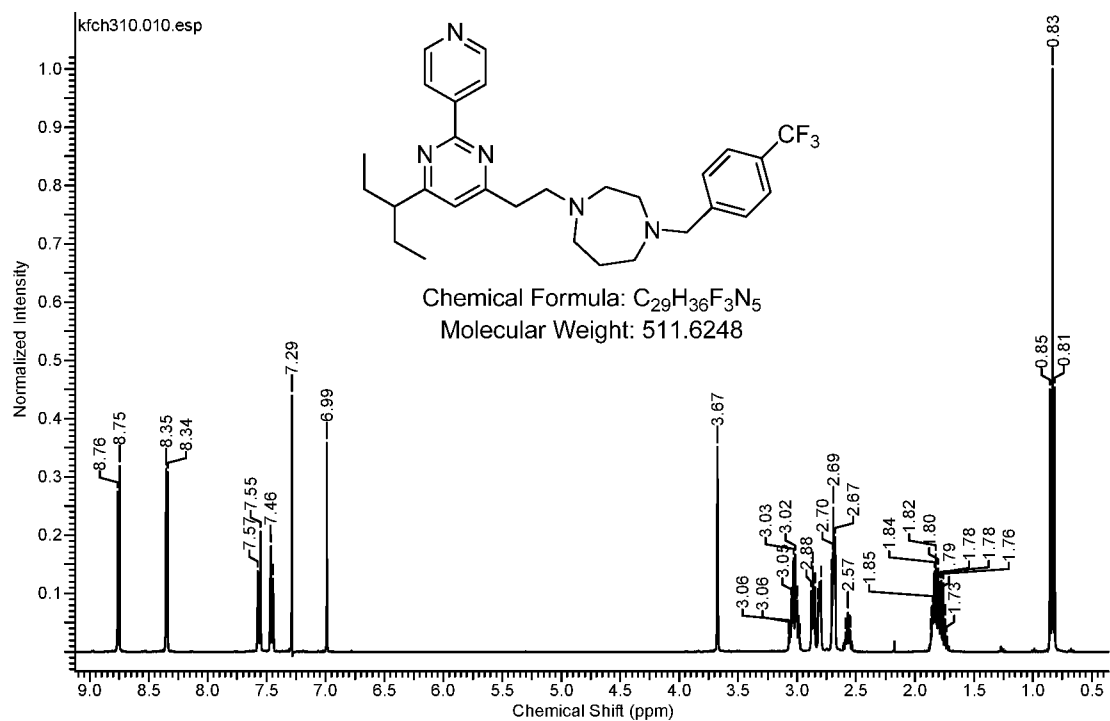
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Figure 13B

