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(54) Title: ACTIVATORS OF CYLINDRICAL PROTEASES

(57) **Abstract:** The present invention is directed to activators of cylindrical proteases ("ACPs"), particularly ClpP, and the role thereof in the diagnosis and treatment of bacterial infections. A number of ACPs were identified that activate caseinolytic protease P ("ClpP"), which independently can only degrade small peptides. In the presence of ACPs, ClpP may be activated to allow it to degrade larger proteins, hence, abolishing the specificity arising from the ATP-dependent chaperones. Members of the ACPs were found to have bactericidal activity. As such, ACPs represent a new classes of compounds that can activate ClpP and that can be developed as potential novel antibiotics.



ACTIVATORS OF CYLINDRICAL PROTEASES

FIELD OF THE INVENTION

0001 The present invention is directed to activators of cylindrical proteases, particularly ClpP, and the role thereof in the diagnosis and treatment of bacterial infections.

0002 This application claims priority from U.S. provisional patent application No. 61/423,953, filed on December 16, 2010.

BACKGROUND OF THE INVENTION

0003 In recent years, there has been an alarming trend of increased bacterial infections caused by strains resistant to most known antibiotics. As a result, diseases that were thought to be controlled by currently available drugs are re-emerging not only in developing countries but also in industrialized nations, especially in clinical settings such as hospitals. Therefore, there is an urgent need for the development of new types of antibiotics that can be used to effectively treat multidrug resistant bacteria. The development of new drugs with novel mechanisms of action is clearly needed to avert an impending crisis.

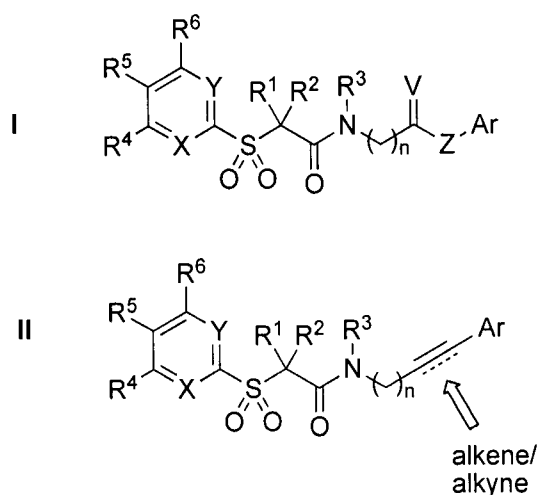
0004 An antibacterial target was identified when it was discovered that the caseinolytic protease P ("ClpP") can be activated by acyldepsipeptides ("ADEPs"), a class of compounds that have been reported to have antibiotic properties. ADEPs were later chemically optimized to address issues related to potency and aqueous solubility. ClpP, a target of the ADEPs, is a tetradecameric serine protease comprised of two stacked heptameric rings which, in *Escherichia coli*, can form complexes with the AAA+ ATPase chaperones ClpX or ClpA. ClpX and ClpA are hexameric chaperones that bind on one or both ends of ClpP. The chaperones bind to target proteins, unfold them, and then thread them into the ClpP proteolytic chamber through axial pores lined by axial loops for degradation. These activities require ATP. In the absence of the ATPase components, ClpP alone can efficiently degrade small peptides of up to about 30 amino acids and can also degrade unstructured proteins albeit with much lower efficiency when compared to ClpXP or ClpAP. It is thought that ADEPs enhance the efficiency of ClpP-dependent degradation of unstructured proteins by opening up the ClpP axial pores.

0005 Thus, there is a need for more efficacious means to address increased bacterial infections, particularly those caused by strains resistant to most known antibiotics.

SUMMARY OF THE INVENTION

0006 An embodiment of the present invention is directed to activators of cylindrical proteases, particularly ClpP, in the diagnosis and treatment of bacterial infections.

0007 An embodiment of the present invention is directed to a compound of formulae (I) or (II).



wherein

R^1 and R^2 are H, CH_3 , a substituted or unsubstituted C_2 - C_8 alkyl, a substituted or unsubstituted C_2 - C_8 cycloalkyl or a substituted or unsubstituted C_2 - C_8 spiro cycloalkyl, when R^1 and R^2 are linked together in a ring;

R^3 is H, CH_3 , a substituted or unsubstituted C_2 - C_8 alkyl, a substituted or unsubstituted C_2 - C_8 cycloalkyl, a substituted or unsubstituted aryl, or a substituted or unsubstituted fused ring, when R^3 is linked together with R^1 / R^2 , V, Z or Ar to form a ring;

R^4 , R^5 and R^6 are H, F, Cl, Br, I, NO_2 , CH_3 , a substituted or unsubstituted C_2 - C_8 alkyl or a substituted or unsubstituted C_2 - C_8 cycloalkyl, CF_3 , CN, a substituted or unsubstituted aryl, $COOH$, $COOR^7$, $CONR^7_2$, COR^7 , OR^7 , NR^7_2 , or SR^7 , wherein R^7 is H, a substituted or unsubstituted C_2 - C_8 alkyl, a substituted or unsubstituted C_2 - C_8 cycloalkyl or a substituted or unsubstituted aromatic group;

X and Y are CH or N;

V is H/H, O, or H/ R^8 , wherein R^8 is CH_3 , a substituted or unsubstituted C_2 - C_8 alkyl, a

substituted or unsubstituted C₂-C₈ cycloalkyl, or substituted or a unsubstituted aryl;

Z is S, CH₂, OC=O, NR⁹C=O, O, or NR⁹, wherein R⁹ is CH₃, a substituted or unsubstituted C₂-C₈ alkyl, a substituted or unsubstituted C₂-C₈ cycloalkyl, or a substituted or unsubstituted aryl;

Ar is a substituted or unsubstituted aromatic, a substituted or unsubstituted fused aromatic, a substituted or unsubstituted heteroaromatic, or a substituted or unsubstituted fused heteroaromatic;

n = 0, 1, 2, 3; and

wherein formulae (I) or (II) do not include ACP1 or ACP2.

0008 Another embodiment is directed to wherein the C₂-C₈ alkyl group is a substituted or unsubstituted ethyl, propyl, isopropyl, butyl, sec-butyl, isobutyl, tert-butyl, pentyl, hexyl, heptyl or octyl.

0009 Yet another embodiment is directed to wherein the C₂-C₈ cycloalkyl group or the C₂-C₈ spiro cycloalkyl is substituted or unsubstituted cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, or cyclooctyl.

0010 Yet another embodiment is directed to wherein the substituted or unsubstituted aryl group is a substituted or unsubstituted phenyl.

0011 Yet another embodiment is directed to wherein the substituted or unsubstituted aromatic group comprises a substituted or unsubstituted phenyl.

0012 Yet another embodiment is directed to wherein the substituted or unsubstituted aromatic group is substituted with *ortho*, *meta* or *para* F, Cl, Br, I, CF₃, CN, NO₂, alkyl, aryl, COOH, COOR¹⁰, CONR¹⁰₂, COR¹⁰, OR¹⁰, or NR¹⁰₂ wherein R¹⁰ is H, CH₃, a substituted or unsubstituted C₂-C₈ alkyl, a substituted or unsubstituted C₃-C₈ cycloalkyl, or a substituted or unsubstituted aryl.

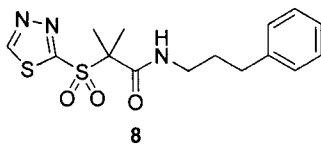
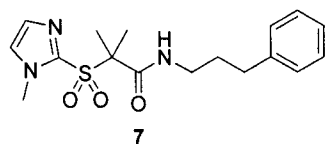
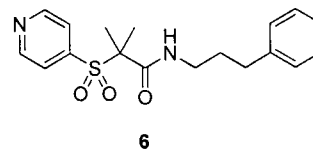
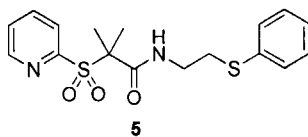
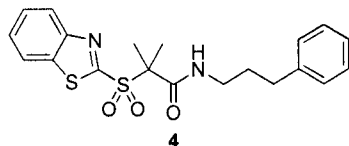
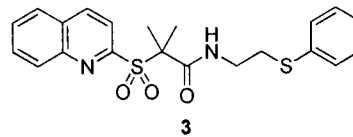
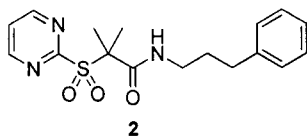
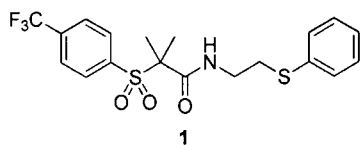
0013 Yet another embodiment is directed to wherein the substituted or unsubstituted fused aromatic group is a substituted or unsubstituted naphthalene or a substituted or unsubstituted indene.

0014 Yet another embodiment is directed to wherein the substituted or unsubstituted heteroaromatic group is substituted or unsubstituted pyridine, substituted or unsubstituted pyrimidine, substituted or unsubstituted pyrazine, substituted or unsubstituted triazine, substituted or unsubstituted thiophene, substituted or unsubstituted furan, substituted or unsubstituted oxazole, substituted or unsubstituted thiazole, substituted or unsubstituted imidazole, substituted or unsubstituted pyrazole, substituted or unsubstituted triazole, substituted or unsubstituted oxadiazole or substituted or unsubstituted tetrazole.

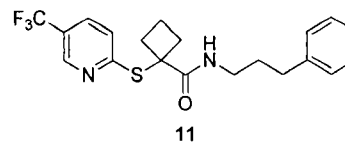
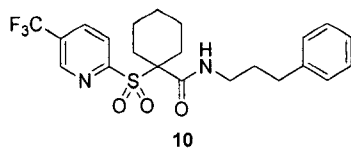
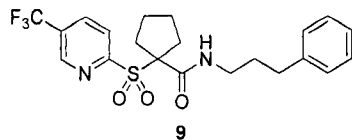
0015 Yet another embodiment is directed to wherein the fused heteroaromatic group comprises substituted or unsubstituted indole, substituted or unsubstituted benzofuran, substituted or unsubstituted benzothiophene, substituted or unsubstituted quinoline, substituted or unsubstituted isoquinoline, substituted or unsubstituted benzimidazole, substituted or unsubstituted benzthiazole, substituted or unsubstituted benzoxazole or substituted or unsubstituted benzpyrazole.

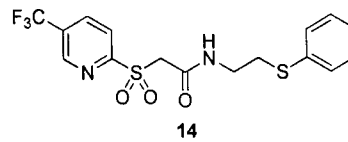
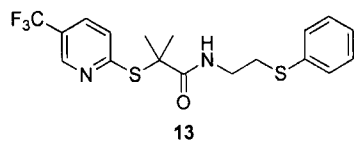
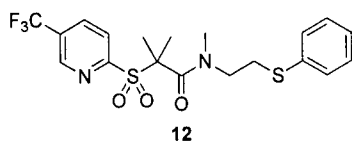
0016 Yet another embodiment is directed to wherein the compound is selected from the group consisting of:

Western Modifications

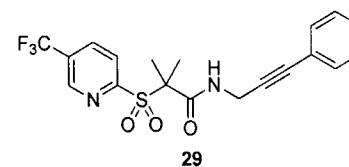
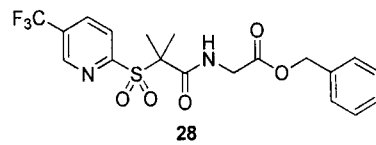
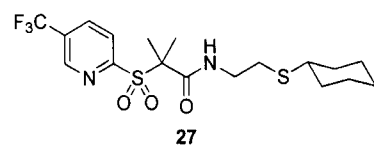
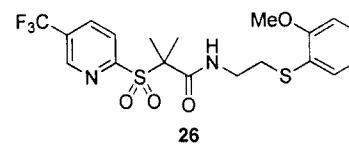
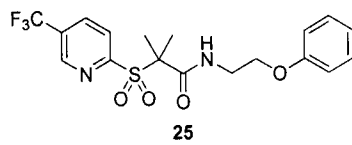
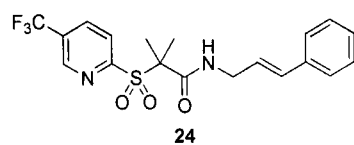
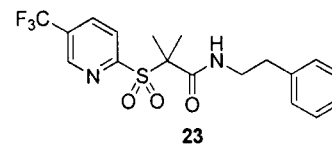
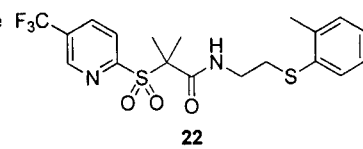
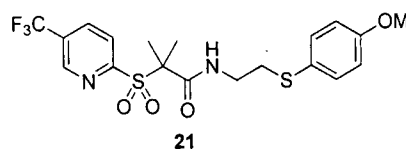
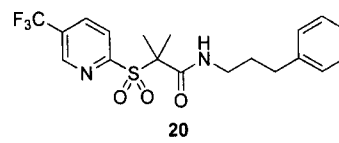
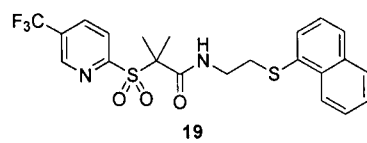
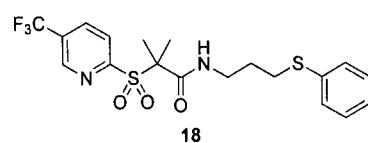
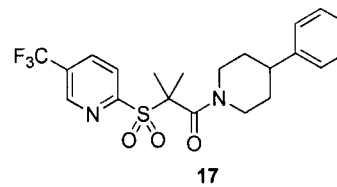
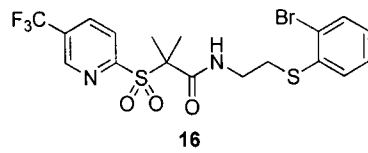
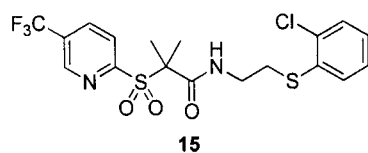


Central Modifications

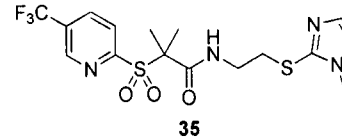
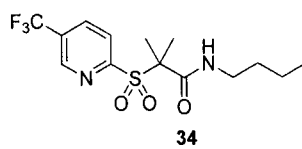
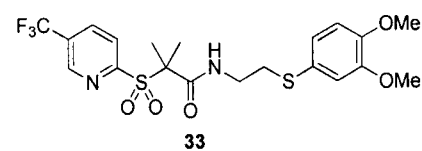
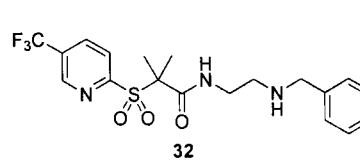
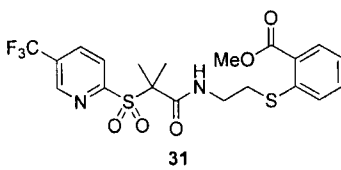
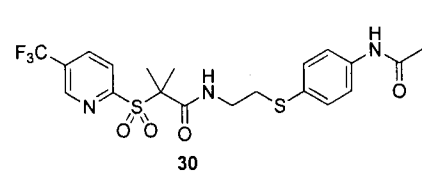


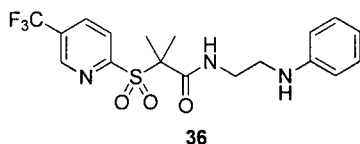


Eastern Modifications

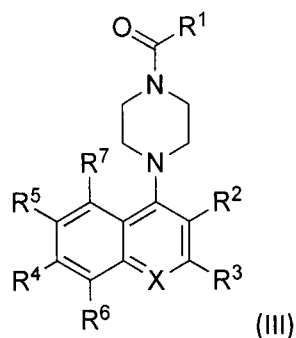


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0017 Yet another embodiment is directed to a compound of the formulae (III).



wherein

R^1 is H, CH_3 , a substituted or unsubstituted C_1 - C_8 alkyl, a substituted or unsubstituted C_3 - C_8 cycloalkyl, a substituted or unsubstituted alkenyl, a substituted or unsubstituted aromatic, a substituted or unsubstituted fused aromatic, a substituted or unsubstituted heteroaromatic, or a substituted or unsubstituted fused heteroaromatic;

R^2 , R^3 , R^4 , R^5 , R^6 and R^7 are H, CH_3 , a substituted or unsubstituted C_1 - C_8 alkyl, a substituted or unsubstituted C_3 - C_8 cycloalkyl, a substituted or unsubstituted alkenyl, a substituted or unsubstituted aromatic, a substituted or unsubstituted fused aromatic, a substituted or unsubstituted heteroaromatic, a substituted or unsubstituted fused heteroaromatic;

X is CH and N; and

wherein formulae (III) does not include ACP3.

0018 Yet another embodiment is directed to wherein the substituted or unsubstituted C_1 - C_8 alkyl group is a substituted or unsubstituted methyl, ethyl, propyl, isopropyl, or butyl, sec-butyl, isobutyl, tert-butyl, pentyl, hexyl, heptyl and octyl.

0019 Yet another embodiment is directed to wherein the substituted or unsubstituted C_3 - C_8 cycloalkyl group or the C_2 - C_8 spiro cycloalkyl is a substituted or unsubstituted cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, or cyclooctyl.

0020 Yet another embodiment is directed to wherein the substituted or unsubstituted alkenyl is vinyl, propenyl or dihalovinyl.

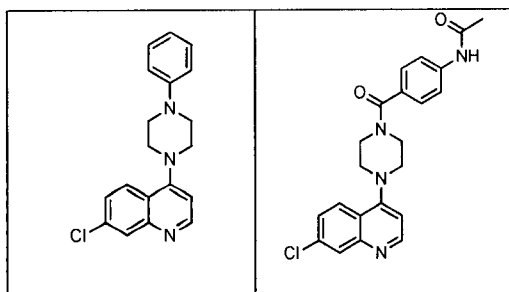
0021 Yet another embodiment is directed to wherein the substituted aromatic is substituted with *ortho*, *meta* or *para* F, Cl, Br, I, CF₃, CN, NO₂, alkyl, aryl, COOH, COOR¹⁰, CONR¹⁰₂, COR¹⁰, OR¹⁰, or NR¹⁰₂ wherein R¹⁰ is H, CH₃, a substituted or unsubstituted C₂-C₈ alkyl, a substituted or unsubstituted C₃-C₈ cycloalkyl, or substituted or unsubstituted aryl.

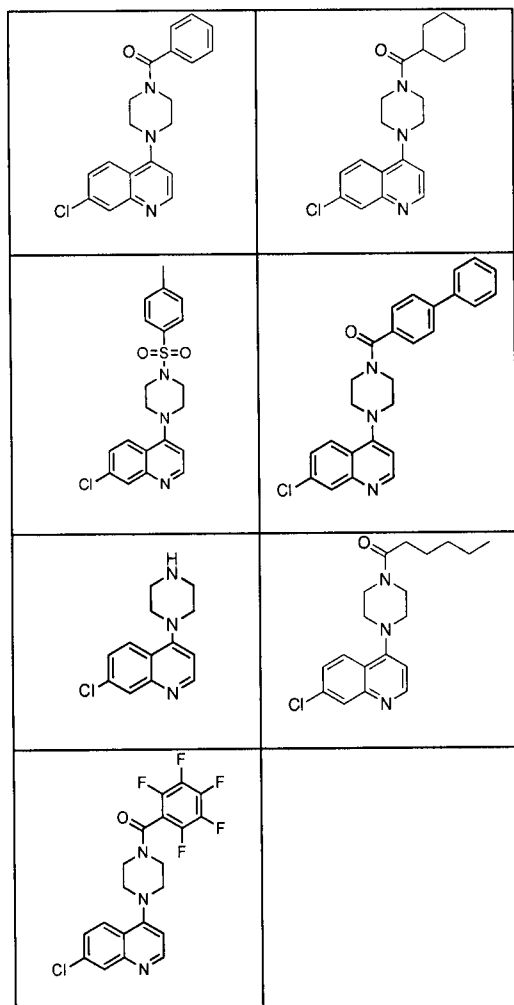
0022 Yet another embodiment is directed to wherein the fused aromatic group is a substituted or unsubstituted naphthalene or a substituted or unsubstituted indene.

0023 Yet another embodiment is directed to wherein the substituted or unsubstituted heteroaromatic group is substituted or unsubstituted pyridine, substituted or unsubstituted pyrimidine, substituted or unsubstituted pyrazine, substituted or unsubstituted triazine, substituted or unsubstituted thiophene, substituted or unsubstituted furan, substituted or unsubstituted oxazole, substituted or unsubstituted thiazole, substituted or unsubstituted imidazole, substituted or unsubstituted pyrazole, substituted or unsubstituted triazole, substituted or unsubstituted oxadiazole or substituted or unsubstituted tetrazole.

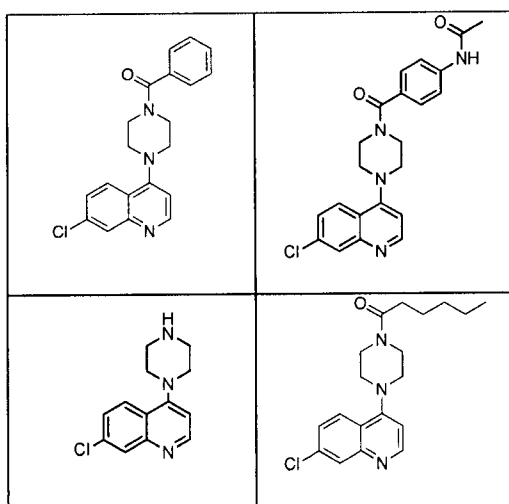
0024 Yet another embodiment is directed to wherein the fused heteroaromatic group comprises substituted or unsubstituted indole, substituted or unsubstituted benzofuran, substituted or unsubstituted benzothiophene, substituted or unsubstituted quinoline, substituted or unsubstituted isoquinoline, substituted or unsubstituted benzimidazole, substituted or unsubstituted benzthiazole, substituted or unsubstituted benzoxazole or substituted or unsubstituted benzpyrazole.