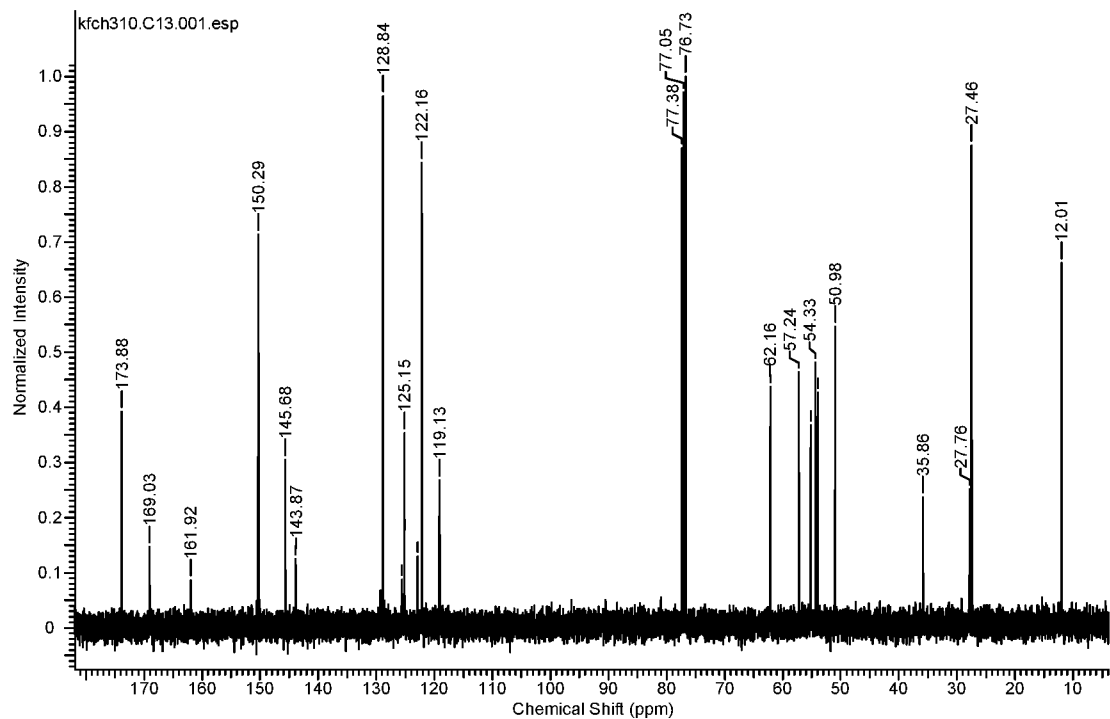


Figure 14A

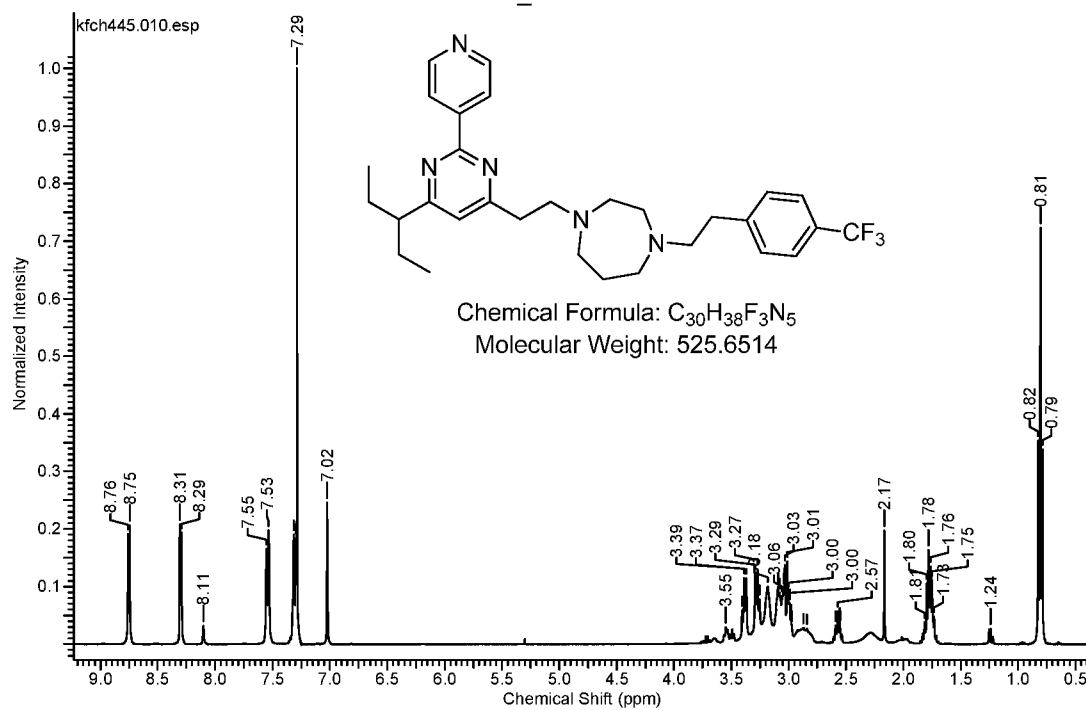
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Figure 14B

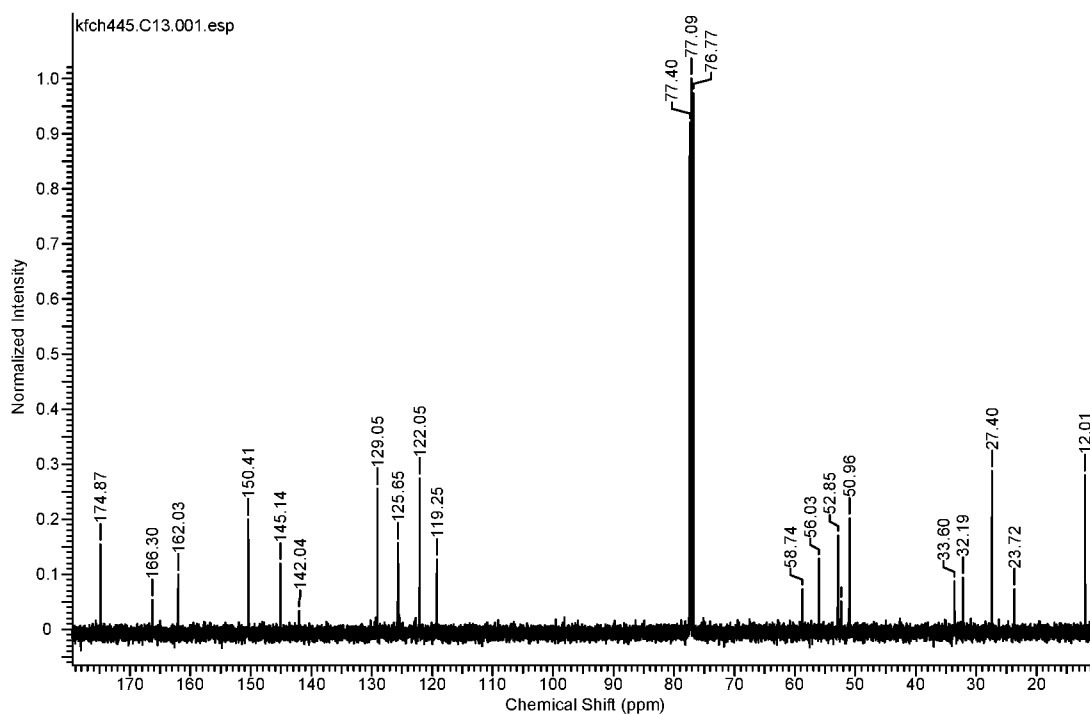


Figure 15A

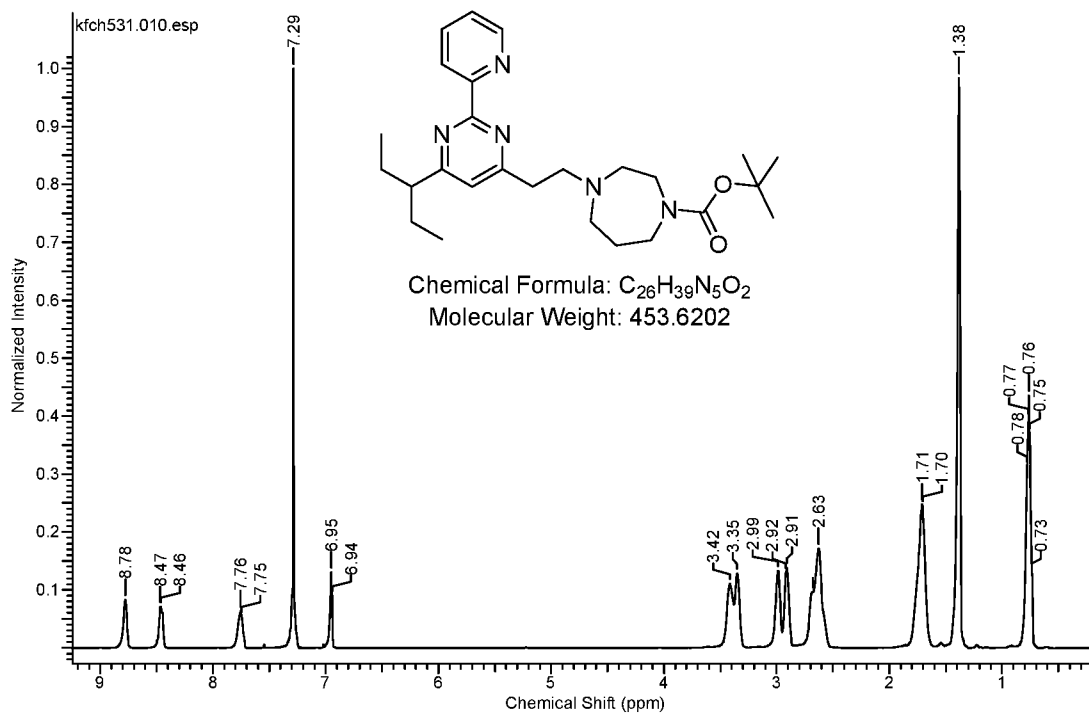
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Figure 15B