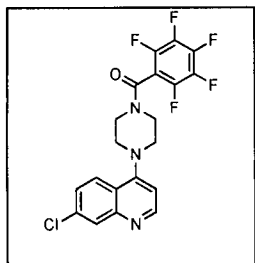
0025 Yet another embodiment is directed to wherein the compound is selected from the group consisting of:



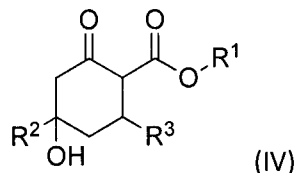


0026 Yet another embodiment is directed to wherein the compound is selected from the group consisting of:





0027 Yet another embodiment is directed to a compound of formulae (IV):



wherein

R¹ is CH₃, a substituted or unsubstituted C₂-C₈ alkyl or a substituted or unsubstituted C₃-C₈ cycloalkyl;

R² and R³ are H, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, a substituted or unsubstituted alkenyl, a substituted or unsubstituted aromatic, a substituted or unsubstituted fused aromatic, a substituted or unsubstituted heteroaromatic, or a substituted or unsubstituted fused heteroaromatic; and

wherein formulae (IV) does not include ACP4 or ACP5.

0028 Yet another embodiment is directed to wherein the substituted or unsubstituted C₁-C₈ alkyl group is a substituted or unsubstituted methyl, ethyl, propyl, isopropyl, or butyl, sec-butyl, isobutyl, tert-butyl, pentyl, hexyl, heptyl and octyl.

0029 Yet another embodiment is directed to wherein the substituted or unsubstituted C₃-C₈ cycloalkyl group is a substituted or unsubstituted cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, and cyclooctyl.

0030 Yet another embodiment is directed to wherein the substituted or unsubstituted alkyl is a substituted or unsubstituted methyl, ethyl, propyl, isopropyl or butyl.

0031 Yet another embodiment is directed to wherein the substituted or unsubstituted cycloalkyl is a substituted or unsubstituted cyclopropyl, cyclobutyl, cyclopentyl or cyclohexyl.

0032 Yet another embodiment is directed to wherein the substituted or unsubstituted alkenyl group is vinyl, propenyl or dihalovinyl.

0033 Yet another embodiment is directed to wherein the substituted aromatic is substituted with *ortho*, *meta* or *para* F, Cl, Br, I, CF₃, CN, NO₂, alkyl, aryl, COOH, COOR¹⁰, CONR¹⁰₂, COR¹⁰, OR¹⁰, or NR¹⁰₂ wherein R¹⁰ is H, CH₃, a substituted or unsubstituted C₂-C₈ alkyl, a substituted or unsubstituted C₃-C₈ cycloalkyl, or a substituted or unsubstituted aryl.

0034 Yet another embodiment is directed to wherein the fused aromatic group is a substituted or unsubstituted naphthalene or a substituted or unsubstituted indene.

0035 Yet another embodiment is directed to wherein the substituted or unsubstituted heteroaromatic group is substituted or unsubstituted pyridine, substituted or unsubstituted pyrimidine, substituted or unsubstituted pyrazine, substituted or unsubstituted triazine, substituted or unsubstituted thiophene, substituted or unsubstituted furan, substituted or unsubstituted oxazole, substituted or unsubstituted thiazole, substituted or unsubstituted imidazole, substituted or unsubstituted pyrazole, substituted or unsubstituted triazole, substituted or unsubstituted oxadiazole or substituted or unsubstituted tetrazole.

0036 Yet another embodiment is directed to wherein the fused heteroaromatic group comprises substituted or unsubstituted indole, substituted or unsubstituted benzofuran, substituted or unsubstituted benzothiophene, substituted or unsubstituted quinoline, substituted or unsubstituted isoquinoline, substituted or unsubstituted benzimidazole, substituted or unsubstituted benzthiazole, substituted or unsubstituted benzoxazole or substituted or unsubstituted benzpyrazole.

0037 Yet another embodiment is directed to an antibacterial compound selected from the group consisting of any of the preceding compounds and pharmaceutically acceptable salts thereof.

0038 Yet another embodiment is directed to an antibacterial compound selected from the group consisting of ACP1, ACP2, ACP3, ACP4, ACP5 and pharmaceutically acceptable salts thereof.

0039 Yet another embodiment is directed to the antibacterial comprising ACP1, ACP3, ACP4, ACP5, more preferably, ACP1 and ACP3, and pharmaceutically acceptable salts thereof.

0040 Yet another embodiment is directed to a cylindrical protease (preferably ClpP) activator for use as an antibacterial compound.

0041 Yet another embodiment is directed to a pharmaceutical composition comprising any one of the preceding compounds or a pharmaceutically acceptable salt thereof in association with one or more pharmaceutically acceptable excipients, diluents and/or carriers.

BRIEF DESCRIPTION OF THE DRAWINGS

0042 The foregoing and other objects, features and advantages of the present invention should become apparent from the following description when taken in conjunction with the accompanying Figures and Tables. The patent or patent application file contains at least one drawing executed in colour. Copies of this patent or patent application publication with colour drawing(s) may be provided by the office upon request and payment of the necessary fee.

0043 **FIG. 1** shows results of the high-throughput compound screen for embodiments of the present invention. (A) shows compounds that were screened to find activators of *E. coli* ClpP. B-score values were calculated for each compound from the increase in fluorescence intensity after a six-hour incubation of casein-FITC with ClpP and the applicable compound. Compounds confirmed as hits, designated ACP1 through ACP5, are indicated by the arrows. (B) shows chemical structures of embodiments of the present invention.

0044 **FIG. 2** shows the relative degradation index of embodiments of the present invention. (A) Shown is the effect of compound concentration on casein-FITC degradation by ClpP after six-hour incubation. Data are the average of three repeats. Error bars represent standard deviations. (B) Comparison of the relative degradation index at 25 μ M compound (RD25) values for the ACP compounds. Data shown represent the average of three repeats. SD is standard deviation.

0045 **FIG. 3** shows the chemical optimization of embodiments of the present invention. (A) General chemical structure of embodiments of the present invention. (B) A schematic of the chemical reaction used to synthesize embodiments using PyBOP mediated amide bond formation between 2-methyl-2-((5-(trifluoromethyl)pyridin-2-yl)thio)propanoic acid and primary amines.

(C) RD25 values of embodiments of the present invention. (D) Shown is the degradation of unlabeled casein by compound-activated ClpP followed on SDS-PAGE gels.

0046 FIG. 4 shows the determination of binding affinity to ClpP of embodiments of the present invention. (A) ITC binding curves for interaction of ClpP and embodiments of the present invention. Results for the fit of the data to one set of an identical independent binding site model is given in the table. Numbers in parentheses refer to standard deviations from the fit. (B) Cooperativity of binding of various embodiments to ClpP was determined by measuring the change in casein degradation rate by compound-activated ClpP as a function of compound concentration.

0047 FIG. 5 shows the effect of embodiments on ClpP oligomeric stability. (A) Sedimentation velocity analytical ultracentrifugation of full length ClpP (46 μ M) and ClpP Δ 31 (51 μ M) at 4°C shown using the continuous distribution model $c(s)$ vs $s_{20,w}$, scaled to initial absorbance. Embodiments of the present invention (at 100 μ M) promote the tetradecamerization of ClpP Δ 31. The table lists the sedimentation coefficients, frictional ratios, and molecular weights corresponding to the various peaks. The peaks of the different curves correspond to the following *MW*: 1, 134 kDa; 2, 140 kDa; 3, 277 kDa; 4, 145 kDa; 5, 297 kDa; 6, 145 kDa; 7, 282 kDa; 8, 148 kDa; 9, 303 kDa. (B) provides sedimentation equilibrium profiles and the corresponding distribution of residuals for 51 μ M ClpP in the absence of compound (top), or in the presence of 100 μ M ACP1a (middle) or 100 μ M ACP1b (bottom). The solid lines represent the best fit to a monomer-dimer model, with the heptameric ClpP (*MW* of 138,544 Da) treated as the monomer. The resulting dissociation constants are given for each data set. The numbers in brackets give the range of K_d values for the 95% confidence interval. (C) The effect of various embodiments (at 100 μ M) on the peptidase activity of ClpP Δ 31 (1 μ M) is shown. The defective peptidase activity of this mutant is partially recovered by the presence of embodiments of the present invention.

0048 FIG. 6 shows possible ACP binding sites. (A) Shown is the inhibition of ClpXP-mediated GFP-ssrA degradation by ACPs and ADEPs added at 100 μ M monitored on SDS-PAGE gels. ClpP was pre-incubated with compound before the addition of ClpX. (B) Surface model of ClpP is shown on the top left. Four neighboring subunits are colored in alternating blue and green. The H pockets are colored in purple while the C pockets are colored in yellow. The

bottom left panel shows a close up view of the predicted compound binding conformations in the two ClpP pockets. Various embodiments are overlaid in the binding pockets. ClpP is shown as a surface model and the compounds are shown as stick models. C, N, O, S, F, Cl, Br, and H in the compounds are colored in gray, blue, red, yellow, cyan, green, purple and white, respectively. The ClpP surface is colored according to the electrostatic potential (red is < -4 kT/e and blue is > 4 kT/e) calculated using DelPhi. Also shown on the right panels are stick models of ACP1 docked into the H and C pockets of ClpP drawn as ribbons colored by chain. For ACP1, C, N, O, S, F, and H are colored in orange, blue, red, yellow, cyan and white, respectively. All molecular graphics figures were prepared using the program PyMOL. (C) Effect of mutations in the H and C pockets on ClpP activation by compounds measured using RD25. Data shown represent the average of three repeats and the standard deviations are represented as error bars.

0049 FIG. 7 shows the confirmation of ClpP activation by the hits identified in the high-throughput screen. The disappearance of unlabeled casein due to degradation by compound-activated ClpP was followed over time on SDS-PAGE gels, which were then stained with Coomassie Brilliant Blue.

0050 FIG. 8 shows the degradation of alternative substrates by compound-activated ClpP. Shown is the degradation of the indicated substrates by compound-activated ClpP followed on SDS-PAGE gels for 6 hours.

0051 FIG. 9 shows the chemical structures of ADEP1A and ADEP1B.

0052 FIG. 10 shows the formation of intermediates during casein degradation by embodiment-activated ClpP. Intermediate species, indicated by the parenthesis, resulting from casein degradation were resolved on 18% percent SDS-PAGE gels.

0053 FIG. 11 shows Surface Plasmon Resonance ("SPR") analysis for the binding of ClpP with different activators. Graphs on the left represent the stacked sensorgrams from SPR experiments of embodiments at various concentrations injected over a biosensor chip surface immobilized with ClpP. Graphs on the right depict the binding curves constructed with the steady state data fit to a one-site Langmuir binding model. The K_d obtained by the fits are listed in the table at the bottom.

0054 FIG. 12 shows the inhibition of ClpXP-mediated GFP-ssrA degradation by ACPs. 100 μ M of ACP1-5 inhibited or reduced GFP-SsrA degradation by ClpXP as monitored on SDS-PAGE gels.

0055 FIG. 13 shows binding conformations of an embodiment of the present invention in the H and C pockets of ClpP. ClpP and compounds are shown as surface and stick models, respectively. The color scheme is the same as that used in Fig. 6B.

0056 FIG. 14 shows the effect of mutations in the H and C pockets on ClpP activity. Peptidase assays comparing the peptide hydrolysis activities of various *E. coli* ClpP mutants against the Suc-LY-AMC peptide. Mutations were made in the H pocket, C pocket, or in combination. The color scheme is the same as that for FIG. 6C. Values were normalized to the wild type ClpP peptide hydrolysis rate. Mutants with peptidase activity less than 70% of WT are grouped on the right. Data shown represent the average of three repeats and the standard deviations are represented by the error bars.

0057 FIG. 15 shows the activity of embodiments, measured using RD25, of the present invention.

0058 Table 1 shows the minimum bactericidal concentration of embodiments of the present invention.

0059 Table 2 shows RD25 values for ClpP Activation by ACP1 Analogs.

0060 Table 3 shows *in vitro* and *in vivo* activity data of embodiments of the present invention.

0061 Table S1 shows ClpP degradation assays with alternative substrates.

0062 Table S2 shows minimum bactericidal concentration of compounds.

0063 Table S3 shows ZINC ID corresponding to ACP4 chiral isomers.

0064 Table S4 shows ZINC ID corresponding to ACP5 chiral isomers.

0065 Table S5 shows Lowest Dock6 score for ACP compounds.

0066 FIG. 16 shows a crystal structure of an embodiment of the present invention (Compound No. 93) bound to an active site of ClpP.

0067 FIG. 17 shows loss of clpP confers resistance to ADEP and ACP in *N. meningitidis* H44/76 and *E. coli* MC4100 (see also FIG. 3). (A) PCR verification of clpP insertional mutagenesis *N. meningitidis* H44/76. Genomic DNA recovered from wild type (W) or erythromycin-resistant *N. meningitidis* H44/76 transformants (A and B, representing two replicate samples) were used as template for PCR with the indicated primer pairs. The schematic depicts relative location of oligonucleotide primer sequences on the genome of the clpP mutant bacteria. (B) Fresh overnight cultures of *N. meningitidis* H44/76 were spread onto the surface of standard growth media, and filter discs impregnated with 256 µg/mL of ADEP1A or ADEP1B were laid on the surface. Zones of clearing on plates cultured with WT meningococci reflect inhibition of bacterial growth, whereas no inhibitory effect of either compound was apparent on plates cultured with the meningococcal clpP mutant. (C) The chemical structures of ADEP1A and ADEP1B. (D) Upper panel shows the growth curves for WT and $\Delta clpP$ *E. coli* MC4100 in the presence of 20 uM CCCP or 20 uM CCCP + 128 ug/mL ACP1B in LB at 30°C. The curves shown represent the average of 3 cultures. The lower panel shows OD600 at 900 minutes for the two strains.

0068 FIG. 18 shows binding of ClpP to ADEP1A, ADEP1B, and ACP1b (see also FIG. 4). (A) ITC binding curves for ClpP-ADEP1A, ADEP1B, or ACP1b interaction. Results for the fit of the data to a one set of identical independent binding site model is given in the table. The number of binding sites, n , were fixed in the fit to the indicated whole number. Numbers in parentheses refer to standard deviations. χ^2/DoF refers to chi-squared divided by the degrees of freedom and indicates the quality of the fit (see also FIG. 4A). (B) Same as A, but n was fixed to 14.

DETAILED DESCRIPTION OF THE INVENTION

0069 In this disclosure, a number of terms and abbreviations are used. The following definitions of such terms and abbreviations are provided.

0070 All references cited in the specification are incorporated herein by reference.

0071 The term "alkyl" includes straight chain and branched hydrocarbons with at least one hydrogen removed to form a radical group. Alkyl groups include, but are not limited to methyl, ethyl, propyl, isopropyl, butyl, isobutyl, t-butyl, 1-methylpropyl, pentyl, isopentyl, sec-pentyl, hexyl, heptyl, octyl, and so on, any of which may be substituted or unsubstituted.

0072 The term "alkenyl" includes straight chain and branched hydrocarbon radicals as above with at least one carbon-carbon double bond. Unless indicated otherwise by the prefix that indicates the number of carbon members, alkenyls include, but are not limited to, ethenyl (or vinyl), prop-1-enyl, prop-2-enyl (or allyl), isopropenyl (or 1-methylvinyl), but-1-enyl, but-2-enyl, butadienyls, pentenyls, hexa-2,4-dienyl, dihalovinyl, and so on, any of which may be substituted or unsubstituted.

0073 Unless indicated otherwise by the prefix that indicates the number of carbon members, "cycloalkyl" includes, but is not limited to, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, and so on, any of which may be substituted or unsubstituted.

0074 Unless indicated otherwise by the prefix that indicates the number of members in the cyclic structure, "heterocyclyl", "heterocyclic" or "heterocycle" is an aromatic, saturated, or partially saturated single or fused ring system that comprises carbon atoms wherein the heteroatoms may be nitrogen, sulfur and oxygen. Examples of heterocyclic groups include, but are not limited to, thiazoyl, thienyl, furyl, pyran, pyranyl, isobenzofuranyl, pyrrolyl, imidazolyl, pyrazolyl, isothiazolyl, isoxazolyl, pyridyl, pyrazinyl, pyrimidinyl, pyridazinyl, indolizyl, isoindolyl, indolyl, indazolyl, purinyl, quinolyl, isoquinolyl, furazanyl, pyrrolidinyl, pyrrolinyl, imidazolidinyl, imidazolyl, pyrazolidinyl, pyrazolinyl, piperidyl, piperazinyl, indolyl, benzothienyl, benzofuranyl, benzimidazolyl, benzoxazolyl, benzothiazolyl, and morpholinyl, any of which may be optionally substituted. Examples of heteroaromatics and fused heteroaromatics include, but are not limited to, naphthalene, indene, quinoline, isoquinoline, benzimidazole, benzthiazole, benzoxazole, furan, pyrrole, pyridine, pyrimidine, pyrazine, triazine, thiophene, furan, oxazole, thiazole, imidazole, any of which may be substituted or unsubstituted.

0075 The term "aryl" or "aromatic", as used herein, unless otherwise indicated, includes an organic radical derived from an aromatic hydrocarbon by removal of one hydrogen, such as, for example, phenyl, naphthyl or indenyl, any of which may be optionally substituted. Unless

indicated otherwise, the terms "heteroaryl" or "heteroaromatic" refer to those heterocycles that are aromatic in nature. Substituted aromatics can include aromatics mono- or polysubstituted at the *ortho*, *meta* or *para* positions by substituents that include, but are not limited to, a halo, CF₃, CN, NO₂, alkyl, aryl, COOH, a fused aromatic, a heteroaromatic, a fused heteroaromatic, COOR, CONR₂, COR, OR, NR₂, where R may be H, CH₃, C₂-C₈ alkyl/cycloalkyl, phenyl, or aryl, any of which may be substituted or unsubstituted.

0076 The term "halo", as used herein, unless otherwise indicated, means fluoro, chloro, bromo or iodo.

0077 It will be understood by a person skilled in the relevant art that some compounds referred to herein are chiral and/or have geometric isomeric centers. The present invention encompasses all such optical isomers, including diastereoisomers and racemic mixtures, atropisomers, and geometric isomers that possess the activity that characterizes the embodiments of this invention. In addition, certain compounds referred to herein can exist in solvated as well as unsolvated forms. It will be understood that this invention encompasses all such solvated and unsolvated forms that possess the activity that characterizes the embodiments of this invention. Embodiments of the present invention that have been modified to be detectable by some analytic technique are also within the scope of this invention. An example of such compounds is an isotopically labeled compound, such as an isotopically labeled compounds that may be used as a probe in detection and/or imaging techniques. Another example of such compounds is an isotopically labeled compound, such as a deuterium and/or tritium labeled compound that may be used in reaction kinetic studies.

0078 The present invention includes within its scope prodrugs of the compounds of this invention. In general, such prodrugs will be functional derivatives of the compounds that are readily convertible *in vivo* into the desired compound. Thus, in the methods of treatment of the present invention, the term "administering" shall encompass the treatment of the various disorders described with the compound specifically disclosed or with a compound that may not be specifically disclosed, but that converts to the specified compound *in vivo* after administration to a subject. Conventional procedures for the selection and preparation of suitable prodrug derivatives are described standard references and are known in pharmaceutical manufacturing.

0079 Reference to a compound herein stands for a reference to any one of: (a) the actually recited form of such compound, and (b) any of the forms of such compound in the medium in which the compound is being considered when named.

0080 "Salt" also comprises the hydrates and solvent addition forms that compounds of the present invention are able to form. Examples of such forms are hydrates, alcoholates, and generally solvates.

0081 "Subject" or "patient" refers to eukaryotic organisms and it includes mammals such as human beings and animals (e.g., dogs, cats, horses, rats, rabbits, mice, non-human primates, etc.) in need of observation, experiment, treatment or prevention in connection with the relevant disease or condition. Preferably, the patient or subject is a human being.

0082 "Pharmaceutically acceptable" means those active agents, salts and esters, and excipients which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of humans and lower animals without undue toxicity, irritation, allergic response and the like, commensurate with a reasonable benefit/risk ratio, and effective for their intended use.

0083 "Composition" includes a product comprising the specified ingredients in effective amounts, as well as any product that results directly or indirectly from combinations of the specified ingredients in the specified amounts.

0084 Compounds according to the present invention and mixtures thereof provide embodiments of the present invention that can be made with excipients and ingredients that would be within the knowledge of a person skilled in the relevant art. Lists of excipients and ingredients for pharmaceutical compositions are available in standard references and are known in pharmaceutical manufacturing.

0085 The examples and preparations provided herein further illustrate and exemplify the compounds of the present invention as well as methods of preparing and testing such compounds. It is to be understood that the scope of the present invention is not limited in any way by the scope of the examples and preparations provided herein. The reactions provided herein can be performed under conventional conditions and following procedures described elsewhere and known to those skilled in the art. It will be further understood that a number of

alternative reaction schemes may be employed to arrive at embodiments of the present invention. All such procedures are considered to be within the scope of the invention.

0086 ClpP is a cylindrical tetradecameric serine protease whose activity is regulated by the unfoldase ATP-dependent chaperones of the AAA+ superfamily. The chaperones act to select substrate proteins, unfold them, and then thread them into the ClpP cylinder for degradation. ClpP on its own can only degrade small peptides.

0087 Using a high-throughput screen, several structurally diverse non-ADEP compounds were identified that activate ClpP to degrade protein substrates in the absence of the ClpX or ClpA ATPases. Embodiments of the present invention are directed to cylindrical protease activators. Further embodiments of the present invention are directed to cylindrical protease activators having antibacterial properties based on the activation and dysregulation of ClpP activity. Preferred embodiments include ACP1 to ACP5 as antibacterial compounds.

0088 Embodiments of the present invention are directed to compounds termed Activators of Self-Compartmentalizing Proteases ("ACPs"). The chemical structures of ACPs identified herein differ significantly from the structures of the previously identified ADEPs. As a result of the techniques provided herein, a number of ACPs have been identified including, but not limited to ACP1 (N1[2(phenylthio)ethyl]2methyl2{[5(trifluoromethyl)2pyridyl]sulfonyl}propanamide), ACP2

(3(tertbutoxy)2{[2[(5(tertbutoxy)2{[(9H9fluorenylmethoxy)carbonyl]amino}5oxopentanoyl)amino]3(tertbutylsulfanyl)propanoyl]amino}butanoic acid), ACP3 ([4(7chloroquinolin4yl)piperazino](cyclohexyl)methanone), ACP4 (ethyl 2-(2,2-dichlorovinyl)-4-hydroxy-4-(3-nitrophenyl)-6-oxocyclohexanecarboxylate), and ACP5 (ethyl 4-(4-bromophenyl)-2-(2,2-dichlorovinyl)-4-hydroxy-6-oxocyclohexanecarboxylate).

0089 Following the identification of the ACPs, analogs thereof were developed through chemical optimization of the identified ACPs, including but not limited to ACP1, which resulted in analogs having the desired bioactivity.

0090 ClpP was used as a target in a high-throughput screen to identify compounds which activate ClpP so as to allow it to degrade larger proteins, hence, abolishing the specificity arising

from the ATP-dependent chaperones. The cylindrical protease activators of the present invention may stabilize the ClpP double ring structure. Hence, the ACPs represent new classes of compounds that can activate ClpP and can be potential antibiotics. It has been found that the compounds of this invention and compositions containing these compounds have antibacterial activities against pathogenic microorganisms, particularly bacterial strains.

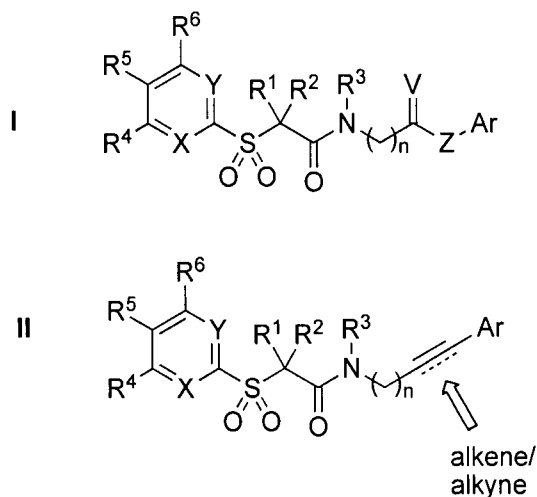
0091 Accordingly, the present invention is also directed to a method of treating a subject having a condition caused by or contributed to by bacterial infection, which comprises administering to said subject a therapeutically effective amount of at least one embodiment of the present invention and/or derivative thereof. The present invention is further directed to a method of preventing a subject from suffering from a condition caused by or contributed to by bacterial infection, which comprises administering to the subject a prophylactically effective amount of at least one embodiment of the present invention and/or derivative thereof.

0092 The invention also features a pharmaceutical composition for treating or preventing bacterial infection in a subject, comprising a therapeutically effective amount of at least one antibacterial agent selected from the embodiments of the present invention, including but not limited to enantiomers, diastereomers, racemates, tautomers, hydrates, solvates thereof, pharmaceutically acceptable salts, amides and esters thereof. In addition, the invention features a pharmaceutical composition for inhibiting bacterial activity or infection in a subject, comprising a therapeutically effective amount of at least one antibacterial agent selected from the embodiments of the present invention, including but not limited to enantiomers, diastereomers, racemates, tautomers, hydrates, solvates thereof, pharmaceutically acceptable salts, amides and esters thereof.

0093 As shown in FIG. 1B, further embodiments of the present invention are directed to compounds representing four different structural classes that can activate ClpP and that have bactericidal properties. With the exception of ACP4 and ACP5, these compounds have no apparent structural similarities to each other or to previously identified ADEPs. The optimization of ACP1 resulted in compounds that had *in vitro* ClpP activation properties similar and even exceeding that of the known ADEPs (see Fig. 3C). Importantly, these additional compounds have good bactericidal properties and further chemical modification and

optimization could unlock their fullest potential, both by improving ClpP affinity and activation as well as by improving compound solubility and cell permeability.

0094 Embodiments of the present invention may be directed to analogs of ACP1 to ACP5. Preferred embodiments of the present invention may be directed to analogs of ACP1. The preferred embodiments of the ACP1 analogs are directed to compounds of formulae (I) or (II).



wherein

R^1 and R^2 may be H, CH_3 , a $\text{C}_2\text{-C}_8$ alkyl / cycloalkyl (preferably, ethyl, propyl, isopropyl, butyl, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl) or a $\text{C}_2\text{-C}_8$ spiro cycloalkyl (i.e., where R^1 / R^2 are linked together in a ring);

R^3 may be H, CH_3 , a $\text{C}_2\text{-C}_8$ alkyl / cycloalkyl (preferably, ethyl, propyl, isopropyl, butyl, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl), an aryl (preferably, phenyl), or a fused ring (i.e., where R^3 may be linked together with R^1 / R^2 , V, Z or Ar to form a ring);

R^4 , R^5 and R^6 may be H, a halo, NO_2 , CH_3 , a $\text{C}_2\text{-C}_8$ alkyl / cycloalkyl (preferably, ethyl, propyl, isopropyl, butyl, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl), CF_3 , CN, an aryl (preferably, phenyl), COOH , COOR^7 , CONR^7_2 , COR^7 , OR^7 , NR^7_2 , or SR^7 wherein R^7 may be H, $\text{C}_2\text{-C}_8$ alkyl / cycloalkyl, phenyl or substituted aromatic;

X and Y may be CH or N;

V may be H/H, O, or H/ R^8 , wherein R^8 may be CH_3 , a $\text{C}_2\text{-C}_8$ alkyl / cycloalkyl

[preferably, ethyl, propyl, isopropyl, butyl, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl], or an aryl [preferably, phenyl]);

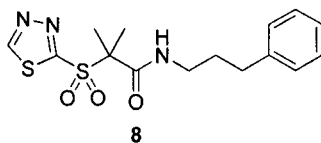
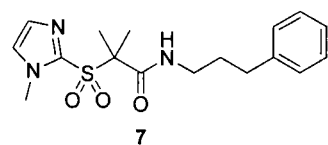
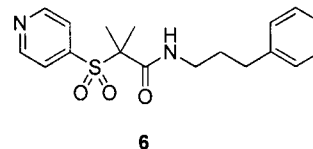
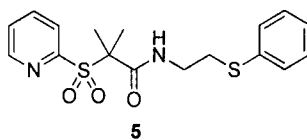
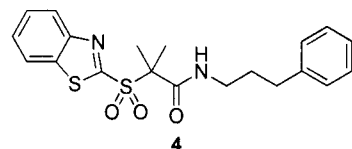
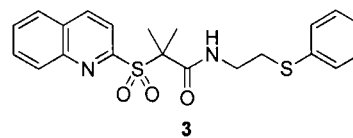
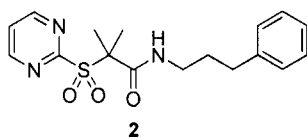
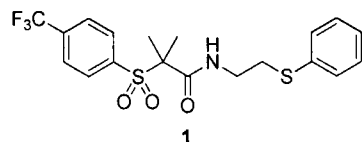
Z may be S, CH₂, OC=O, NR⁹C=O, O, or NR⁹, wherein R⁹ may be CH₃, a C₂-C₈ alkyl / cycloalkyl [preferably, ethyl, propyl, isopropyl, butyl, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl], or an aryl [preferably, phenyl]);

Ar may be phenyl, a substituted aromatic, preferably, with *ortho*, *meta* or *para* F, Cl, Br, I, CF₃, CN, NO₂, alkyl, aryl, COOH, a fused aromatic (preferably, naphthalene, indene), a heteroaromatic (preferably, pyridine, pyrimidine, pyrazine, triazine, thiophene, furan, oxazole, thiazole, imidazole), or a fused heteroaromatic (preferably, quinoline, isoquinoline, benzimidazole, benzthiazole, benzoxazole), COOR¹⁰, CONR¹⁰₂, COR¹⁰, OR¹⁰, NR¹⁰₂, where R¹⁰ may be H, CH₃, a C₂-C₈ alkyl/cycloalkyl, phenyl, an aryl; and

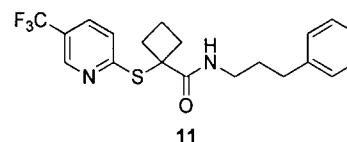
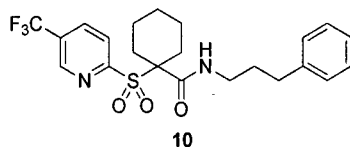
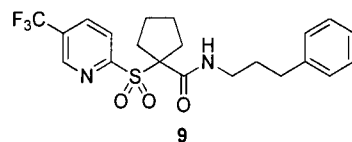
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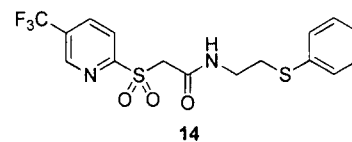
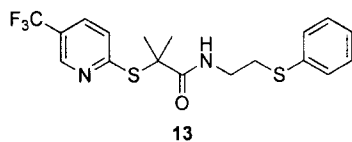
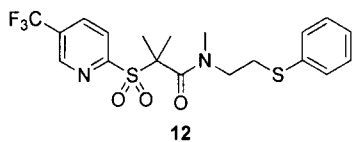
0095 More preferred embodiments of the present invention are set out below:

Western Modifications

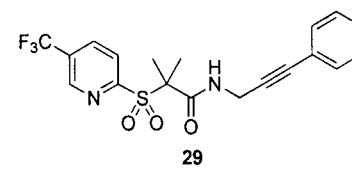
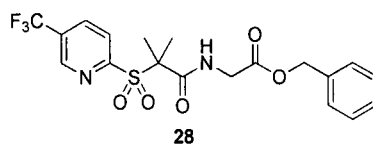
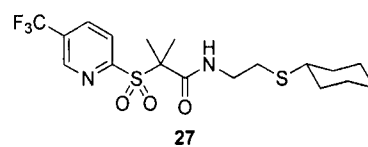
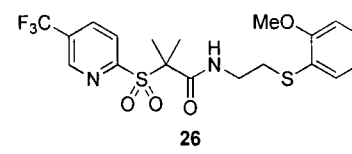
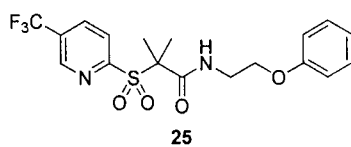
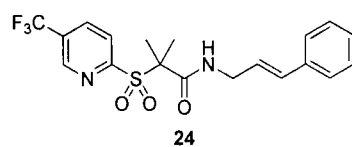
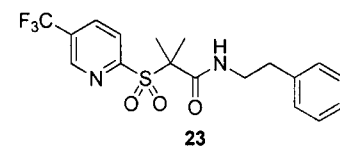
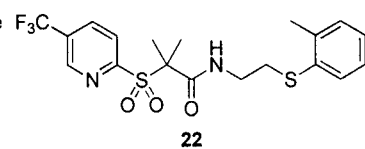
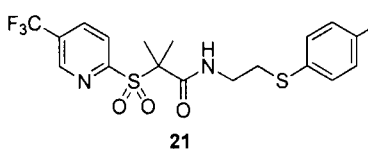
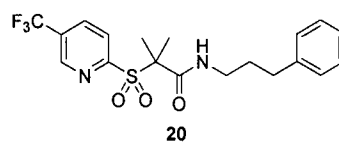
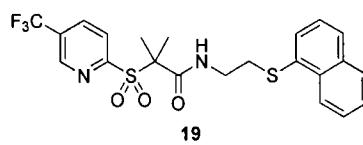
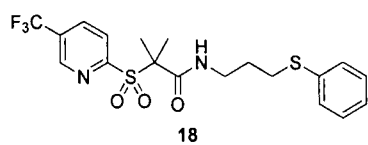
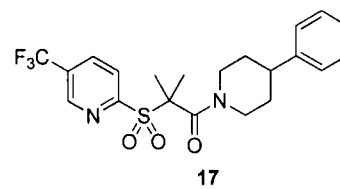
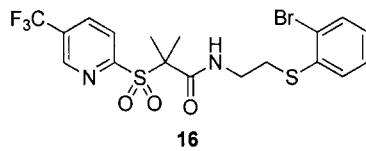
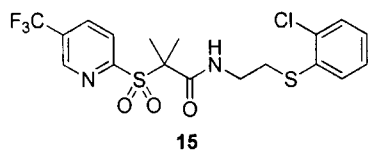


Central Modifications

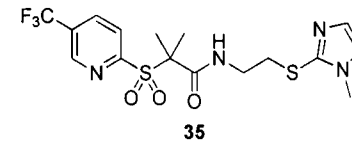
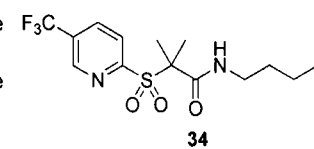
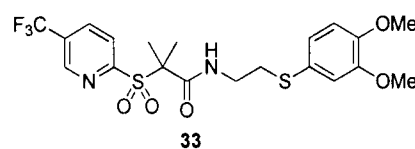
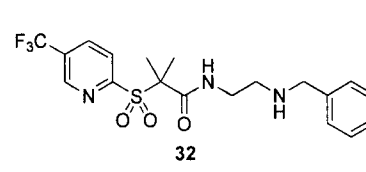
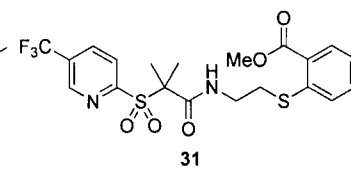
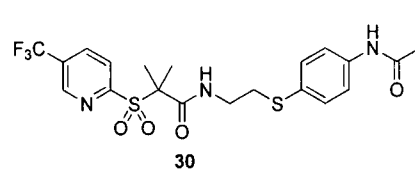


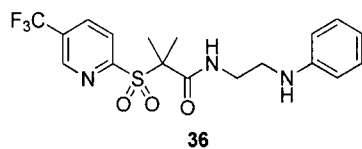


Eastern Modifications



O





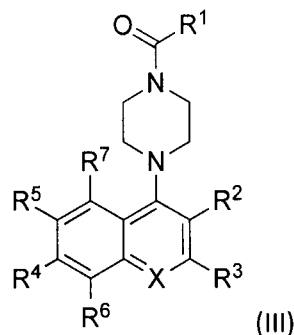
0096 The biologic activity of embodiments of the present invention can be seen in Table 2.

<u>Table 2</u>			
<u>ClpP Activation by ACP1 Analogs</u>			
Category	Label	RD ₂₅	Std. Deviation
Natural Product	ADEP1A	0.78	0.01
	ADEP1B	0.60	0.06
Parent Compound	ACP1	0.53	0.02
Western Mod.	1	0.77	n/a
	2	0.50	0.13
	3	0.13	0.03
	4	0.03	n/a
	5	0.03	0
	6	0	0
	7	0	0
	8	0	0
Central Mod.	9	0.69	n/a
	10	0.38	0.03
	11	0.32	0.01
	12	0.06	0
	13	0.02	0
	14	0.01	0

Table 2ClpP Activation by ACP1 Analogs

Eastern Mod.	15	0.85	0.01
	16	0.83	0.01
	17	0.76	n/a
	18	0.64	0.04
	19	0.59	0.03
	20	0.58	0.04
	21	0.51	0.01
	22	0.51	0.01
	23	0.49	0.03
	24	0.41	0.01
	25	0.39	0.06
	26	0.38	0
	27	0.36	n/a
	28	0.36	0.02
	29	0.32	0.02
	30	0.19	0.05
	31	0.09	0.03
	32	0.02	0
	33	0.02	0
	34	0.01	0.01
	35	0	0
	36	0	0

0097 Embodiments of the present invention may be directed to analogs of ACP3. The preferred embodiments of the ACP3 analogs are directed to compounds of formulae (III).



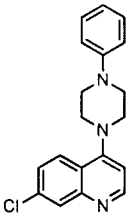
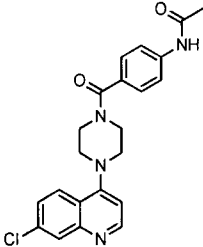
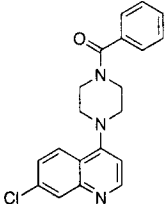
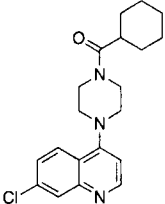
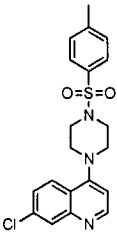
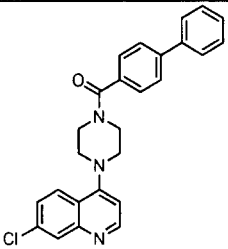
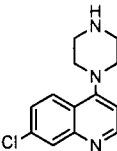
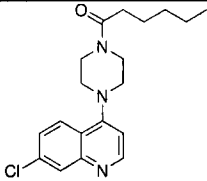
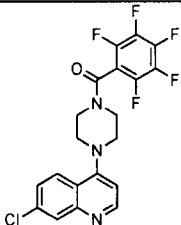
wherein

R^1 may be H, CH_3 , other C_2 - C_8 alkyl / cycloalkyl (preferably, methyl, ethyl, propyl, isopropyl, butyl, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl), alkenyl or substituted alkenyl (e.g., vinyl, propenyl, dihalovinyl), phenyl, substituted aromatic (preferably, with *ortho*, *meta* or *para* F, Cl, Br, I, CF_3 , CN, NO_2 , alkyl, aryl, COOH, $COOR^8$, $CONR^8_2$, COR^8 , OR^8 , NR^8_2 , wherein R^8 may be H, methyl, C_2 - C_8 alkyl / cycloalkyl, phenyl or substituted aromatic]), a fused aromatic (e.g., naphthalene, indene), a heteroaromatic (preferably, pyridine, pyrimidine, pyrazine, triazine, thiophene, furan, oxazole, thiazole, imidazole), or a fused heteroaromatic (preferably, quinoline, isoquinoline, benzimidazole, benzthiazole, benzoxazole);

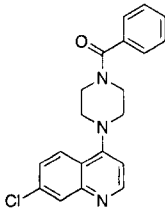
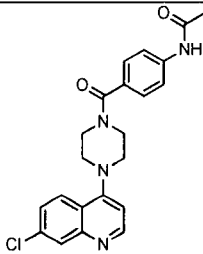
R^2 , R^3 , R^4 , R^5 , R^6 and R^7 may be H, CH_3 , a C_2 - C_8 alkyl / cycloalkyl (preferably, methyl, ethyl, propyl, isopropyl, butyl, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl), alkenyl or substituted alkenyl (e.g., vinyl, propenyl, dihalovinyl), phenyl, a substituted aromatic (preferably, with *ortho*, *meta* or *para* F, Cl, Br, I, CF_3 , CN, NO_2 , alkyl, aryl, COOH, $COOR^9$, $CONR^9_2$, COR^9 , OR^9 , NR^9_2 , wherein R^9 may be H, methyl, C_2 - C_8 alkyl / cycloalkyl, phenyl or substituted aromatic]), a fused aromatic (e.g., naphthalene, indene), a heteroaromatic (preferably, pyridine, pyrimidine, pyrazine, triazine, thiophene, furan, oxazole, thiazole, imidazole), or a fused heteroaromatic (preferably, quinoline, isoquinoline, benzimidazole, benzthiazole, benzoxazole); and

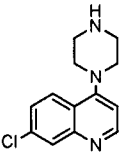
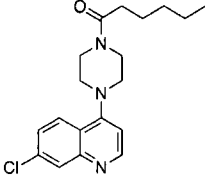
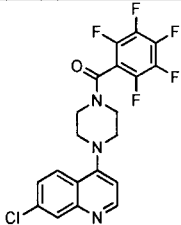
X may be CH and N.