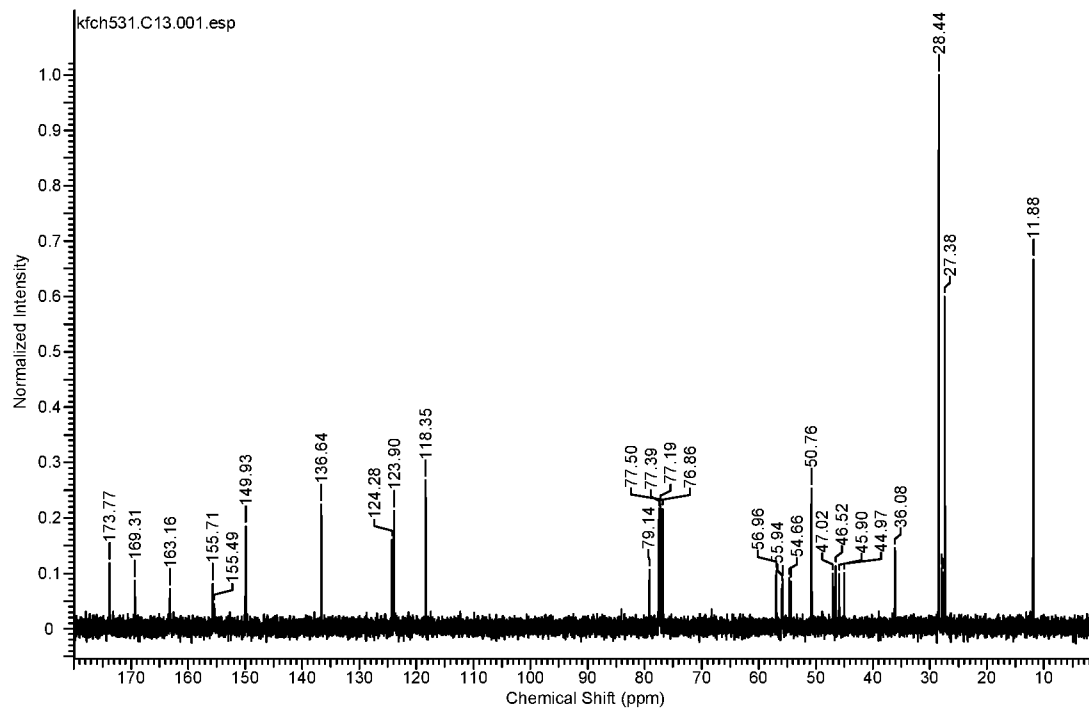


Figure 16A

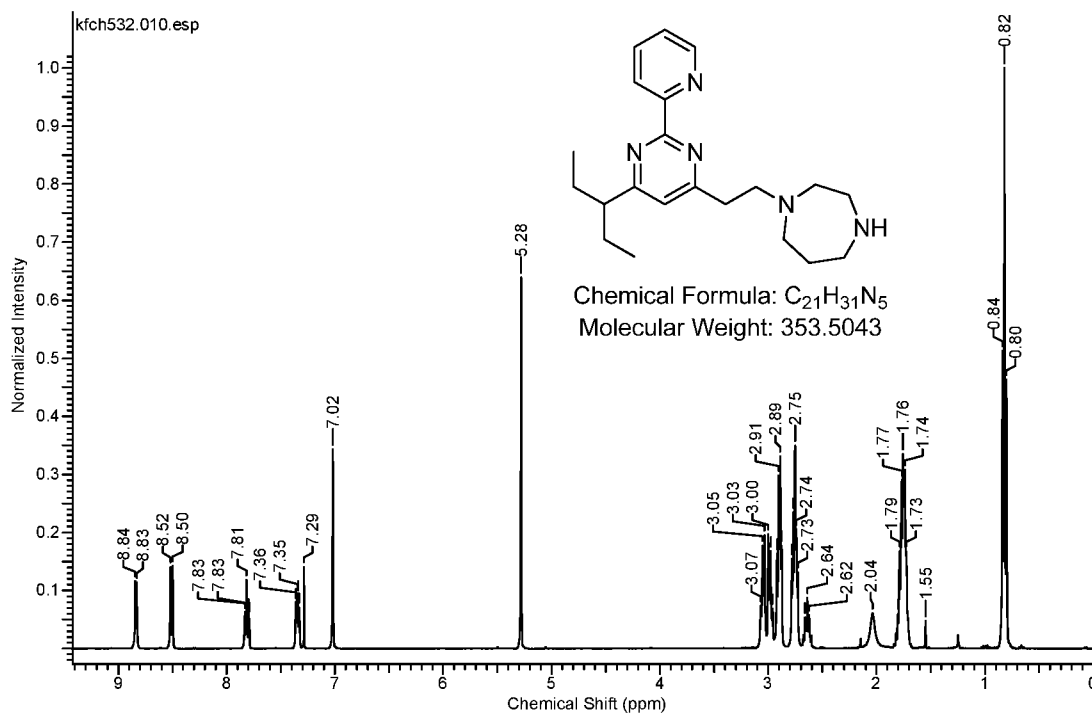
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Figure 16B

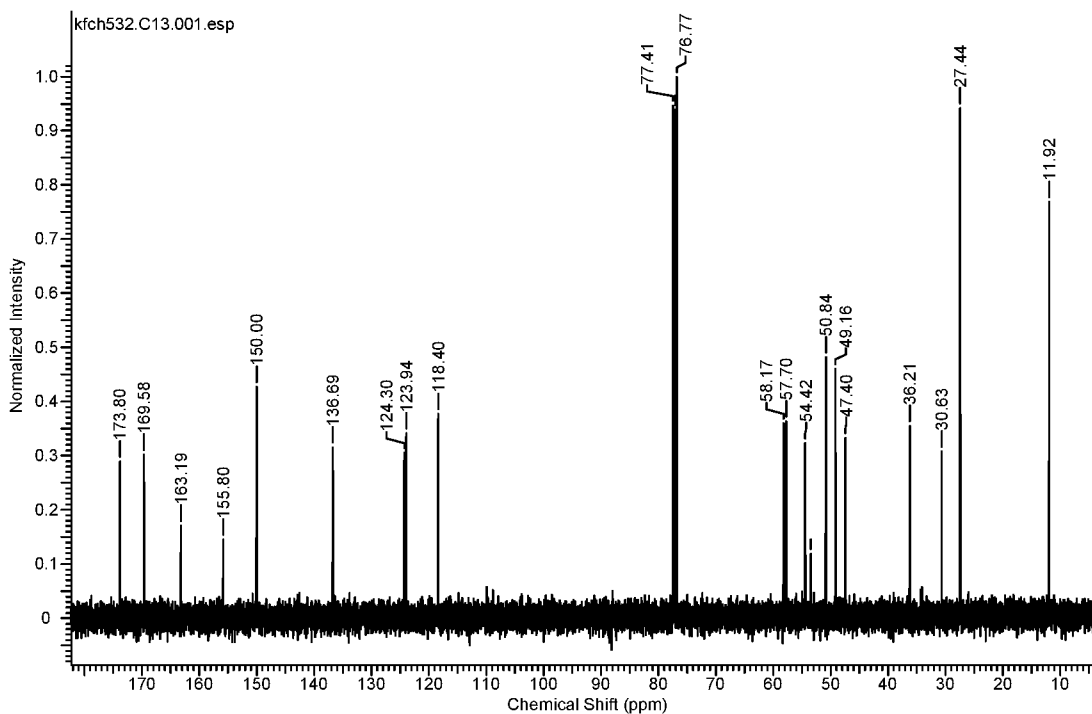


Figure 17A

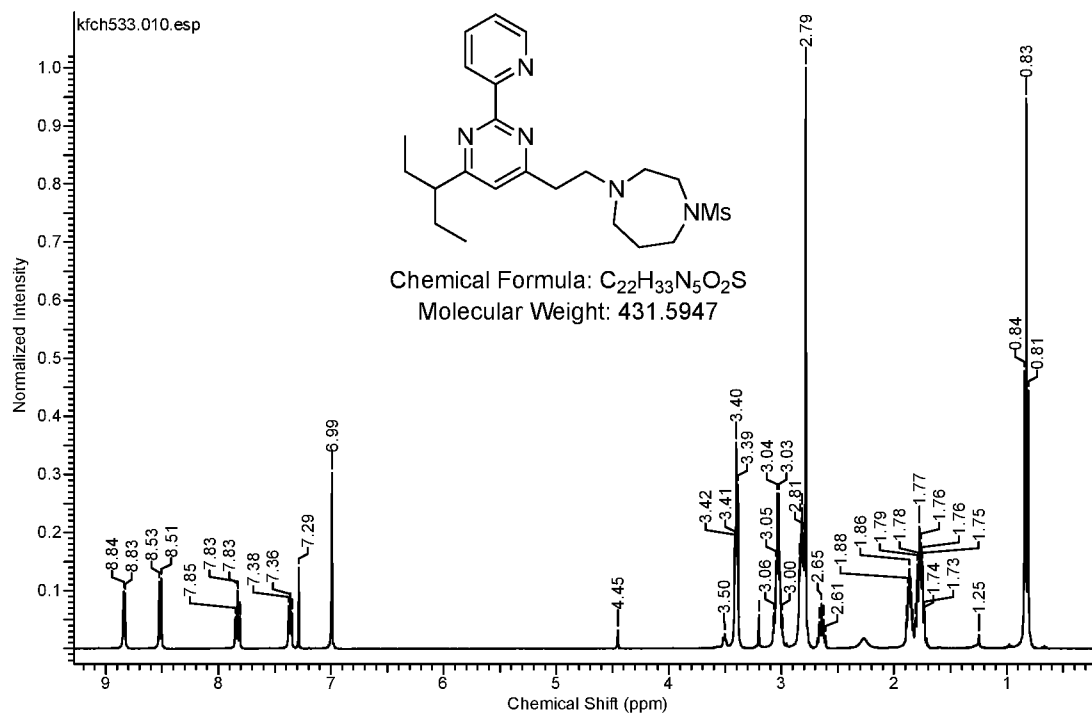
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Figure 17B