0098 More preferred embodiments of the present invention are set out below:

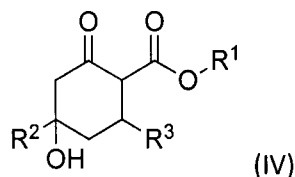
AJ09		AJ24	
AJ10		ACP3 AJ27	
AJ16		AJ28	
AJ17		AJ29	
AJ23			

0099 Still more preferred embodiments of the present invention are set out below:

AJ10		AJ24	
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AJ17		AJ29	
AJ23			

00100 Embodiments of the present invention may be directed to analogs of ACP4 and ACP5. The preferred embodiments of the ACP4/5 analogs are directed to compounds of formulae (IV):



wherein

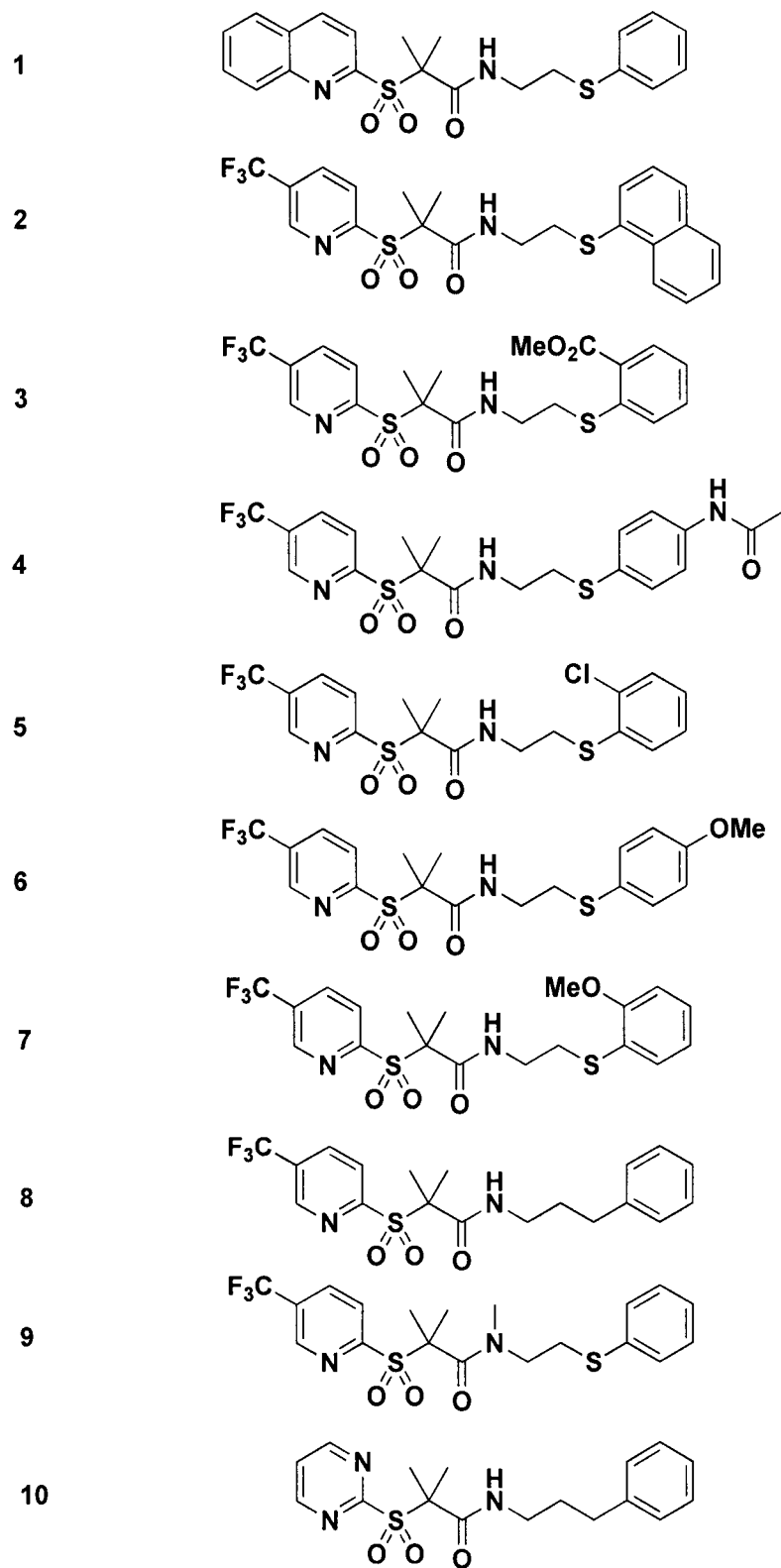
R^1 may be CH_3 or a C_2 - C_8 alkyl / cycloalkyl (preferably, ethyl, propyl, isopropyl, butyl, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl).

and

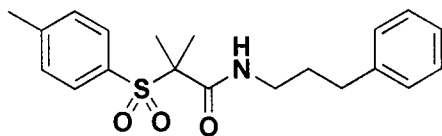
R^2 and R^3 may be H, alkyl / cycloalkyl (preferably, methyl, ethyl, propyl, isopropyl, butyl, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl), alkenyl or substituted alkenyl (e.g., vinyl, propenyl, dihalovinyl), phenyl, substituted aromatic (preferably, with *ortho*, *meta* or *para* F, Cl, Br, I, CF_3 , CN, NO_2 , alkyl, aryl, COOH, COOR, $CONR_2$, COR, OR, NR_2 wherein R may be H, methyl, a C_2 - C_8 alkyl / cycloalkyl, phenyl or substituted aromatic, fused aromatic (preferably, naphthalene or indene), a heteroaromatic (preferably, pyridine, pyrimidine, pyrazine, triazine, thiophene, furan, oxazole, thiazole, imidazole), a fused heteroaromatic (preferably, quinoline, isoquinoline, benzimidazole, benzthiazole, benzoxazole).

00101 Further embodiments of the present invention are provided below.

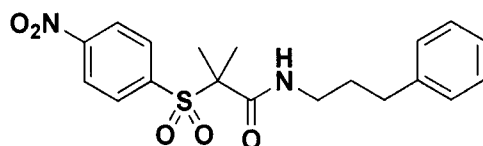
ACP1 Analogs:



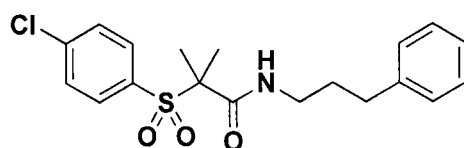
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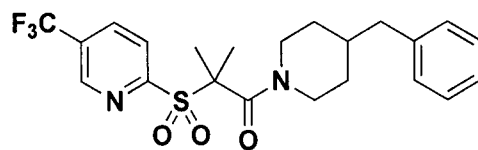
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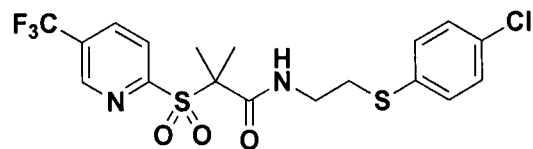
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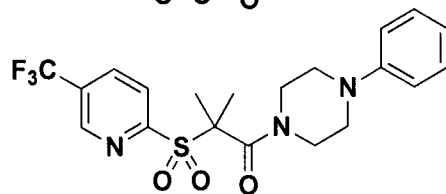
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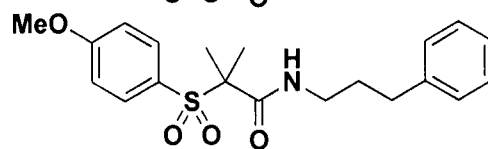
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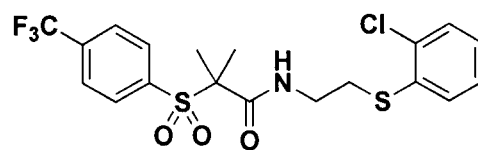
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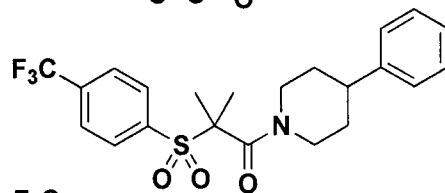
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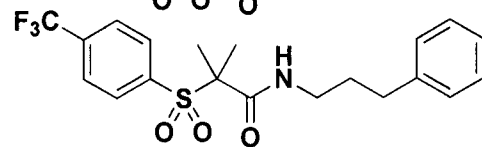
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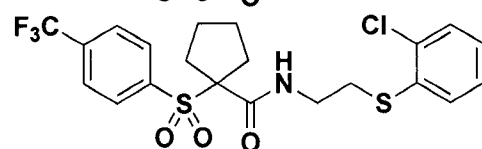
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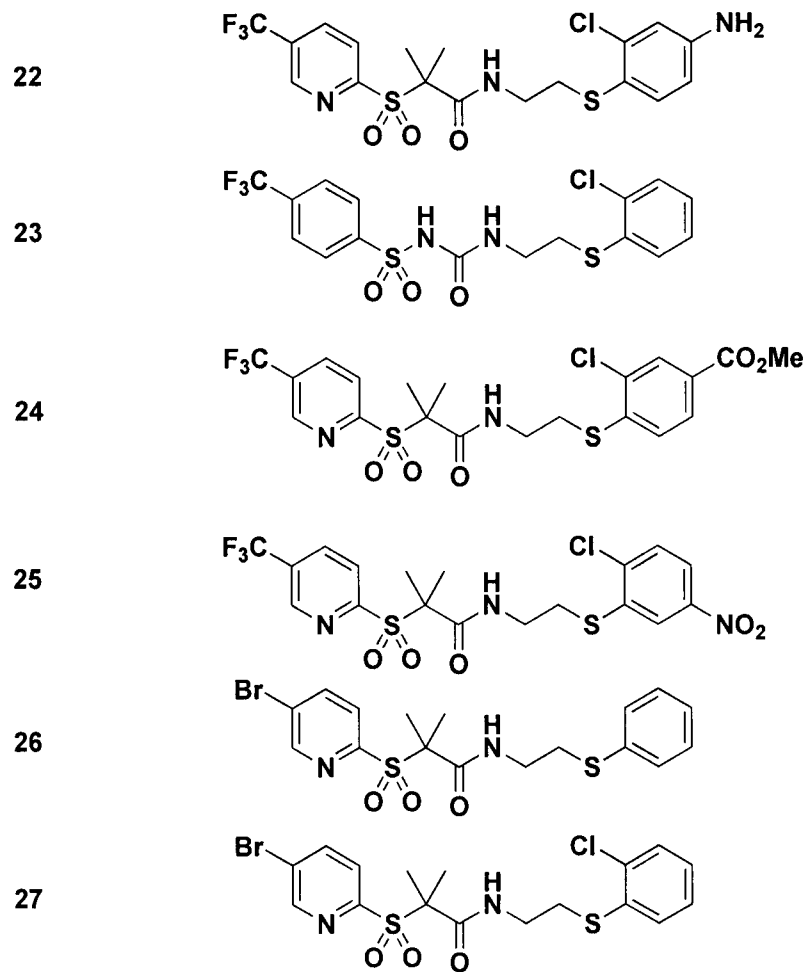


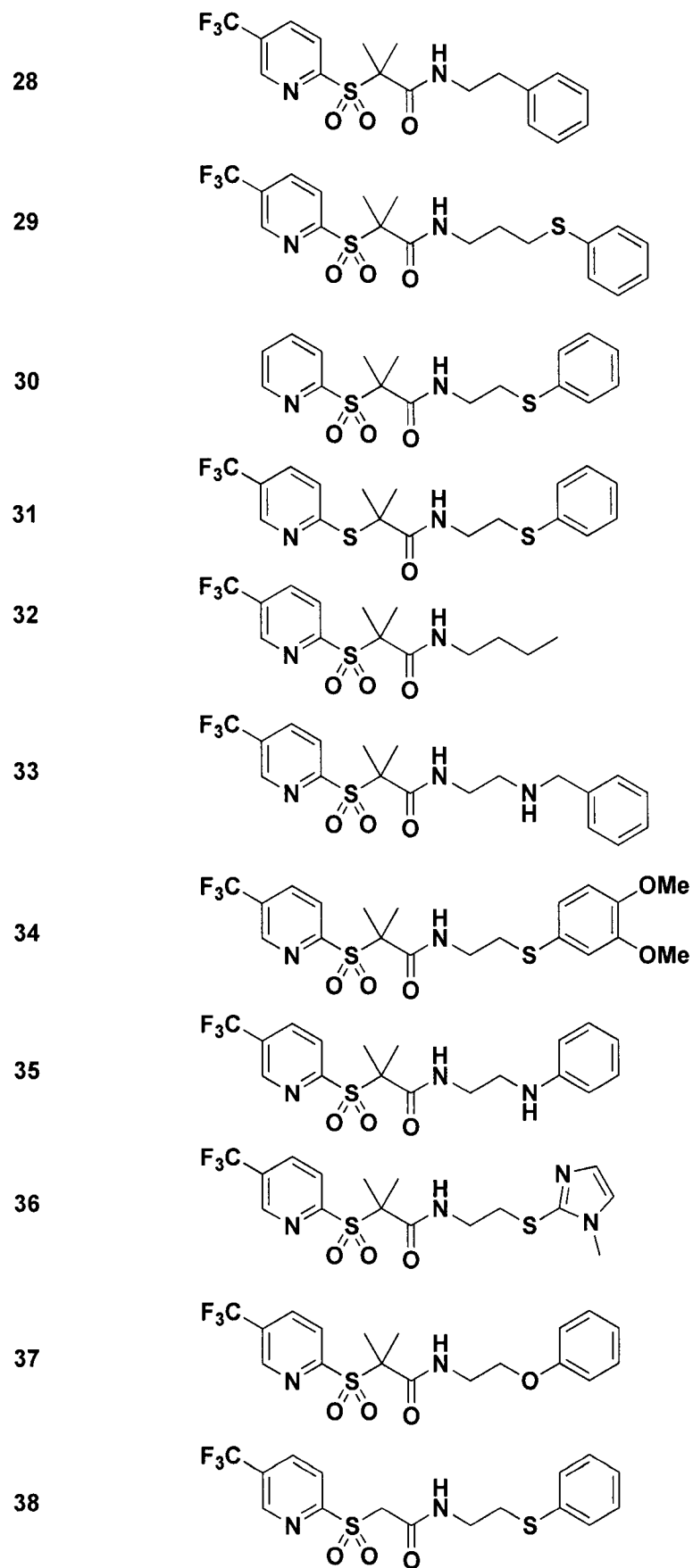
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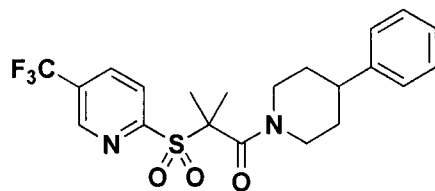
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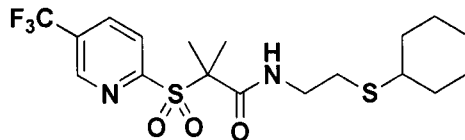




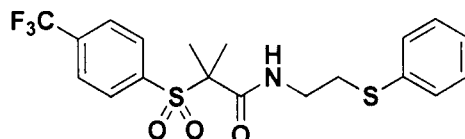
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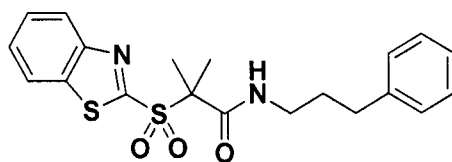
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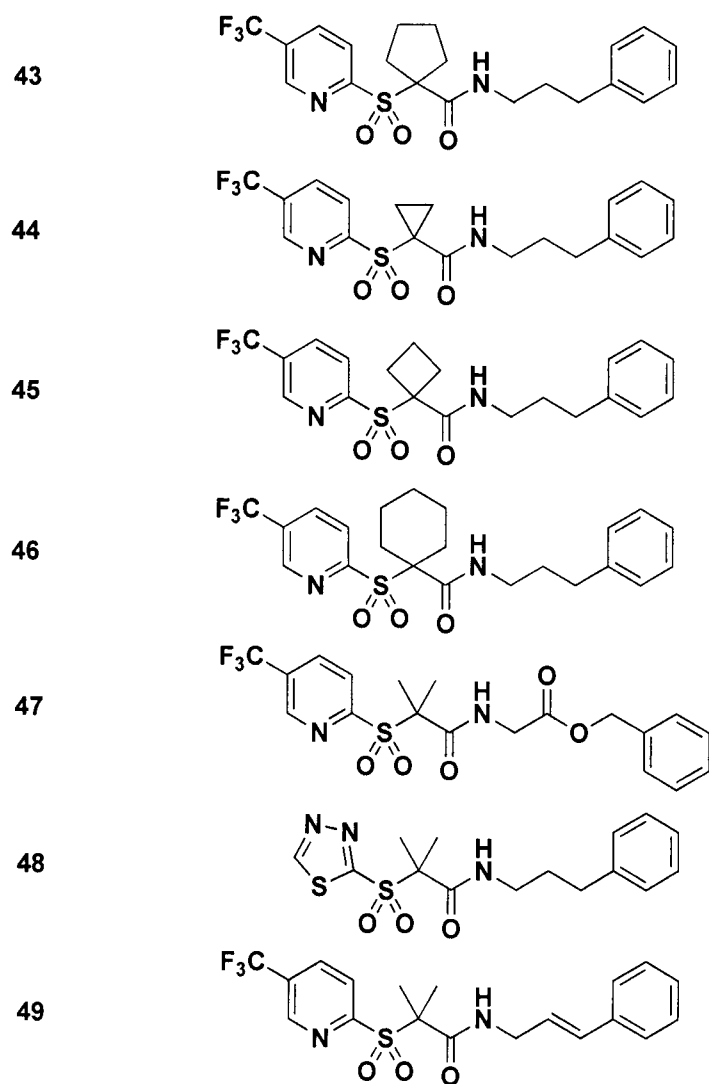


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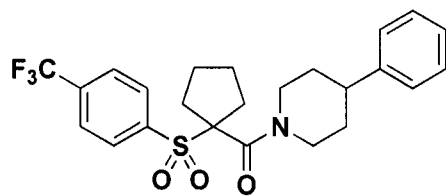


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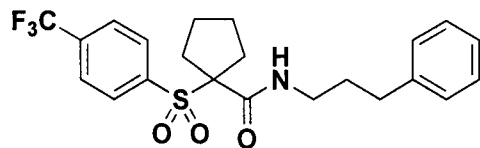




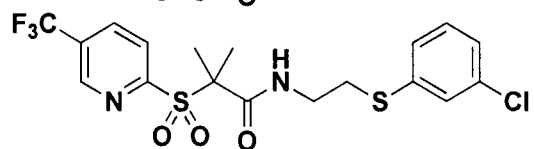
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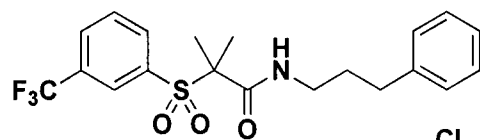
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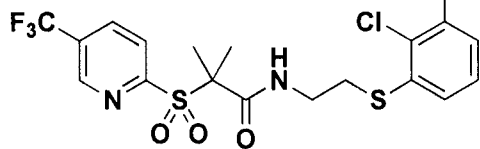
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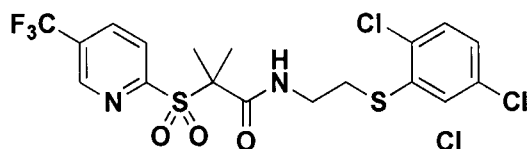
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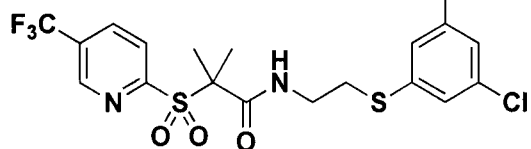
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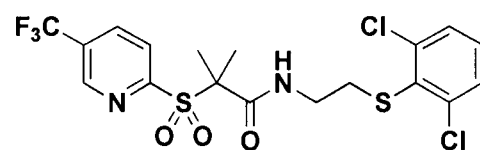
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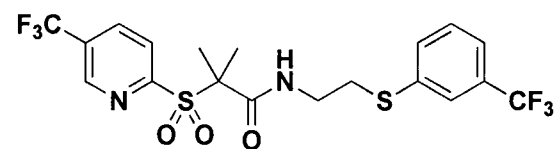
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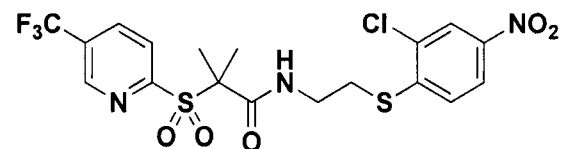
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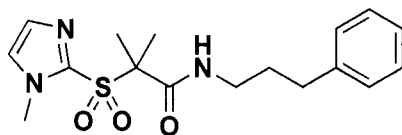
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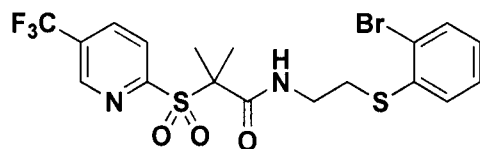
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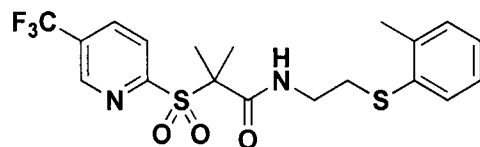
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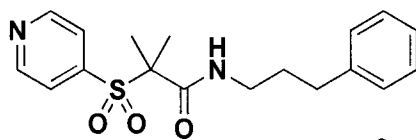
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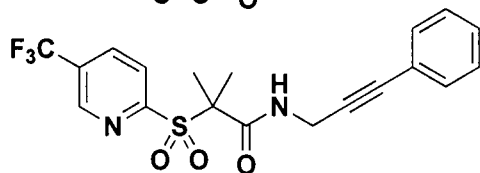
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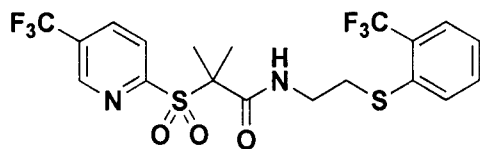
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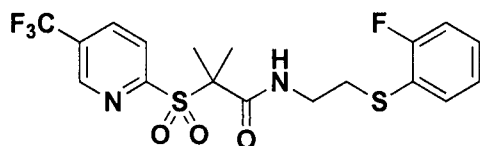
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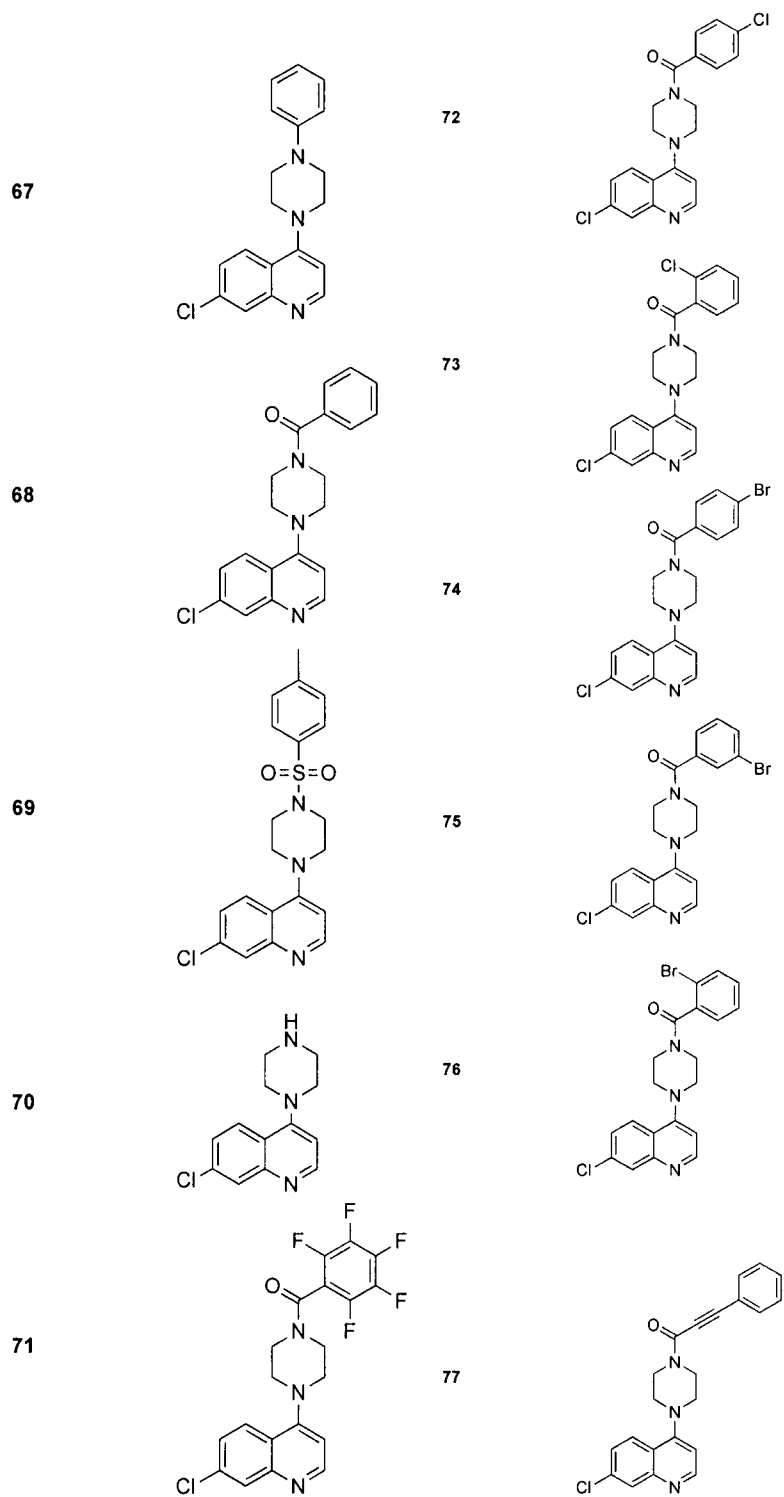
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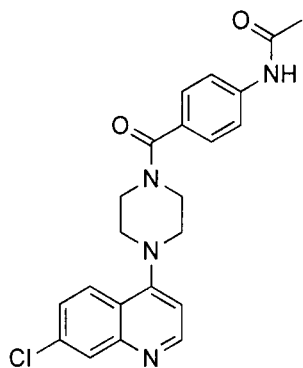
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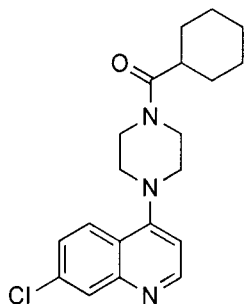
ACP 3 Analogs:



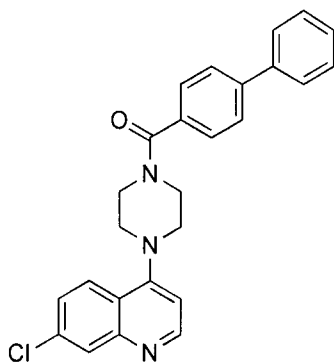
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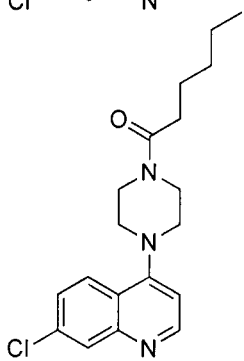
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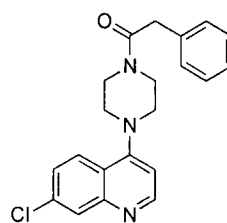
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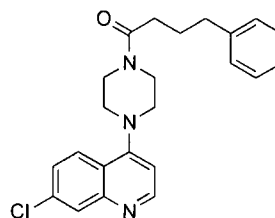
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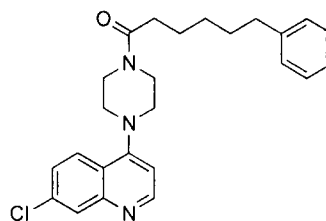
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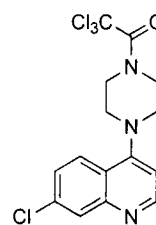
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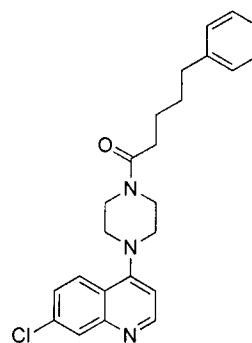
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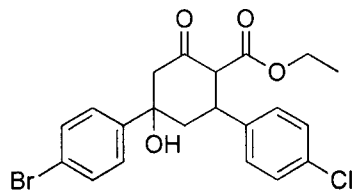
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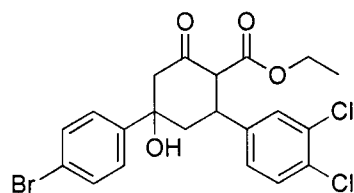
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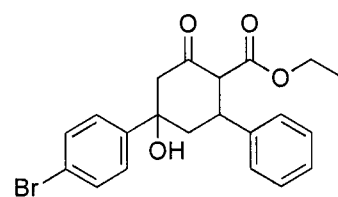
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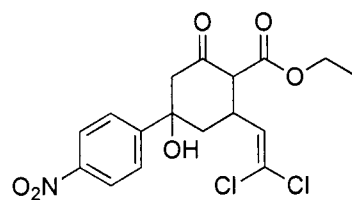
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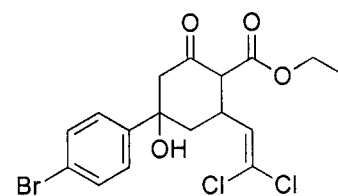
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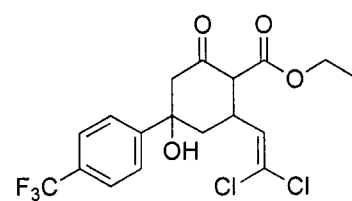
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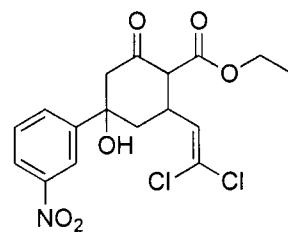
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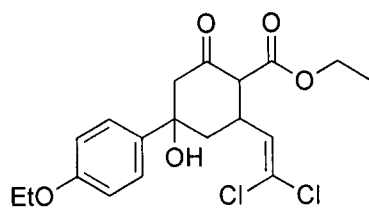


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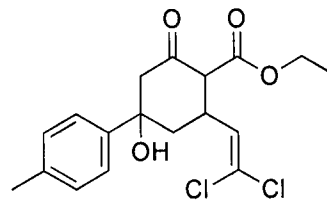


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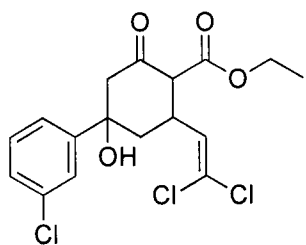
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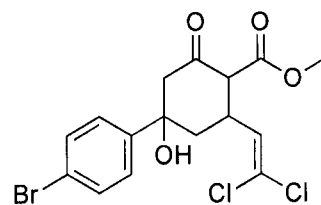
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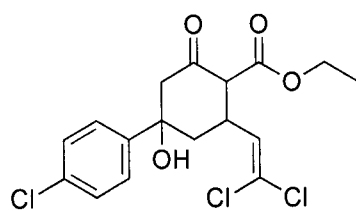
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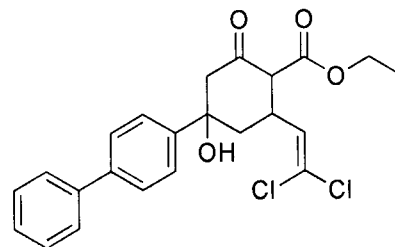
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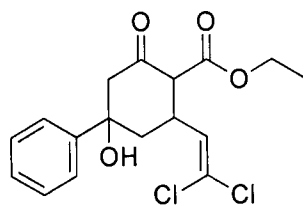
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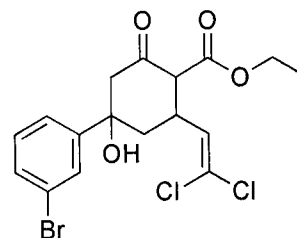
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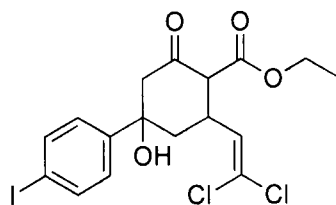
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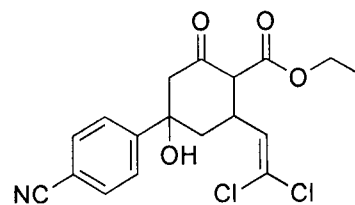
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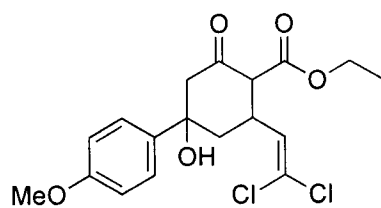
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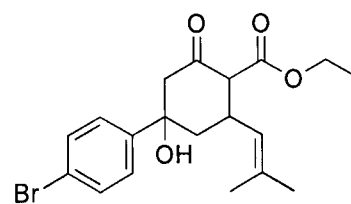
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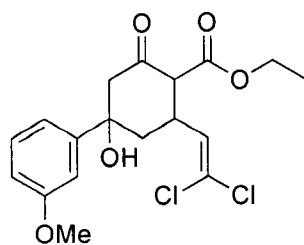
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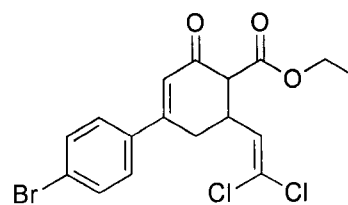
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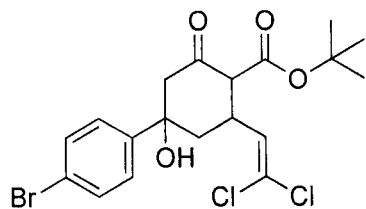
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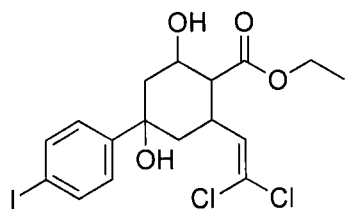
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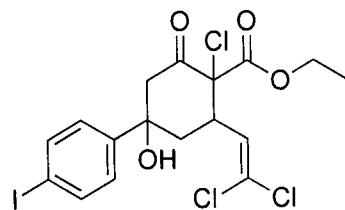
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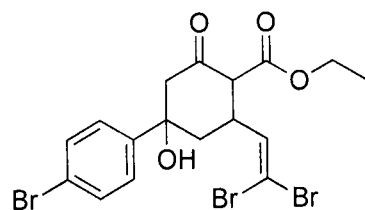
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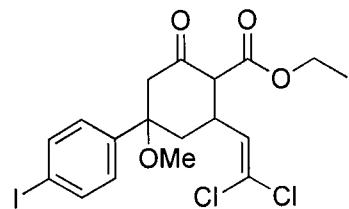
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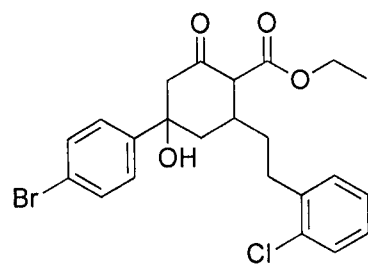
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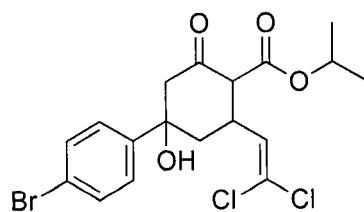
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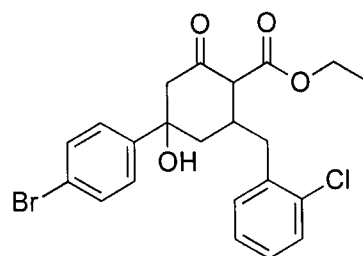
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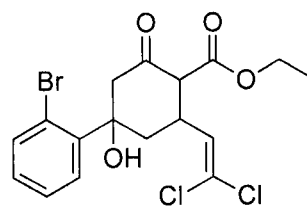
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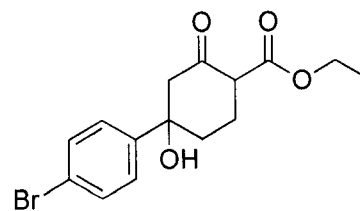
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00102 As set out in Table 3, there is provided *in vitro* and *in vivo* activity data of the embodiments of the present invention.

EXAMPLES

00103 In order to illustrate the invention, the following examples are provided. These examples do not limit the invention. They are meant to illustrate embodiments of the invention. Those skilled in the art may find additional embodiments in light of the teachings and examples provided herein, additional embodiments that are deemed to be within the scope of this invention.

00104 To identify embodiments of the present invention that activate ClpP, a high-throughput screening assay was developed with a fluorescence-based readout. The assay employed fluorescein isothiocyanate-labeled casein (casein-FITC) as the proteolytic target of the *E. coli* ClpP. When casein-FITC is intact, FITC fluorescence is quenched; protease-catalyzed hydrolysis of casein-FITC relieves this quenching, yielding highly fluorescent dye-labeled peptides. The high-throughput screening assay may be used to select compounds that resulted in increased fluorescence upon incubation of casein-FITC with ClpP. Preliminary tests performed in the presence and absence of the unfoldase chaperone ClpA revealed a 5-fold dynamic range after 30-minute incubation and intra- and inter-assay variability, expressed as coefficient of variation, of 2 and 5%, respectively. In light of ClpP stability over several hours at 37°C, reactions were typically monitored every 15 min for 6 hours to rule out time-dependent effects.

00105 With the initial intent of exploring drug repositioning opportunities, 4,500 chemicals composed of biologically- and pharmacologically-active entities were tested, of which approximately 45% were marketed drugs or drug candidates evaluated in clinical trial stages. None of these compounds were active at a concentration of 10 μ M, suggesting the necessity to significantly broaden chemical diversity to explore ClpP druggability and the likelihood to activate ClpP using small molecules. The screening campaign was expanded to include an additional ~60,000 chemicals. This undertaking was carried out using a final compound concentration of 20 μ M. Results were normalized and corrected for systematic errors using the B-score method (Fig. 1A) and positive hits were defined as the compounds whose signals were at least three standard deviations (99.73% confidence interval) from the mean of the general sample

population. An excellent quality of the screening setup was shown by the dimensionless parameters Z'- and Z-factors, which were consistently in the 0.7 range throughout the entire screening campaign, thereby indicating an effective combination of dynamic range, variability, and hit rate.

00106 This chemical screen led to the selection of five confirmed hits (Fig. 1B), that were named Activators of Self-Compartmentalizing Proteases (“ACPs”) 1 to 5. Interestingly, two of them (ACP4 and ACP5) were analogous molecules, with identical structures that only differed in the modification of an aromatic group (Fig. 1B).

00107 Characterization of ACP-mediated ClpP activation. To compare the potency of ACPs, dose-response analyses were carried out following the degradation of casein-FITC for six hours (Fig. 2A). The results were evaluated using a quantitative measure referred to as the relative degradation index (RD) and defined as Equation (1):

$$RD = \frac{(\Delta\phi_{\text{ClpP+compound}})_{\text{after 6 hrs}} - (\Delta\phi_{\text{ClpP}})_{\text{after 6 hrs}}}{(\Delta\phi_{\text{E. coli ClpAP}})_{\text{after 6 hrs}} - (\Delta\phi_{\text{E. coli ClpP}})_{\text{after 6 hrs}}} \dots (1)$$

00108 $\Delta\phi$ is the change in fluorescence after 6 hours of starting the reaction, measured using 485 nm excitation and 535 nm emission, which primarily detects the signal from casein-FITC. To measure the RD index, each reaction consisted of 3.6 μM ClpP and a specified amount of compound in buffer A (25 mM TrisHCl, pH 7.5, and 100 mM KCl). The reactions were pre-incubated for 10 minutes at 37°C before 4.5 μM casein-FITC and 15.5 μM unlabelled casein (to ensure that the substrate is in excess) were added. ClpAP-dependent degradation of the same casein-FITC substrate was used as a control. Each ClpAP reaction contained 3.6 μM ClpP, 3 μM ClpA and 0.3 mM ATP in buffer B (25 mM HEPES pH 7.5, 20 mM MgCl_2 , 30 mM KCl, 0.03% Tween 20, and 10% glycerol), and an ATP-regenerating system (13 units/mL of creatine kinase and 16 mM creatine phosphate). Reactions were incubated at 37°C and the fluorescence (485 nm excitation, 535 nm emission) was monitored every 15 minutes for 6 hours on a PHERASar detection system. Casein-FITC degradation by compound-activated ClpP after 6 hours at 37°C

was compared to the ClpAP-dependent casein-FITC degradation after 6 hours at 37°C, using equation (1). The number next to RD refers to the final concentration, in μM , of the compound in the reaction mixture. For example, RD25 refers to the RD value measured using 25 μM of final compound concentration. *E. coli* ClpAP is used as a benchmark for maximum ClpP proteolytic activity.

00109 The activation of ClpP by the various ACPs was further confirmed by direct observation of unlabeled casein degradation on SDS-PAGE gels (Fig. 7), which also indicated that the activation of embodiment-mediated ClpP activation was consistent with the observations obtained from fluorometric determinations.

00110 In order to verify that the activators were acting on ClpP rather than on the casein substrate directly, the ability of embodiment-activated ClpP to degrade proteins from various organisms was tested using a variety of model substrates and unrelated proteins (Fig. 8 and Table S1). It will be understood based on Table S1 that some activators may induce ClpP to degrade a wider variety of protein substrates than the other activators with no apparent specificity. However, the proteins subject to extensive proteolytic cleavage, such as Casein, Tah1, CFTR R-region, reduced carboxymethylated α -lactalbumin (RCMLa), λN protein, and α -synuclein are either considered to be unstable or disordered proteins or to have disordered regions. Conversely, well-folded substrates, such as GFP-SsrA and creatine kinase, were degraded to a lesser extent or not at all, likely due to the stability of their structures. Proteins which were only clipped by compound-activated ClpP (α -M protein, ClpA, and ClpX) were all larger sized proteins.

Table S1. CIP degradation assays with alternative substrates.

Substrate	MW (kDa)	Activator						
		ADEP1A	ADEP1B	ACP1	ACP2	ACP3	ACP4	ACP5
Bovine α -casein	35	++	++	++	++	++	++	++
Yeast Tah1	13	++	++	++	++	++	++	++
Human CFTR R-Domain	33	++	++	++	++	++	++	++
Bovine reduced carboxymethylated α -lactalbumin	14	++	++	++	++	++	++	++
Bacteriophage AN-M ₆	15	++	++	++	++	-	++	++
Human α -synuclein	16	++	++	++	-	-	++	++
Bacteriophage λ O	33	-	-	-	-	-	-	-
<i>Neisseria</i> α -M protein	44	-	-	-	-	-	-	-
<i>E. coli</i> CIP A	84	-	-	-	-	-	-	-
<i>E. coli</i> CIP X	50	-	-	-	-	-	-	-
Jellyfish green fluorescent protein-ssrA	27	-	-	-	-	-	-	-
Rabbit creatine kinase	43	-	-	-	-	-	-	-

The double plus (++) denotes complete substrate degradation, the single plus (+) denotes partial degradation or clipping of the substrate, and the hyphen (-) denotes no visible degradation. The original SDS-PAGE gels are available in Fig. S2.

00111 Chemical optimization of ACP1. When tested for bactericidal properties against 10 different bacteria, the embodiments showed minimum bactericidal concentrations (“MBC”) at relatively low concentrations (Table S2). Based on the list provided, it will be understood by a person skilled in the relevant art that the embodiments of the present invention would have bactericidal activities against a broad range of bacteria, including, but not limited to, the families *Neisseriaceae*, *Pasteurellaceae*, *Enterobacteriaceae*, *Pseudomonadaceae*, *Staphylococcaceae*, *Streptococcaceae*, *Listeriaceae*, and *Mycobacteriaceae*. It will be further understood that each of the families listed encompasses several species of bacteria.

Table S2. Minimum bactericidal concentration of compounds.

PMBN	ACP1		ACP2		ACP3		ACP4		ACP5	
	+	-	+	-	+	-	+	-	+	-
<i>N. gonorrhoeae</i>	>256	>256	>256	64	>256	>256	32	32	>256	>256
<i>N. meningitidis</i>	64	64	32	32	>256	>256	4	4	>256	>256
<i>H. influenzae</i>	64	>256	8	>256	>256	>256	4	128	32	>256
<i>E. coli</i>	>256	>256	8	>256	>256	>256	16	>256	16	>256
<i>S. typhimurium</i>	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
<i>P. aeruginosa</i>	>256	>256	8	>256	>256	>256	>256	>256	>256	>256
<i>S. aureus</i>	N/A	>256	N/A	>256	N/A	>256	N/A	>256	N/A	>256
<i>S. pneumoniae</i>	N/A	>256	N/A	16	N/A	>256	N/A	8	N/A	>256
<i>L. monocytogenes</i>	N/A	>256	N/A	32	N/A	>256	N/A	>256	N/A	>256
<i>M. smegmatis</i>	>256	>256	>256	>256	>256	>256	128	128	256	256

4-8
16-64
128 or more

00112 The chemical structures of ACP1–5 (Fig. 1B) show little obvious structural similarities with the ADEPs (Fig. 9) or with each other, except for ACP 4 and 5. As a preferred embodiment, ACP1 was selected for further optimization since it had the higher RD25 value than other embodiments (Fig. 2B).

00113 ACP1 satisfies Lipinski's rule of five and has a topological polar surface area of 75.6, calculated molar refractivity of 10.4, and Clog P of 3.98. The structure of ACP1 consists of a central β -amido sulfone core appended with a western electron deficient pyridyl ring and an eastern hydrophobic tail incorporating a phenylthioether group (Fig. 1B and 3A). The natural synthetic disconnection point chosen for the synthesis of analogs of ACP1 was the amide linkage (Fig 3A). Analogs were synthesized using a late-stage amide-bond forming reaction between the eastern amine and the western β -sulfonyl carboxylic acid (Fig 3B) using well-known synthetic protocols and, subsequently, evaluated by measuring their RD25 values. We used ADEP1 factor A and B (Fig. 9) isolated from *Streptomyces hawaiiensis* as a reference. ADEP1A has a higher ClpP activation activity than ADEP1B (Fig. 3C).