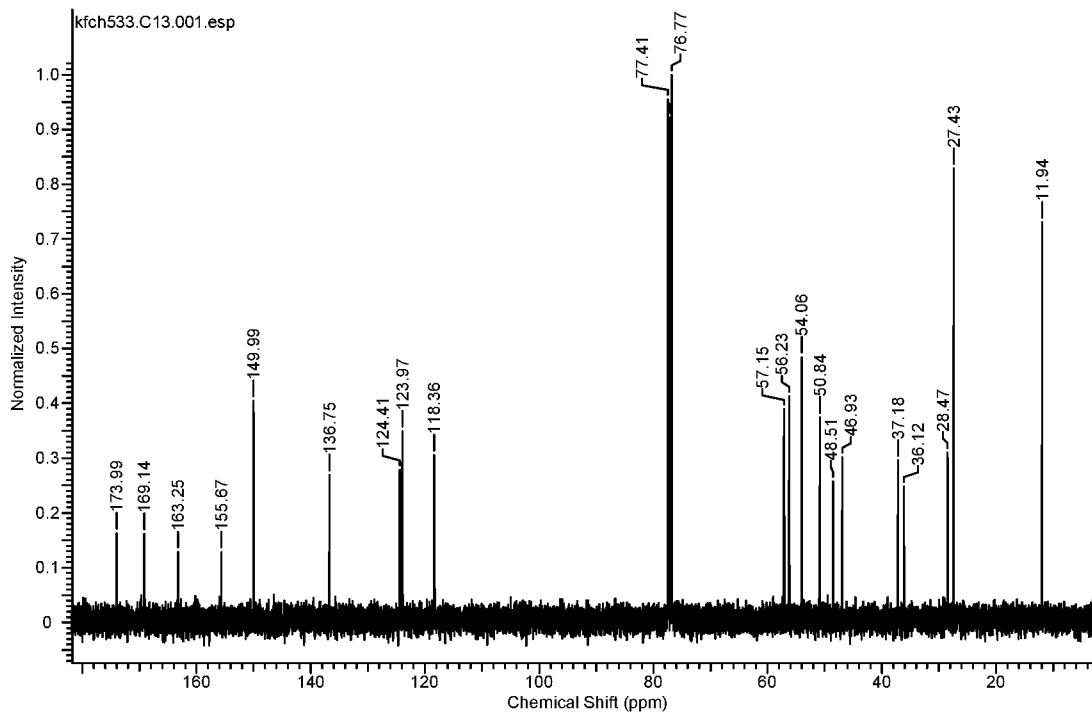


Figure 18A

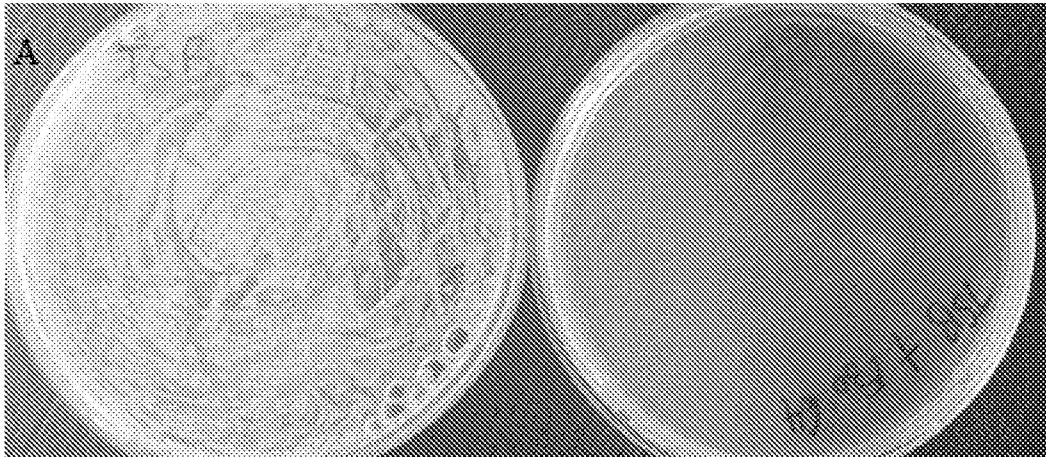


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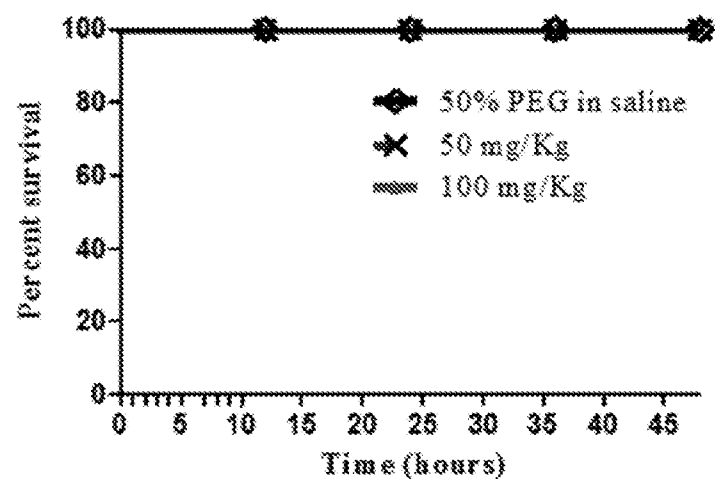