00114 A screen of analogs identified a preferred embodiment, ACP1b (Fig. 1B) that had an RD25 value slightly higher than that of ADEP1A (Fig. 3C). For comparison, another analog, ACP1a, that had a lower RD25 value than that of ACP1b, but comparable to that of ADEP1B (Fig. 3C), is shown in Fig. 1B. ACP1a incorporates a sulfur to methylene substitution in the eastern tail, and ACP1b has an *ortho*-chloro substituent in the arylthioether ring (Fig. 1B).

00115 The degradation of casein upon activation of ClpP by these compounds is shown in Fig. 3D and reflects the RD25 results. It has previously been shown that ADEPs activate *E. coli* ClpP to degrade casein with reduced processivity. This was also true for the ACPs (Fig. 10). The patterns of appearance and disappearance of the degradation intermediates may suggest similarities between the general mechanisms of activation by the different compounds.

00116 Among the different bacterial species tested, ACP1b had the lowest MBC value against *H. influenzae* compared to ACP1 and ACP1a; the MBC of ACP1b was similar to that of ADEP1A (see Table 1). ACP1b also had an improved MBC value against *N. meningitidis* compared to ACP1 and ACP1a, but not ADEP1A (see Table 1). These results suggest that the optimization efforts improved the antibacterial properties of ACP1.

Table 1. Minimum bactericidal concentration of compounds.

	ADEP1A	ACP1	ACP1a	ACP1b
<i>N. meningitidis</i>	0.25	64	64	16
<i>H. influenzae</i>	8	16	32	8

00117 Determination of binding affinity and stoichiometry of ACP1b. The dissociation constant (K_d) for the binding of ACP1b to ClpP was measured using isothermal titration calorimetry (ITC) and was found to be about 3 μ M, which is comparable, within errors, to that of ADEP1A (0.3 μ M) and ADEP1B (0.7 μ M) (Fig. 4A). The results suggest that there may be 14 binding sites for the ADEPs and ACP1b on ClpP₁₄. In comparison, ACP1 has a dissociation constant for ClpP of about 130 μ M as determined by Surface Plasmon Resonance (SPR) measurements (Fig. S5). ITC could not be used to measure ACP1 affinity for ClpP due to weak binding, but the K_d 's obtained for the ADEPs by SPR (Fig. S5) are similar to those obtained by ITC (Fig. 4A).

00118 Measurement of casein degradation by compound-activated ClpP as a function of ADEP1A or ACP1b concentration resulted in a saturation curve with $S_{0.5}$ of 2 – 3 μ M and a Hill coefficient, h , of about 4 (Fig. 4B). The data may suggest that these compounds act on ClpP in a similar manner and bind to the protease cooperatively or promote cooperative allosteric transitions within the protease structure.

00119 ClpP Tetradecamer Stabilization by ACP Binding. Thermal melt experiments indicated that the embodiments of the present invention enhanced ClpP stability. An N-terminal truncation mutant of *E. coli* ClpP was constructed to open the central pore of the protease in an attempt to mimic a proposed mechanism of compound activation. However, this mutant, ClpP Δ 31, mainly eluted as a heptameric single ring upon size exclusion chromatography and had no peptidase activity. In order to verify the oligomeric state of this protein, sedimentation velocity analytical ultracentrifugation was employed (Fig. 5A, top panel). The results indicate that the ClpP Δ 31 mutant had a molecular weight corresponding to that of a heptamer; by comparison, WT ClpP is mostly tetradecameric with a small proportion of heptamers (Fig. 5A, bottom panel). Addition of ACP1, ACP1a, or ACP1b promoted the formation of tetradecameric ClpP Δ 31 (Fig. 5A top panel) and resulted in a catalytically active ClpP Δ 31 (Fig. 5B). The proportion of tetradecamers formed correlated with the binding affinity of the compounds, with ACP1 resulting in much less

tetradecamers being formed compared to ACP1b (Fig. 5B, top panel). These results strongly suggest that the compounds stabilize ClpP by promoting its tetramer formation.

00120 ACP binding sites on ClpP. The ADEPs have been found to bind in a hydrophobic pocket (the “H pocket”) on ClpP apical surface in which the IGF loop of the ClpX/ClpA ATPases also binds. As a result, the presence of the ADEPs inhibited or reduced the ClpXP/ClpAP-mediated degradation of GFP-ssrA by interfering with the binding of ClpX/ClpA to ClpP. As shown in Fig. 6A and 12, a similar effect was also seen for embodiments of the present invention suggesting that these compounds may bind to or allosterically modulate the H pocket of ClpP.

00121 Two binding pockets on ClpP of equal probability were predicted by computational procedures implemented in DOCK6.3 software (Fig. 6B and Tables S3-S5): the H pocket and a separate pocket, which we have named as the C pocket, featuring a larger number of charged residues, referred to herein as the C pocket. The H pocket is composed of residues V42, F44, L62, Y74, Y76, I104, F126, L203, and R206 from one subunit, and L37 and F96 from the neighboring subunit. The C pocket comprises residues Y90, M94, Q95, D100, V101, and H170 of one subunit and residues H205, N207 of the neighboring subunit. Interestingly, the H and C pockets are separated by residues at the very C-terminus of ClpP, corresponding to amino acids 203 – 207 (using Swiss-Prot numbering). While ADEPs co-crystallized with ClpP were found bound to the H pocket, all the ACP compounds were well docked to both the H and C pockets (Fig. S7), and their docking scores differed little between the two pockets (Table S5).

Table S3. ZINC ID corresponding to ACP4 chiral isomers.

ZINC ID ^a	X ₁	X ₂	X ₃
ZINC15636003	S	S	S
ZINC04011799	S	S	R
ZINC01234770	S	R	S
ZINC04011798	S	R	R
ZINC15636001	R	S	S
ZINC00753769	R	S	R
ZINC15636005	R	R	S
ZINC04011797	R	R	R

^a ACP4 is ethyl (1X₁,2X₂,4X₃)-2-(2,2-dichloroethenyl)-4-hydroxy-4-(3-nitrophenyl)-6-oxocyclohexane-1-carboxylate in IUPAC nomenclature, where X₁, X₂, and X₃ can adopt either S or R chirality.

Table S4. ZINC ID corresponding to ACP5 chiral isomers.

ZINC ID ^a	X ₁	X ₂	X ₃
ZINC15635969	S	S	S
ZINC35327726	S	S	R
ZINC15635993	S	R	S
ZINC35327717	S	R	R
ZINC01234768	R	S	S
ZINC00752024	R	S	R
ZINC15635991	R	R	S
ZINC35327713	R	R	R

^aACP5 is ethyl (1X₁,2X₂,4X₃)-4-(4-bromophenyl)-2-(2,2-dichloroethenyl)-4-hydroxy-6-oxocyclohexane-1-carboxylate in IUPAC nomenclature, where X₁, X₂, and X₃ can adopt either S or R chirality.

Table S5. Lowest Dock6 score for ACP compounds.

Name of Compound	Dock6 Score	
	H pocket	C pocket
ACP1	-43.36	-42.05
ACP2	-74.50	-71.10
ACP3	-37.91	-39.72
ACP4 ^a	-40.75	-42.14
ACP5 ^b	-38.78	-40.02

^aZINC15636003 and ZINC04011797 showed the lowest Dock6 score values on H and C pockets, respectively.

^bZINC00752024 and ZINC35327717 showed the lowest Dock6 score values on H and C pockets, respectively.

00122 As shown in Figs. 4-6, the identified ACP compounds may share a similar mechanism of ClpP activation as the ADEPs, including, but not limited to stabilizing ClpP and promoting the formation of the double ring structure. The embodiments may confer on ClpP similar degradation patterns (Fig. 10). The embodiments of the present invention may prevent the binding of ClpP to its associated unfoldase (Figs. 6A and 12), and may, at the same time, activate nonspecific proteolysis.

00123 The co-crystal structures of ADEP bound to *B. subtilis* and *E. coli* ClpP show ADEP binding to the H pocket of the protease. Here we have identified another binding pocket, the C pocket, which may also be involved in compound binding, as supported by the mutational analysis provided in Fig. 6C. The binding of compounds in the H pocket may mimic the binding of ATPase chaperones and may cause conformational changes in the ClpP axial pore, which may normally occur with the binding of the Clp ATPase.

GENERAL EXPERIMENTAL DETAILS

00124 Subcloning and mutagenesis. The plasmids pET9a ClpP and pET9a ClpA(M169T) were used for overexpressing the respective *E. coli* proteins. The M169T mutation in ClpA removes an internal translation initiation site. ClpA(M169T) is referred to as ClpA.

00125 The coding sequences for genes to overexpress *E. coli* ClpP Δ 31 were amplified from the pET9a ClpP plasmid and inserted between the NdeI and BamHI sites of pET3a. Full length ClpP point mutants were constructed using mutagenic primers to the pET9a ClpP plasmid and the QuickChangeTM system (StratageneTM).

00126 Protein expression and purification. All proteins were expressed from IPTG inducible promoters. ClpP constructs were expressed in BL21(DE3)1146D strain, which lacks the gene for chromosomal *E. coli* ClpP. All other constructs were expressed in BL21(DE3)Gold (StratageneTM). Untagged wild type and mutant *E. coli* ClpP, ClpX, and GFP-SsrA were expressed and purified using known methods. All His-tagged proteins were purified on Ni-NTA agarose resin (QiagenTM) according to the manufacturer's protocols. If possible, the tag was removed using the tobacco etch virus (TEV) protease. Protein concentrations were determined by absorbance at 280 nm with extinction coefficients calculated using ProtParamTM.

00127 Tah1 and λ O were purified as previously described. pHF010 plasmid encoding H₆- λ N was used to produce and the protein was purified according to protocols described elsewhere.

00128 Isolation of ADEP1A and ADEP1B from *Streptomyces hawaiiensis* NRRL 15010. Bacterial strain *Streptomyces hawaiiensis* NRRL 15010 was obtained from the US Agricultural Research Service Culture Collection (NRRL). Amberlite XAD16 resin and Diaion HP-20 resin were purchased from Sigma-Aldrich (St. Louis, MO); all other chemicals and solvents were from Fisher Scientific (Pittsburgh, PA) and its associated providers. A54556 factor A and B, referred to as ADEP1A and ADEP1B, respectively, were purified to at least 95% homogeneity from the fermentation culture of *S. hawaiiensis*. Specifically, 20 liters of fermentation medium (0.4% D-glucose, 0.4% yeast extract, 1.0% malt extract, 0.1% CaCO₃, 1.0% Diaion HP-20 resin, and 1.0% Amberlite XAD16 resin in tap water, pH 7.2) was inoculated with 200 mL of overnight seed culture, divided into 40 flasks (500 ml per 2-L flask), cultivated for 4 days at 30°C on a

rotary shaker (200 rpm). Culture filtrate and residue (resin, mycelia and cell debris) were separated by filtration. The filtrate was extracted with equal volume of ethyl acetate for 3 times and the residue was extracted with 2-fold volume of ethyl acetate for 5 times. The ethyl acetate extracts were combined and evaporated under reduced pressure to an oily dense residue. The residue was dissolved in methanol and subjected sequentially to normal phase silica gel gravity chromatography (eluting with chloroform/methanol), C-18 reverse phase silica gel (ODS) gravity chromatography (eluting with methanol/water), and C-18 reverse phase silica gel preparative high performance liquid chromatography (HPLC; eluting with methanol/water). Fractions containing the target compounds were monitored by electro-spray ionization mass spectrometry (ESI-MS). The final compound preparations were verified by mass spectrometry and nuclear magnetic resonance analyses, and stored as amorphous dry powder.

00129 Chemical libraries and high-throughput screening. The libraries employed for the screening campaigns were composed of experimental bioactives, pharmacologically-active chemicals and natural products, off-patent marketed drugs, and small molecules with drug-like properties. Samples were obtained from the following, commercially-available collections: LOPAC 1280™ (Sigma, USA, 1280 samples), Prestwick Chemical library® (Prestwick Chemical, France, 1120 samples), SPECTRUM collection (MicroSource, USA, 2000 samples), Maybridge Screening collection (Maybridge-Thermo Fisher Scientific, UK, 50,000 samples), and Chembridge DIVERSet™ (ChemBridge Corp, USA, 10,000 samples). In all instances, samples stored in 384-well plates as 1 mM or 5 mM solutions in 100% DMSO were transferred to assay plates in a fixed volume of 200 nL by a pin-tool (V&P Scientific, USA). Screens were conducted using a fully automated procedure run on a DIM4 flipmover platform (Thermo Electron Corp) equipped with a Biomek FX liquid handler (Beckman, USA) and a PHERAStar detection system (BMG Labtech, Germany). The reaction for the screening assay contained 20 µM compound, 3.6 µM ClpP, and 4.5 µM casein-FITC in buffer A (25 mM TrisHCl, pH 7.5, and 100 mM KCl) at 37°C.

00130 Activity assays. To measure the RD index, each reaction consisted of 3.6 µM ClpP and a specified amount of the applicable the applicable compound in buffer A. The reactions were pre-incubated for 10 minutes at 37°C before 4.5 µM casein-FITC and 15 µM unlabelled casein were added. ClpAP-dependent degradation of the same casein-FITC substrate was used as a positive

control. Each ClpAP reaction contained 3.6 μ M ClpP, 3 μ M ClpA and 0.3 mM ATP in buffer B (25 mM HEPES pH 7.5, 20 mM MgCl₂, 30 mM KCl, 0.03% Tween 20, and 10% glycerol), and an ATP-regenerating system (13 units/mL of creatine kinase and 16 mM creatine phosphate). Reactions were incubated at 37°C and the fluorescence (485 nm excitation, 535 nm emission) was monitored every 15 minutes for 6 hours on a PHERAStar detection system. Casein-FITC degradation by compound-activated ClpP after 6 hours at 37°C was compared to the ClpAP-dependent casein-FITC degradation after 6 hours at 37°C, using equation (1) set out herein.

00131 The effects of the compounds on the degradation of GFP-ssrA by ClpXP were also analyzed on SDS-PAGE gels. Each reaction contained 1.2 μ M ClpP, 3.9 μ M GFP-ssrA, 3mM ATP and 100 μ M compound in buffer C (25 mM HEPES pH 7.5, 5 mM MgCl₂, 5 mM KCl, 0.03% Tween 20, and 10% glycerol), and an ATP-regenerating system. The reaction mixtures were pre-incubated for 3 minutes at 37°C before 1 μ M ClpX was added. Samples were taken at various time points, stopped by boiling in 2% SDS, and resolved on 12% SDS-PAGE gels.

00132 Peptidase activity of ClpP was measured by the ability of ClpP to cleave the dipeptide Suc-LY-AMC. Each reaction contained 1 μ M ClpP in buffer D (50 mM TrisHCl, pH 8, 200 mM KCl, and 1 mM DTT). ClpP was incubated for 3 minutes at 37°C before Suc-LY-AMC was added to a final concentration 0.5 mM. Fluorescence (350 nm excitation, 460 nm emission) of the released AMC was detected on the PHERAStar system.

00133 For the Hill plot analysis of Fig. 4B, each reaction consisted of 3.6 μ M ClpP and specified amount of the identified compound in buffer A. The reactions were pre-incubated for 10 minutes at 37°C before 4.5 μ M casein-FITC was added. Reactions were incubated at 37°C and the fluorescence (485 nm excitation, 535 nm emission) was monitored every 3 seconds for 5 minutes on an EnSpire Multilabel Plate Reader (PerkinElmer™).

00134 Surface Plasmon Resonance (SPR) measurements. SPR measurements were conducted on a BiacoreX instrument (GE Healthcare) at 25°C. ClpP was immobilized on CM5 chips (GE Healthcare) using the Biacore amine coupling kit (GE Healthcare), following the manufacturer's protocols. One flow cell was immobilized with ClpP, while the other was activated and deactivated without protein immobilization. The sensorgrams in the sham activated-deactivated control surface were subtracted from the corresponding sensorgrams in the ClpP immobilized

flow cell to remove the effect of nonspecific binding to the chip surface and the bulk effect from the buffer. Binding experiments were performed at 20 $\mu\text{L}/\text{minute}$ in running buffer E (20 mM TrisHCl, pH 7.5, 100 mM KCl, 3 mM EDTA, 0.005% P20 surfactant, and 5% DMSO). An association phase of a 60 or 120 seconds injection of compound was followed by a dissociation phase of 60 or 120 seconds of running buffer flow before the chip and sample loop were washed at high flow rate. The surface was regenerated between injections with a one minute pulse of 10% DMSO in running buffer E. The steady state responses were plotted versus the corresponding analyte concentrations and fit to one-site Langmuir binding models using BiaEvaluation 4.1 software (GE Healthcare).

00135 Isothermal Titration Calorimetry (ITC). Experiments were performed at 37°C using a Microcal VP-ITC isothermal titration calorimeter (GE Healthcare). The 1.4 mL sample cell was filled with 10 μM ClpP in buffer F (25 mM TrisHCl, pH 7.5, 100 mM KCl, and 1% DMSO) and stirred constantly at 270 rpm. The syringe was filled with 250 μM compound and titrated into the sample cell in one 1 μL injection, followed by 10 μL injections at 170 seconds intervals. After the background heats of dilution from the titration of compound into buffer were subtracted from the experimental data, the net binding data were fit by least squares regression to a model corresponding to one set of identical independent binding sites using Origin 7.0 software (OriginLab, MA, USA).

00136 Thermal denaturation of ClpP. Thermal melting of ClpP in the presence and absence of activating compound was performed on a Jasco J-810 circular dichroism spectrophotometer. Proteins and compounds were diluted into buffer A to a concentration of 25 μM ClpP and 80 μM compound. DMSO (from the compound stock solution) accounted for no more than 0.16% v/v of the solution.

00137 Sedimentation velocity analytical ultracentrifugation. Sedimentation velocity experiments were carried out at the Ultracentrifugation Service Facility at the Department of Biochemistry, University of Toronto. 1 mg/mL ClpP was exchanged into buffer B containing 100 μM of the applicable compound by dialysis. Samples were spun at 45,500 g at 4°C in a Beckman Optima Model XL-A analytical ultracentrifuge equipped with an An-60 Ti rotor.

00138 The density and viscosity of buffer and the partial specific volumes of proteins used were calculated using SEDNTERP. The sedimentation data were fit to a continuous distribution model $c(s)$ using SEDFIT. The observed sedimentation coefficients obtained from the fitting were corrected to the density and viscosity of water at 20°C to obtain $s_{20, w}$.

00139 Determination of minimum bactericidal concentrations. Bacterial strains used included both gram-negative and gram-positive bacteria: *Escherichia coli* DH5 α , *Salmonella typhimurium* SL1344, *Pseudomonas aeruginosa* PAO1, *Haemophilus influenzae* H2192, *Neisseria gonorrhoeae* N.279, *Neisseria meningitidis* MC58, *Staphylococcus aureus* ATCC 29213, *Streptococcus pneumoniae* ATCC 49619, and *Listeria monocytogenes* EGD. Due to the low water solubility of the ADEP and ACP compounds, minimum bactericidal concentrations (MBC) values were determined by plating compound-treated bacteria on agar plates without compound. All compounds were diluted in Brain Heart Infusion (BHI) medium with 1% Isovitale X (Becton Dickinson). All bacteria were also inoculated into BHI. A two-fold dilution series of each compound was created in 96-well plates, with and without 120 $\mu\text{g/mL}$ of the membrane permeabilizing agent polymyxin B nonapeptide, PMBN (Sigma-Aldrich). All bacterial suspensions were pelleted, resuspended in BHI, and added to the compound containing media. *H. influenzae*, *N. gonorrhoeae*, *N. meningitidis* were incubated 18 to 20 hours, while the remaining strains were incubated 12 to 16 hours. *H. influenzae*, *N. gonorrhoeae*, *N. meningitidis*, and *S. pneumoniae* were incubated in the presence of 5% CO₂. 2 μL of culture from the incubations were then plated onto compound-free agar plates (*H. influenzae*, *N. gonorrhoeae*, *N. meningitidis* for 18 to 20 hours; remaining strains for 12-16 hours) to determine the bactericidal activity. *H. influenzae* was grown on chocolate agar; *N. gonorrhoeae* on GC agar base; *N. meningitidis*, *S. aureus*, and *L. monocytogenes* on BHI agar; *S. pneumoniae* on 5% sheep blood agar; and *E. coli*, *S. typhimurium*, and *P. aeruginosa* on LB agar. The lowest concentration of compound at which no bacterial growth was seen was designated as the minimum bactericidal concentration.

00140 Antimycobacterial activities of the ClpP activating compounds were investigated using a modified 96 well plate broth microdilution method, followed by plating for viable bacteria. Compounds were dissolved in 100% DMSO. *M. smegmatis* mc²155 cultures were grown to an OD₆₀₀ 1-1.3 in Middlebrook 7H9 broth (Difco, BD Biosciences) supplemented with 2% oleic

acid, albumin, dextrose, and catalase (OADC enrichment, BD Biosciences) as well as 0.2% glycerol and 0.5% Tween-80 to avoid clumping of bacteria. Cultures were then diluted 10 fold and 5 μ L were used to inoculate 100 μ L of a two-fold dilution series of compounds in the range of 1 μ g/mL to 256 μ g/mL in 7H9 broth supplemented with OADC and 0.2% glycerol in the presence or absence of 12.5 μ g/mL polymyxin B. Polymyxin B is known to enhance mycobacterial permeability to hydrophobic compounds. The solvent control, DMSO at 2% or less showed no inhibitory effects on *M. smegmatis* growth. Plates were incubated in a CO₂ incubator for 8 days. Following incubation, dilutions of sample aliquots were spread on Middlebrook 7H11 plates (Difco, BD biosciences) supplemented with 2% OADC and 0.5% glycerol for determination of bacterial viability.