Figure 18C

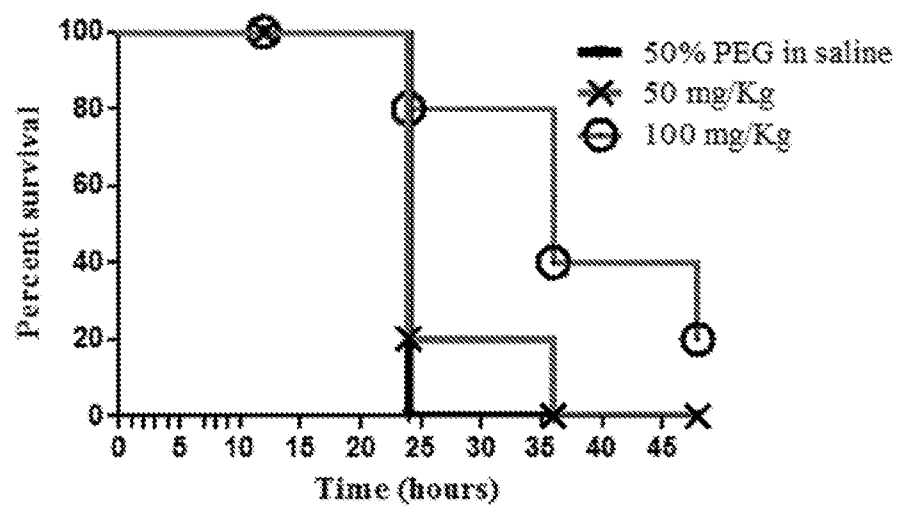


Figure 19A

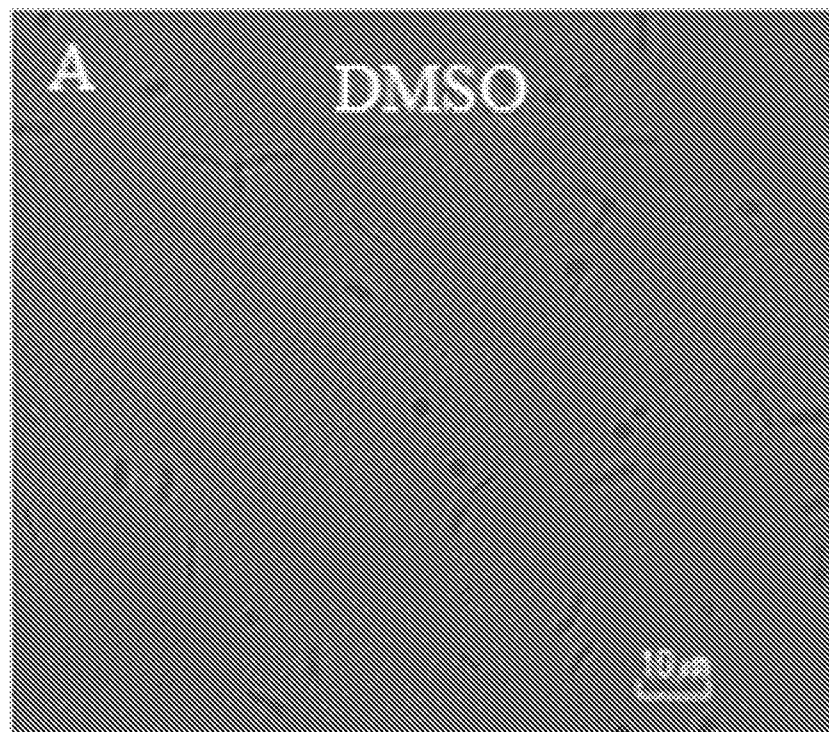


Figure 19B

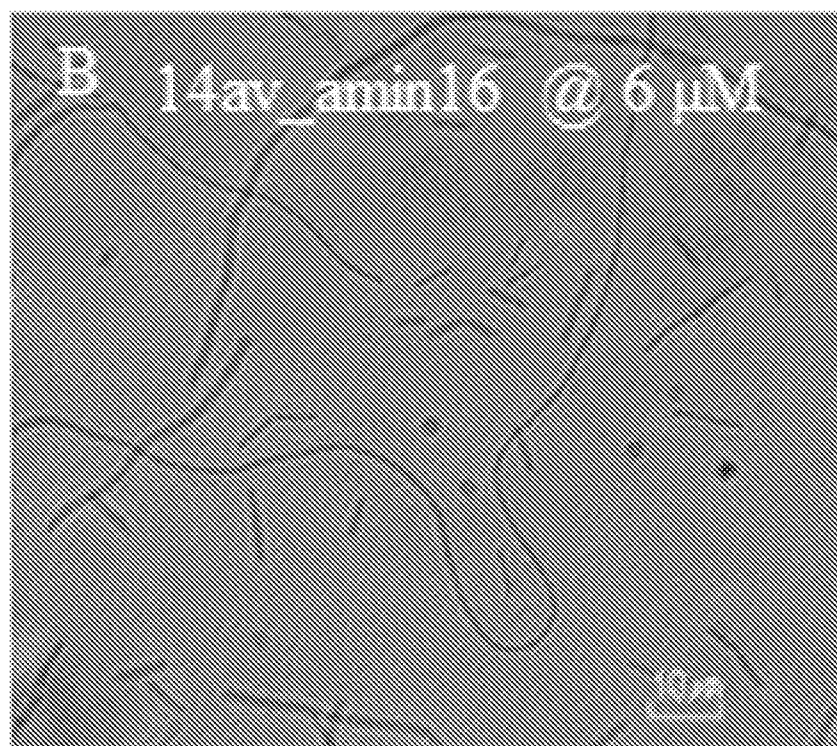


Figure 19C

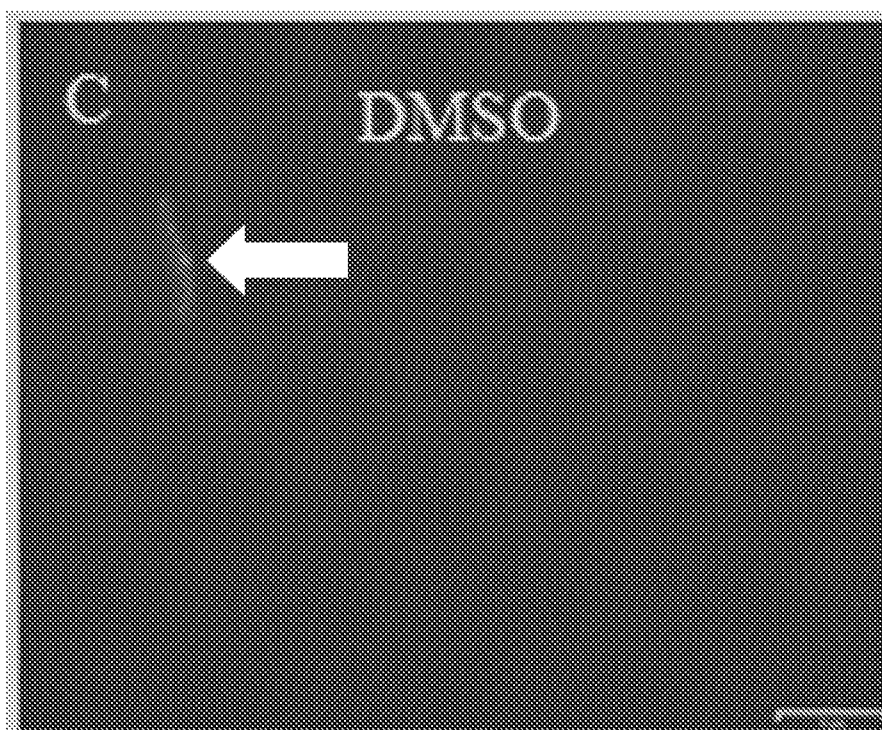


Figure 19D

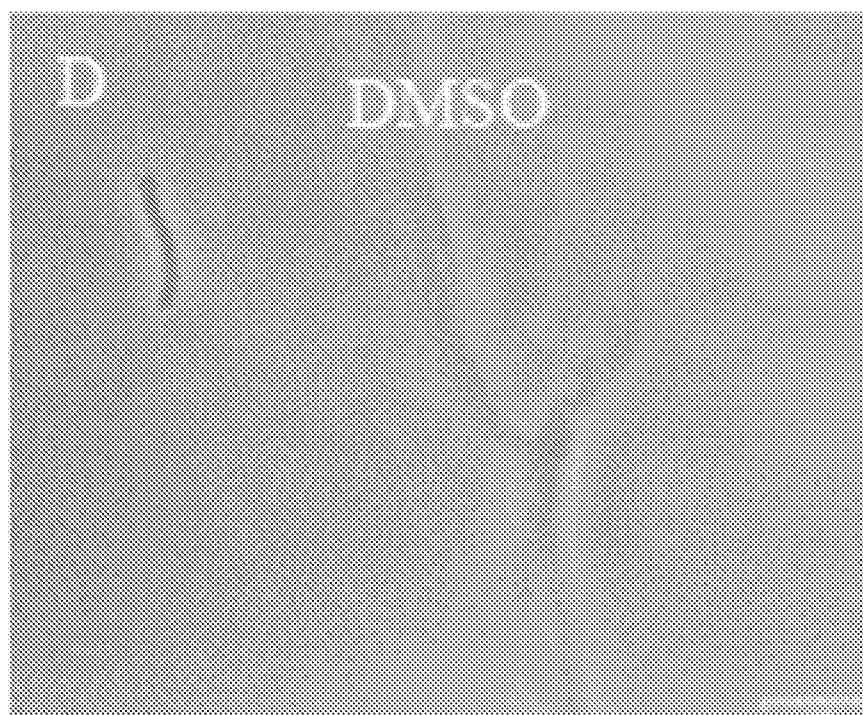


Figure 19E

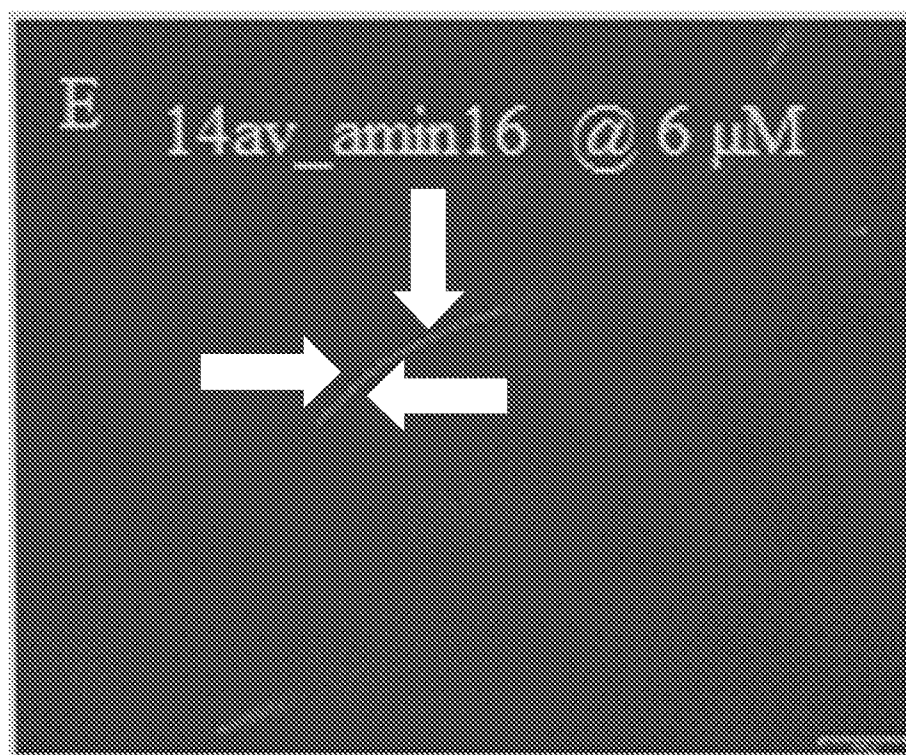
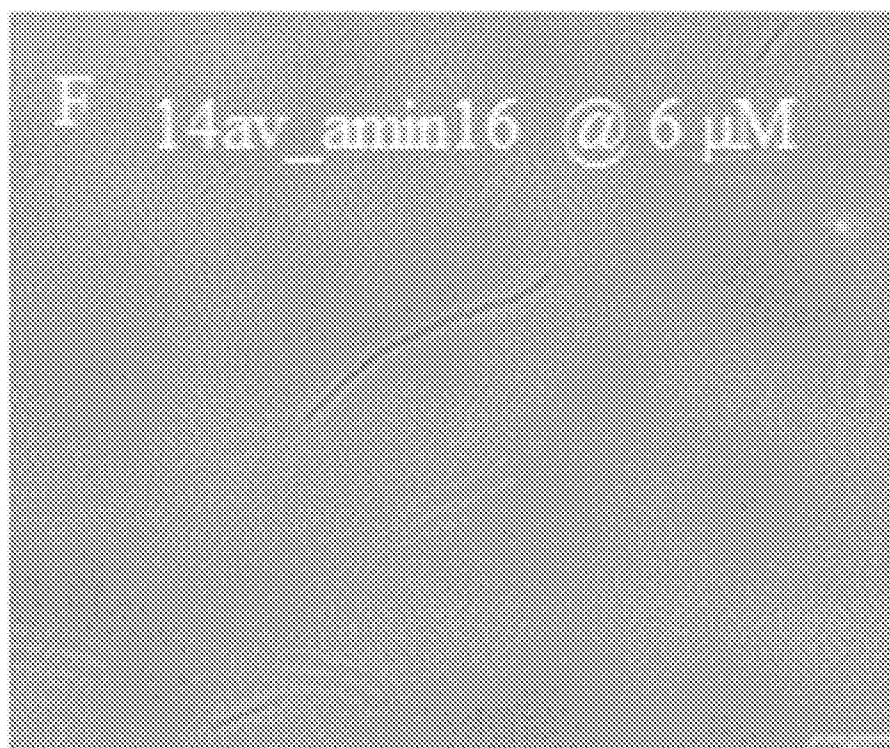


Figure 19F



INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB2016/055839

A. CLASSIFICATION OF SUBJECT MATTER		
C07D 239/02(2006.01)i; A61K 31/7064(2006.01)i; A61K 9/00(2006.01)i; A61K 9/06(2006.01)i; A61K 9/08(2006.01)i; A61K 9/14(2006.01)i; A61K 9/16(2006.01)i; A61K 9/20(2006.01)i; A61K 9/40(2006.01)i; A61K 9/48(2006.01)i; A61K 9/72(2006.01)i; A61P 31/04(2006.01)i		
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) C07D,A61K,A61P		
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) WPI, EPODOC, CPRS(CN), CNKI, CTMPD(CN), CHEMICAL PHARMACEUTICAL ABSTRACT(CPA), STN: pyrimidine, pyridin,diazepane,bacteria,,filamenting temperature-sensitive mutant Z, FtsZ, structure search on the basis of claims		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Fung-Yi Chan et al. "Identification of a New Class of FtsZ Inhibitors by Structure-Based Design and in Vitro Screening" <i>Journal of Chemical Information and Modeling</i> , Vol. 53, 12 July 2017 (2017-07-12), pages 2131-2140, especially the abstract and table 2	1-20
☐ 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 23 December 2016		Date of mailing of the international search report 12 January 2017
Name and mailing address of the ISA/CN STATE INTELLECTUAL PROPERTY OFFICE OF THE P.R.CHINA 6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing 100088 China		Authorized officer FAN,Chanjuan
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