00141 Docking Procedure. Initial structures of ACP1, ACP2 and ACP3 were obtained from the PubChem database. Those of ACP4 and ACP5 were retrieved from the ZINC database. ACP4 and ACP5 have 8 chiral isomers each (see Tables S3 and S4), arising from three chiral carbons in the oxocyclohexane moiety. Structures for all 8 isomers were available in the Zinc database. The 19 resulting ACP structures (one each for ACP1, ACP2, and ACP3, and 8 chiral structures for each of ACP4 and ACP5) were minimized using the Steepest Decent algorithm and the MMFF94 force field implemented in the OpenBabel software package. The structure of ClpP used in the docking procedure was that of *E. coli* ClpP (PDB code 1yg6). A dimer of the neighboring chains A and G in the PDB entry were extracted from one heptameric ring of ClpP and used for docking procedures.

00142 The DOCK6.3 package was used to dock the flexible conformations of the five compounds into the structure of ClpP, which was kept rigid. For each compound the ‘anchor and grow’ method was used with default options. The docking poses for each compound structure were ranked using the standard DOCK scoring function (see Table S5), which is based on the AMBER molecular mechanics force field. This scoring function includes contributions from van der Waals and Coulomb interactions. The latter were screened using a distance dependent dielectric constant ($\epsilon=4r$, where r is the interatomic distance), but no cut-off was used for the non-bonded interactions. The 10 highest ranking poses and conformations were retained for further analysis.

00143 Prior to executing the docking calculations, the SPHGEN algorithm was used to identify ligand binding pockets on the surface of the ClpP dimer facing ClpX. Two pockets were predicted: one largely hydrophobic pocket corresponding to the ClpX IGF loop binding cleft was designated as the H pocket. The second pocket, located nearby, was designated as the C pocket. Docking calculations were performed separately for the H and C pockets, with boxes for the grid-based scoring function (grid spacing of 0.3Å) generated from the output of SPHGEN for each pocket.

00144 Chemical Synthesis of ACP1 and Analogs. Embodiments of the present inventions were typically prepared using conventional amide bond-coupling conditions between the acid and amine components. PyBOP ((benzotriazol-1-yl-oxy)tripyrrolidinophosphonium hexafluorophosphate) was established as the most effective reagent for amide couplings. The precursor acid and amines were synthesized using standard methods.

00145 A solution of 2-methyl-2-((5-(trifluoromethyl)pyridin-2-yl)sulfonyl)propanoic acid (20 mg, 0.067 mmol) in anhydrous DMF (2.0 mL) was treated with a solution of PyBOP (38 mg, 0.074 mmol) in anhydrous DMF (0.5 mL) followed by diisopropylethylamine (35 µL, 0.20 mmol). A solution of the amine (0.067 mmol) in anhydrous DMF (0.5 mL) was then added dropwise. The resulting yellow solution was stirred at room temperature for 1 hr. The DMF was removed *in vacuo* and the crude product was absorbed onto silica gel. The product was purified by column chromatography (silica gel, EtOAc/hexanes).

00146 2-Methyl-*N*-(3-phenylpropyl)-2-((5-(trifluoromethyl)pyridin-2-yl)sulfonyl)propanamide (ACP1a): Obtained in 88 % yield as a white solid from ethyl acetate and hexanes: R_f = 0.26 (25 % v/v ethyl acetate in hexanes), mp 83–86°C; ^1H NMR (300 MHz, CDCl_3) δ 8.90 (1H, s), 8.20–8.16 (2H, m), 7.35–7.25 (2H, m), 7.25–7.15 (3H, m), 7.06–6.90 (1H, br m), 3.38–3.28 (2H, m), 2.71 (2H, t, J = 7.5 Hz), 1.92 (2H, quintet, J = 7.5 Hz), 1.62 (6H, s); ^{13}C NMR (75 MHz, CDCl_3) δ 167.4, 158.3 (q, J = 1.4 Hz), 147.3 (q, J = 3.9 Hz), 141.4, 135.6 (q, J = 3.6 Hz), 130.3 (q, J = 34.0 Hz), 128.6, 128.5, 126.2, 124.6, 122.5 (q, J = 273.1 Hz), 67.9, 40.1, 33.2, 30.7, 20.6; IR (thin film) $\tilde{\nu}$ 3394 (br), 3063 (w), 3028 (w), 2939 (m), 2862 (w), 1670 (s), 1531 (s), 1327 (s) cm^{-1} ; LRMS (ESI, % base peak) m/z 453.1 (2, $[\text{MK}]^+$), 437.1 (57, $[\text{MNa}]^+$), 415.1 (100, $[\text{MH}]^+$),

351.2 (10), 334.0 (4), 312.1 (8), 280.0 (33); HRMS (ESI) m/z calc'd. for $C_{19}H_{22}F_3N_2O_3S$ $[MH]^+$ 415.1297, found 415.1296.

00147 Disc Assay Protocols. *N. meningitidis* protocol: Fresh overnight bacterial plates of *N. meningitidis* H44/76 (grown on BHI (Brain Heart Infusion) agar plates at 37°C and 5%CO₂) were resuspended in BHI broth and diluted to a concentration of 1.0×10^6 bacteria/ml whereupon 200 µl of said dilution was spread evenly onto a fresh BHI plate and allowed to soak in for 20 mins. Meanwhile, the filter discs were impregnated with 10 µl of 256 µg/mL of the compound to be tested (for example, ADEP1A or ADEP1B) diluted in BHI broth. The impregnated discs were then aseptically laid on the surface and the plates were incubated at 37°C and 5%CO₂ overnight. Zones of clearing on plates were measured with an analytical ruler and recorded as the diameter of clearance.

00148 As shown in the FIGS. and Tables (more preferably Table 3) of the present application, zones of clearing on plates cultured with WT meningococci reflected inhibition of bacterial growth, whereas no inhibitory effect of either compound was apparent on plates cultured with the meningococcal ClpP mutant.

00149 *S. aureus* protocol: Fresh overnight bacterial plates of *S. aureus* (grown on BHI agar plates at 37°C and 5%CO₂) were resuspended in BHI broth and diluted to a concentration of 1.0×10^7 bacteria/ml whereupon 200 µl of said dilution was spread evenly onto a fresh BHI plate and allowed to soak in for 20 mins. Meanwhile, the filter discs were impregnated with 10 µl of 256 µg/mL of the compound to be tested (for example, ADEP1A or ADEP1B) diluted in BHI broth. The impregnated discs were then aseptically laid on the surface and the plates were incubated at 37°C and 5%CO₂ overnight. Zones of clearing on plates were measured with an analytical ruler and recorded as the diameter of clearance.

00150 *E. Coli* protocol: Fresh overnight bacterial plates of *E. Coli* (grown on either LB or BHI) agar plates at 37°C) were resuspended in BHI broth and diluted to a concentration of 1.0×10^6 bacteria/ml whereupon 200 µl of said dilution was spread evenly onto fresh BHI or LB plates and allowed to soak in for 20 mins. Meanwhile, the filter discs were impregnated with 10 µl of 256 µg/mL of the compound to be tested (for example, ADEP1A or ADEP1B) diluted in BHI broth. The impregnated discs were then aseptically laid on the surface and the plates were

incubated at 37°C overnight. Zones of clearing on plates were measured with an analytical ruler and recorded as the diameter of clearance.

00151 As shown in the FIGS. and Tables (more preferably Table 3) of the present application, regardless of the media plates utilized, no observable differences were obtained for the *E. coli* experiments.

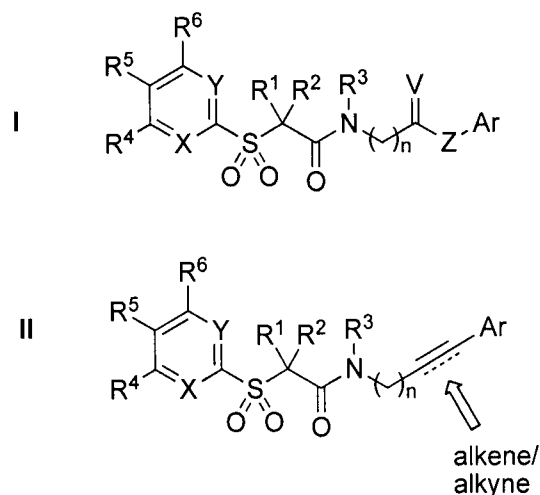
00152 Leung *et al.*, *Activators of Cylindrical Proteases as Antimicrobials: Identification and Development of Small Molecule Activators of ClpP Protease*, **Chemistry & Biology** 18, 1167-1178, (2011) is hereby incorporated by reference.

00153 Having described the invention in specific detail and exemplified the manner in which it may be carried into practice, it will be apparent to those skilled in the art that innumerable variations, applications, modifications, and extensions of the basic principles involved may be made without departing from its spirit or scope. It is to be understood that the foregoing is merely exemplary and the present invention is not to be limited to the specific form or arrangements of parts herein described and shown.

00154 More generally, the scope of the invention is defined by the following claims.

CLAIMS:

1. A compound of formulae (I) or (II).



wherein

R^1 and R^2 are H, CH_3 , a substituted or unsubstituted $\text{C}_2\text{-C}_8$ alkyl, a substituted or unsubstituted $\text{C}_2\text{-C}_8$ cycloalkyl or a substituted or unsubstituted $\text{C}_2\text{-C}_8$ spiro cycloalkyl, when R^1 and R^2 are linked together in a ring;

R^3 is H, CH_3 , a substituted or unsubstituted $\text{C}_2\text{-C}_8$ alkyl, a substituted or unsubstituted $\text{C}_2\text{-C}_8$ cycloalkyl, a substituted or unsubstituted aryl, or a substituted or unsubstituted fused ring, when R^3 is linked together with R^1 / R^2 , V, Z or Ar to form a ring;

R^4 , R^5 and R^6 are H, F, Cl, Br, I, NO_2 , CH_3 , a substituted or unsubstituted $\text{C}_2\text{-C}_8$ alkyl or a substituted or unsubstituted $\text{C}_2\text{-C}_8$ cycloalkyl, CF_3 , CN, a substituted or unsubstituted aryl, COOH , COOR^7 , CONR^7_2 , COR^7 , OR^7 , NR^7_2 , or SR^7 , wherein R^7 is H, a substituted or unsubstituted $\text{C}_2\text{-C}_8$ alkyl, a substituted or unsubstituted $\text{C}_2\text{-C}_8$ cycloalkyl or a substituted or unsubstituted aromatic group;

X and Y are CH or N;

V is H/H, O, or H/R^8 , wherein R^8 is CH_3 , a substituted or unsubstituted $\text{C}_2\text{-C}_8$ alkyl, a substituted or unsubstituted $\text{C}_2\text{-C}_8$ cycloalkyl, or substituted or a unsubstituted aryl;

Z is S, CH_2 , OC=O , $\text{NR}^9\text{C=O}$, O, or NR^9 , wherein R^9 is CH_3 , a substituted or unsubstituted $\text{C}_2\text{-C}_8$ alkyl, a substituted or unsubstituted $\text{C}_2\text{-C}_8$ cycloalkyl, or a

substituted or unsubstituted aryl;

Ar is a substituted or unsubstituted aromatic, a substituted or unsubstituted fused aromatic, a substituted or unsubstituted heteroaromatic, or a substituted or unsubstituted fused heteroaromatic;

n = 0, 1, 2, 3; and

wherein formulae (I) or (II) do not include ACP1 or ACP2.

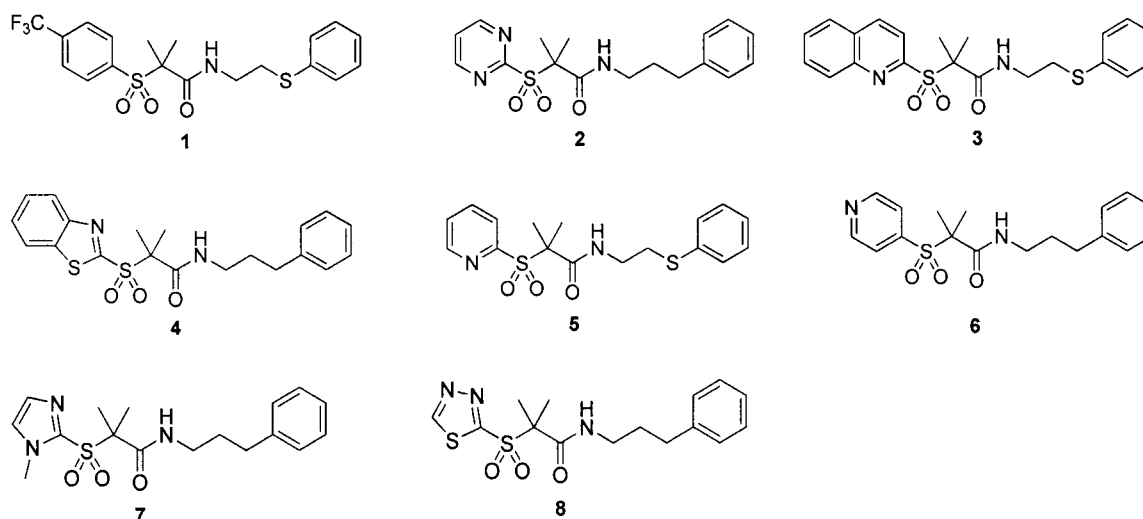
2. The compound of claim 1, wherein the C₂-C₈ alkyl group is a substituted or unsubstituted ethyl, propyl, isopropyl, butyl, sec-butyl, isobutyl, tert-butyl, pentyl, hexyl, heptyl or octyl.
3. The compound of claims 1 or 2, wherein the C₂-C₈ cycloalkyl group or the C₂-C₈ spiro cycloalkyl is substituted or unsubstituted cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, or cyclooctyl.
4. The compound of any one of claim 1 to 3, wherein the substituted or unsubstituted aryl group is a substituted or unsubstituted phenyl.
5. The compound of any one of claim 1 to 4, wherein the substituted or unsubstituted aromatic group comprises a substituted or unsubstituted phenyl.
6. The compound of any one of claims 1 to 5, wherein the substituted or unsubstituted aromatic group is substituted with *ortho*, *meta* or *para* F, Cl, Br, I, CF₃, CN, NO₂, alkyl, aryl, COOH, COOR¹⁰, CONR¹⁰₂, COR¹⁰, OR¹⁰, or NR¹⁰₂ wherein R¹⁰ is H, CH₃, a substituted or unsubstituted C₂-C₈ alkyl, a substituted or unsubstituted C₃-C₈ cycloalkyl, or a substituted or unsubstituted aryl.
7. The compound of any one of claims 1 to 6, wherein the substituted or unsubstituted fused aromatic group is a substituted or unsubstituted naphthalene or a substituted or unsubstituted indene.
8. The compound of any one of claims 1 to 7, wherein the substituted or unsubstituted heteroaromatic group is substituted or unsubstituted pyridine, substituted or unsubstituted pyrimidine, substituted or unsubstituted pyrazine, substituted or unsubstituted triazine, substituted or unsubstituted thiophene, substituted or unsubstituted furan, substituted or

unsubstituted oxazole, substituted or unsubstituted thiazole, substituted or unsubstituted imidazole, substituted or unsubstituted pyrazole, substituted or unsubstituted triazole, substituted or unsubstituted oxadiazole or substituted or unsubstituted tetrazole.

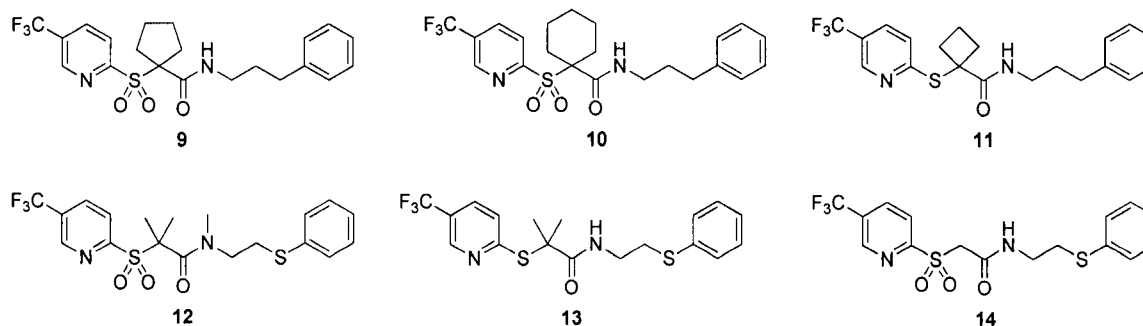
9. The compound of any one of claims 1 to 8, wherein the fused heteroaromatic group comprises substituted or unsubstituted indole, substituted or unsubstituted benzofuran, substituted or unsubstituted benzothiophene, substituted or unsubstituted quinoline, substituted or unsubstituted isoquinoline, substituted or unsubstituted benzimidazole, substituted or unsubstituted benzthiazole, substituted or unsubstituted benzoxazole or substituted or unsubstituted benzpyrazole.

10. The compound of claim 1, wherein the compound is selected from the group consisting of:

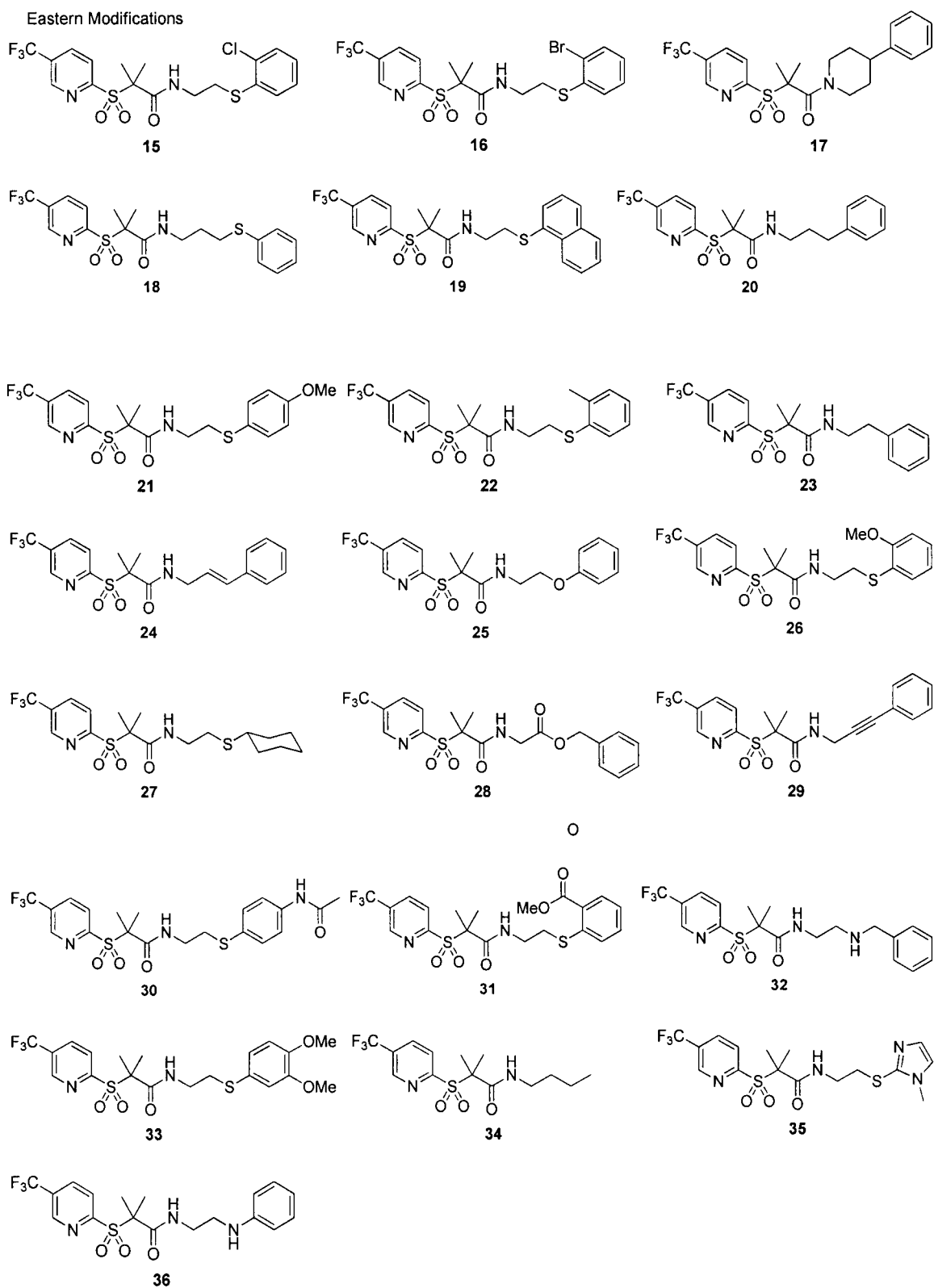
Western Modifications



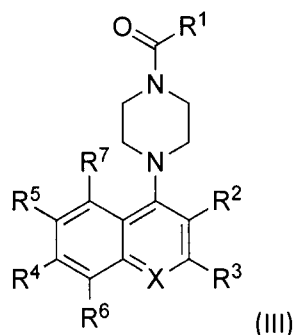
Central Modifications



Eastern Modifications



11. A compound of the formulae (III).



wherein

R^1 is H, CH_3 , a substituted or unsubstituted $\text{C}_1\text{-C}_8$ alkyl, a substituted or unsubstituted $\text{C}_3\text{-C}_8$ cycloalkyl, a substituted or unsubstituted alkenyl, a substituted or unsubstituted aromatic, a substituted or unsubstituted fused aromatic, a substituted or unsubstituted heteroaromatic, or a substituted or unsubstituted fused heteroaromatic;

R^2 , R^3 , R^4 , R^5 , R^6 and R^7 are H, CH_3 , a substituted or unsubstituted $\text{C}_1\text{-C}_8$ alkyl, a substituted or unsubstituted $\text{C}_3\text{-C}_8$ cycloalkyl, a substituted or unsubstituted alkenyl, a substituted or unsubstituted aromatic, a substituted or unsubstituted fused aromatic, a substituted or unsubstituted heteroaromatic, a substituted or unsubstituted fused heteroaromatic;

X is CH and N; and

wherein formulae (III) does not include ACP3.

12. The compound of claim 11, wherein the substituted or unsubstituted $\text{C}_1\text{-C}_8$ alkyl group is a substituted or unsubstituted methyl, ethyl, propyl, isopropyl, or butyl, sec-butyl, isobutyl, tert-butyl, pentyl, hexyl, heptyl and octyl.

13. The compound of claim 11 or 12, wherein the substituted or unsubstituted $\text{C}_3\text{-C}_8$ cycloalkyl group or the $\text{C}_2\text{-C}_8$ spiro cycloalkyl is a substituted or unsubstituted cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, or cyclooctyl.

14. The compound of any one of claims 11 to 13, wherein the substituted or unsubstituted alkenyl is vinyl, propenyl or dihalovinyl.

15. The compound of any one of claims 11 to 14, wherein the substituted aromatic is substituted with *ortho*, *meta* or *para* F, Cl, Br, I, CF_3 , CN, NO_2 , alkyl, aryl, COOH , COOR^{10} ,

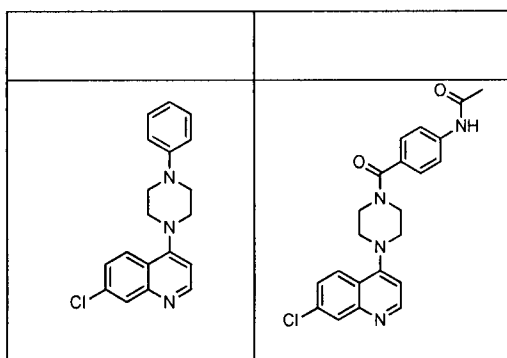
CONR¹⁰₂, COR¹⁰, OR¹⁰, or NR¹⁰₂ wherein R¹⁰ is H, CH₃, a substituted or unsubstituted C₂-C₈ alkyl, a substituted or unsubstituted C₃-C₈ cycloalkyl, or substituted or unsubstituted aryl.

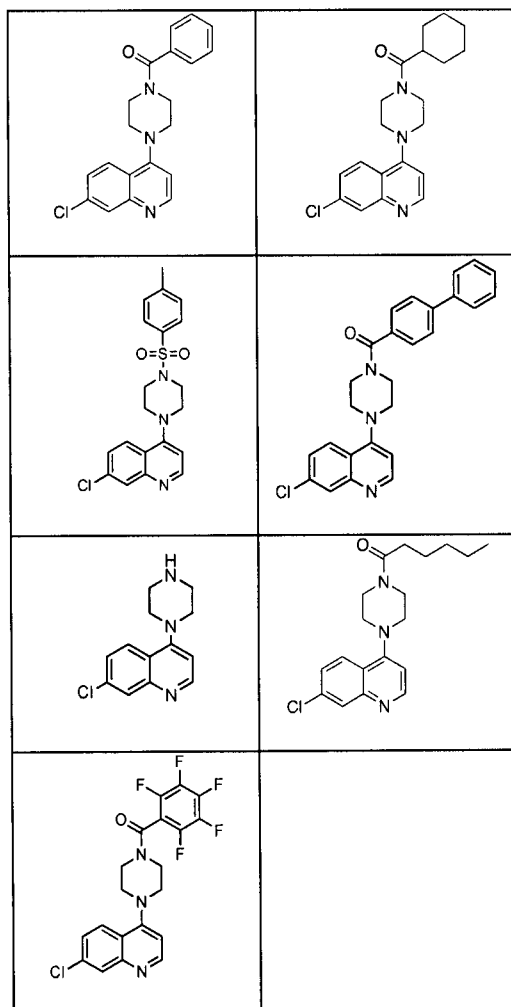
16. The compound of any one of claims 11 to 15, wherein the fused aromatic group is a substituted or unsubstituted naphthalene or a substituted or unsubstituted indene.

17. The compound of any one of claims 11 to 16, wherein the substituted or unsubstituted heteroaromatic group is substituted or unsubstituted pyridine, substituted or unsubstituted pyrimidine, substituted or unsubstituted pyrazine, substituted or unsubstituted triazine, substituted or unsubstituted thiophene, substituted or unsubstituted furan, substituted or unsubstituted oxazole, substituted or unsubstituted thiazole, substituted or unsubstituted imidazole, substituted or unsubstituted pyrazole, substituted or unsubstituted triazole, substituted or unsubstituted oxadiazole or substituted or unsubstituted tetrazole.

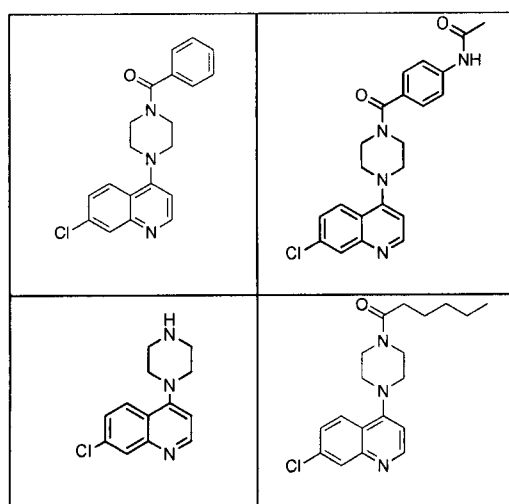
18. The compound of any one of claims 11 to 17, wherein the fused heteroaromatic group comprises substituted or unsubstituted indole, substituted or unsubstituted benzofuran, substituted or unsubstituted benzothiophene, substituted or unsubstituted quinoline, substituted or unsubstituted isoquinoline, substituted or unsubstituted benzimidazole, substituted or unsubstituted benzthiazole, substituted or unsubstituted benzoxazole or substituted or unsubstituted benzpyrazole.

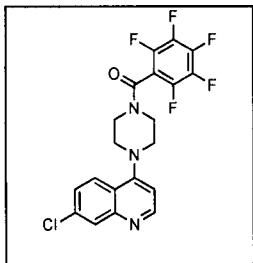
19. The compound of claim 11, wherein the compound is selected from the group consisting of:



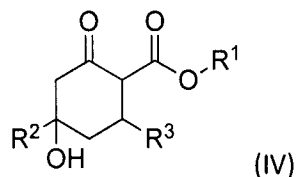


20. The compound of claim 19, wherein the compound is selected from the group consisting of:





21. A compound of formulae (IV):



wherein

R¹ is CH₃, a substituted or unsubstituted C₂-C₈ alkyl or a substituted or unsubstituted C₃-C₈ cycloalkyl;

R² and R³ are H, a substituted or unsubstituted alkyl, a substituted or unsubstituted cycloalkyl, a substituted or unsubstituted alkenyl, a substituted or unsubstituted aromatic, a substituted or unsubstituted fused aromatic, a substituted or unsubstituted heteroaromatic, or a substituted or unsubstituted fused heteroaromatic; and

wherein formulae (IV) does not include ACP4 or ACP5.

22. The compound of claim 21, wherein the substituted or unsubstituted C₁-C₈ alkyl group is a substituted or unsubstituted methyl, ethyl, propyl, isopropyl, or butyl, sec-butyl, isobutyl, tert-butyl, pentyl, hexyl, heptyl and octyl.

23. The compound of claim 21 or 22, wherein the substituted or unsubstituted C₃-C₈ cycloalkyl group is a substituted or unsubstituted cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, and cyclooctyl.

24. The compound of any one of claims 21 to 23, wherein the substituted or unsubstituted alkyl is a substituted or unsubstituted methyl, ethyl, propyl, isopropyl or butyl.

25. The compound of any one of claims 21 to 24, wherein the substituted or unsubstituted cycloalkyl is a substituted or unsubstituted cyclopropyl, cyclobutyl, cyclopentyl or cyclohexyl.

26. The compound of any one of claims 21 to 25, wherein the a substituted or unsubstituted alkenyl group is vinyl, propenyl or dihalovinyl.
27. The compound of any one of claims 21 to 26, wherein the substituted aromatic is substituted with *ortho*, *meta* or *para* F, Cl, Br, I, CF₃, CN, NO₂, alkyl, aryl, COOH, COOR¹⁰, CONR¹⁰₂, COR¹⁰, OR¹⁰, or NR¹⁰₂ wherein R¹⁰ is H, CH₃, a substituted or unsubstituted C₂-C₈ alkyl, a substituted or unsubstituted C₃-C₈ cycloalkyl, or a substituted or unsubstituted aryl.
28. The compound of any one of claims 21 to 27, wherein the fused aromatic group is a substituted or unsubstituted naphthalene or a substituted or unsubstituted indene.
29. The compound of any one of claims 21 to 28, wherein the substituted or unsubstituted heteroaromatic group is substituted or unsubstituted pyridine, substituted or unsubstituted pyrimidine, substituted or unsubstituted pyrazine, substituted or unsubstituted triazine, substituted or unsubstituted thiophene, substituted or unsubstituted furan, substituted or unsubstituted oxazole, substituted or unsubstituted thiazole, substituted or unsubstituted imidazole, substituted or unsubstituted pyrazole, substituted or unsubstituted triazole, substituted or unsubstituted oxadiazole or substituted or unsubstituted tetrazole.
30. The compound of any one of claims 21 to 29, wherein the fused heteroaromatic group comprises substituted or unsubstituted indole, substituted or unsubstituted benzofuran, substituted or unsubstituted benzothiophene, substituted or unsubstituted quinoline, substituted or unsubstituted isoquinoline, substituted or unsubstituted benzimidazole, substituted or unsubstituted benzthiazole, substituted or unsubstituted benzoxazole or substituted or unsubstituted benzpyrazole.
31. An antibacterial compound selected from the group consisting of ACP1, ACP2, ACP3, ACP4, ACP5 and pharmaceutically acceptable salts thereof.
32. The antibacterial compound of claim 31 comprising ACP1, ACP3, ACP4, ACP5 and pharmaceutically acceptable salts thereof.
33. The antibacterial compound of claim 32 comprising ACP1 and ACP3.
34. A cylindrical proteases activator for use as an antibacterial compound.

35. The use of claim 34 wherein the cylindrical protease is ClpP.
36. A pharmaceutical composition comprising a compound according to any one of claims 1 to 37 or a pharmaceutically acceptable salt thereof in association with one or more pharmaceutically acceptable excipients, diluents and/or carriers.

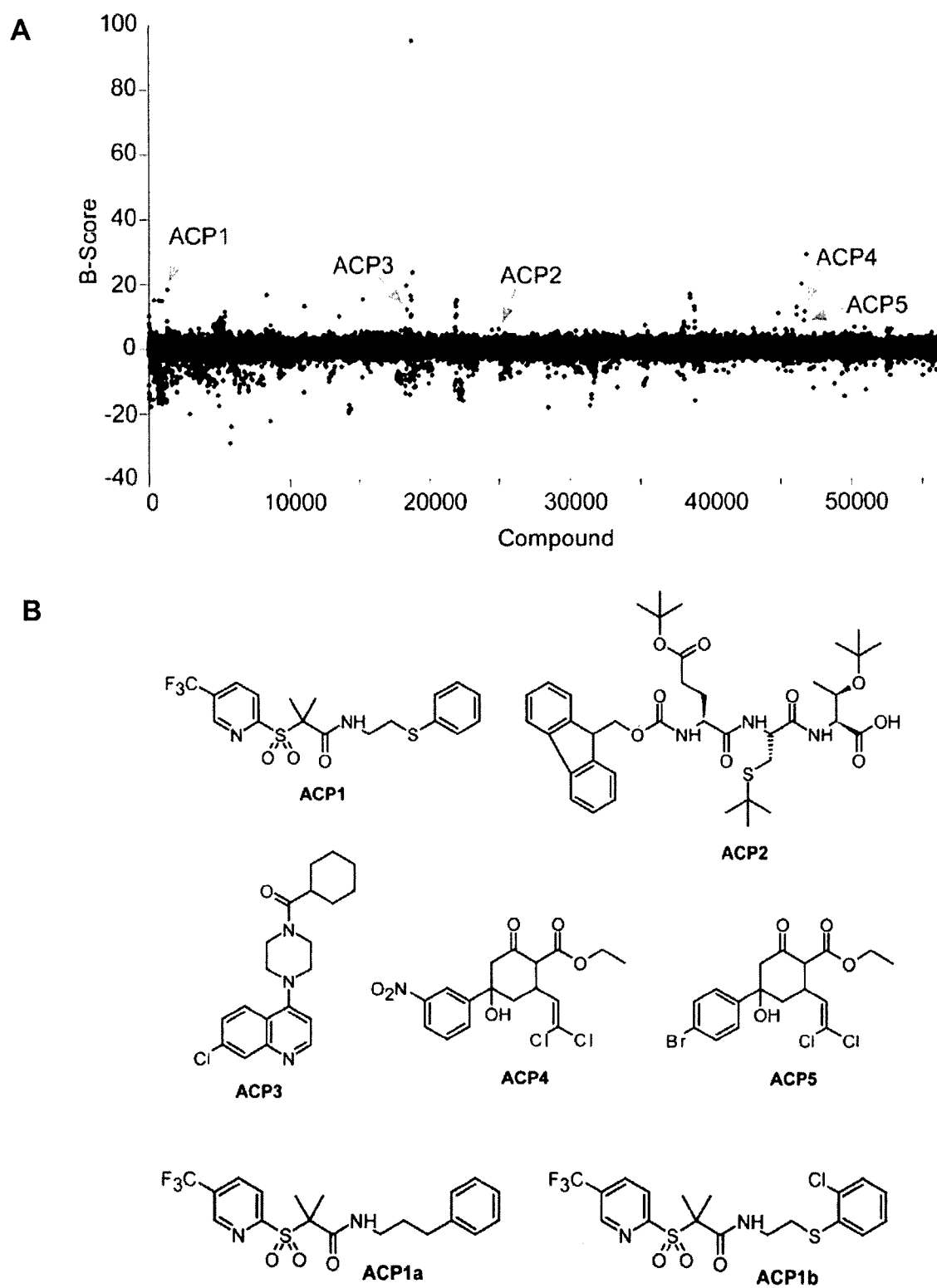
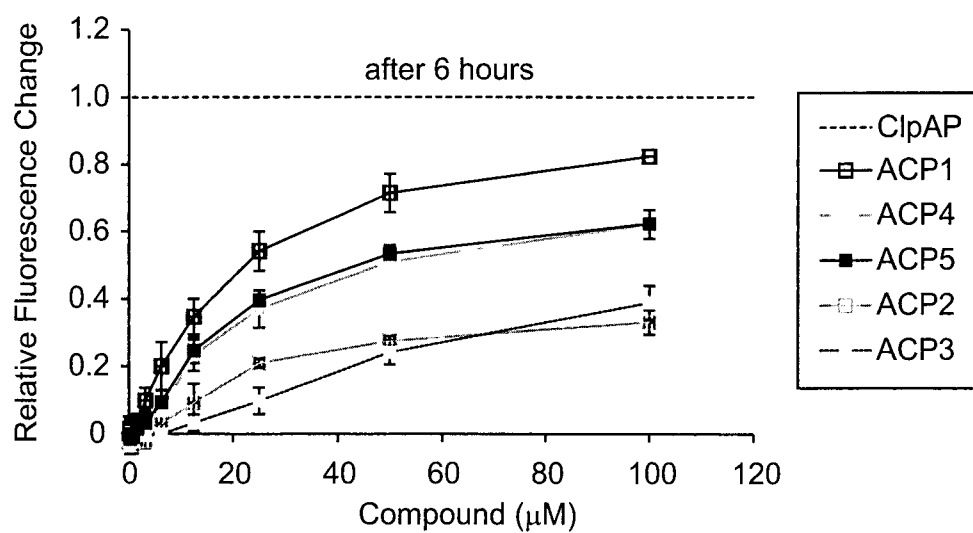


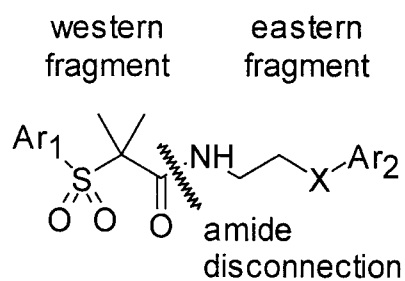
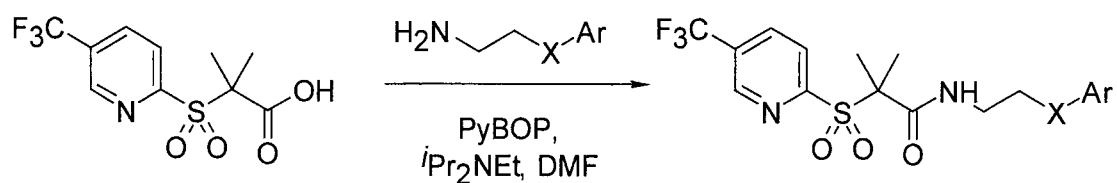
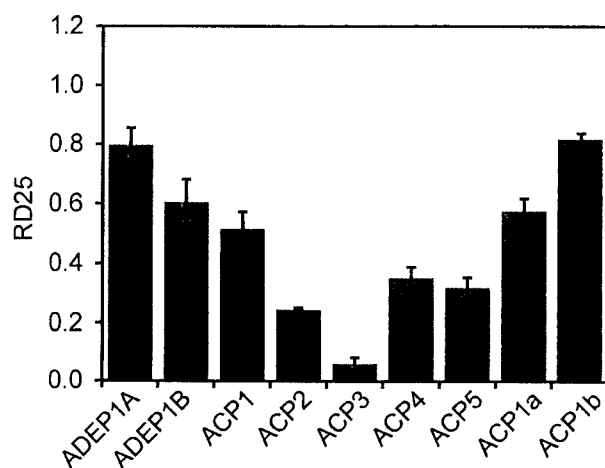
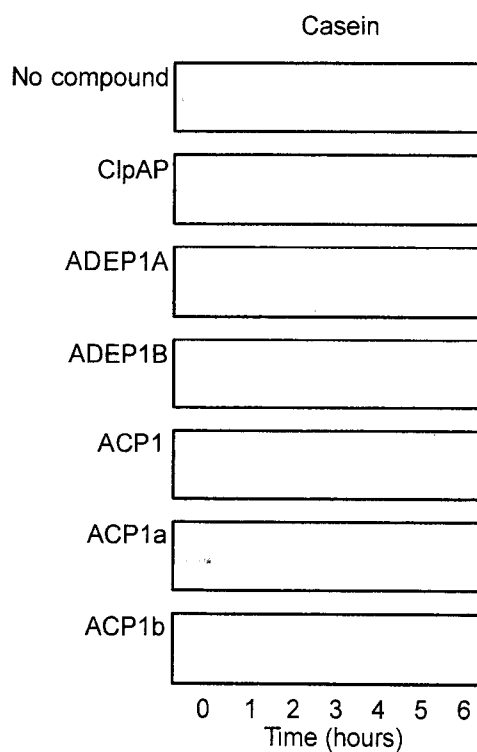
Figure 1

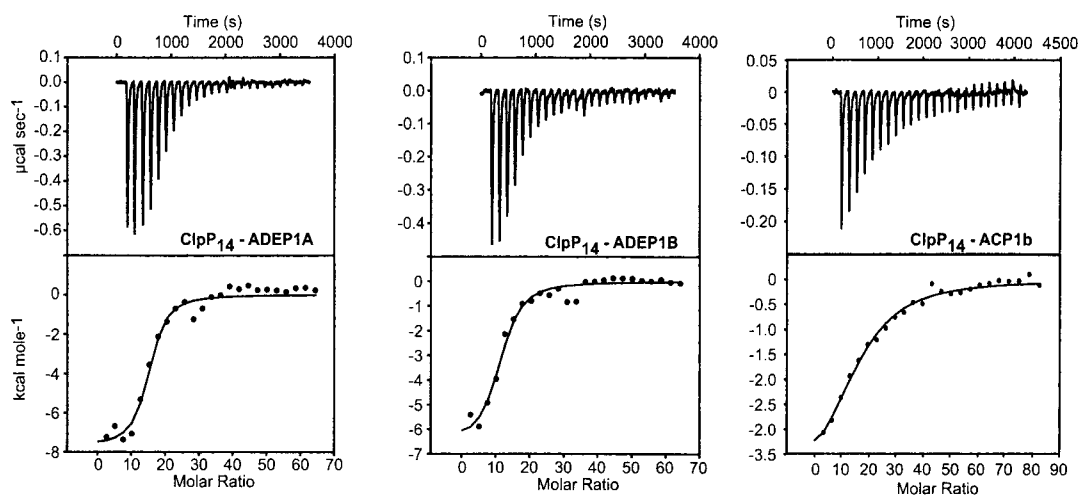
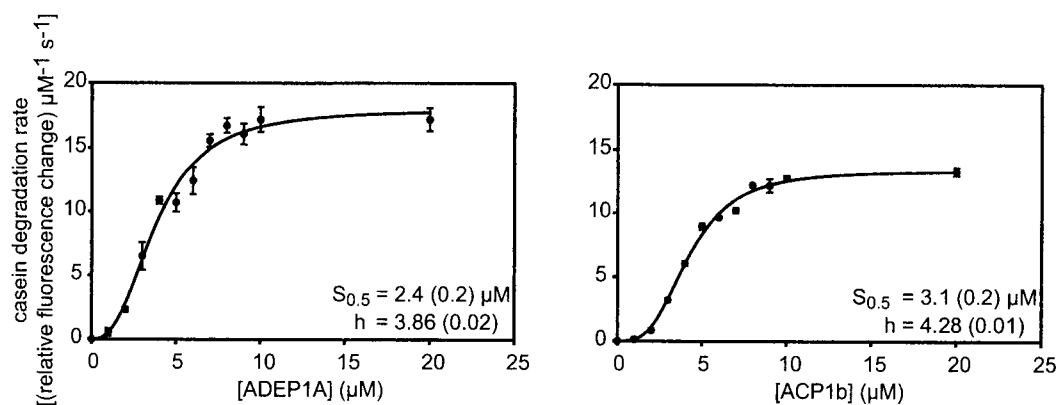
A**B**

Compound	RD25	SD
ACP1	0.53	0.04
ACP2	0.20	0.01
ACP3	0.10	0.04
ACP4	0.37	0.05
ACP5	0.39	0.04

Figure 2

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A**B****C****D****Figure 3**

A**B****Figure 4**

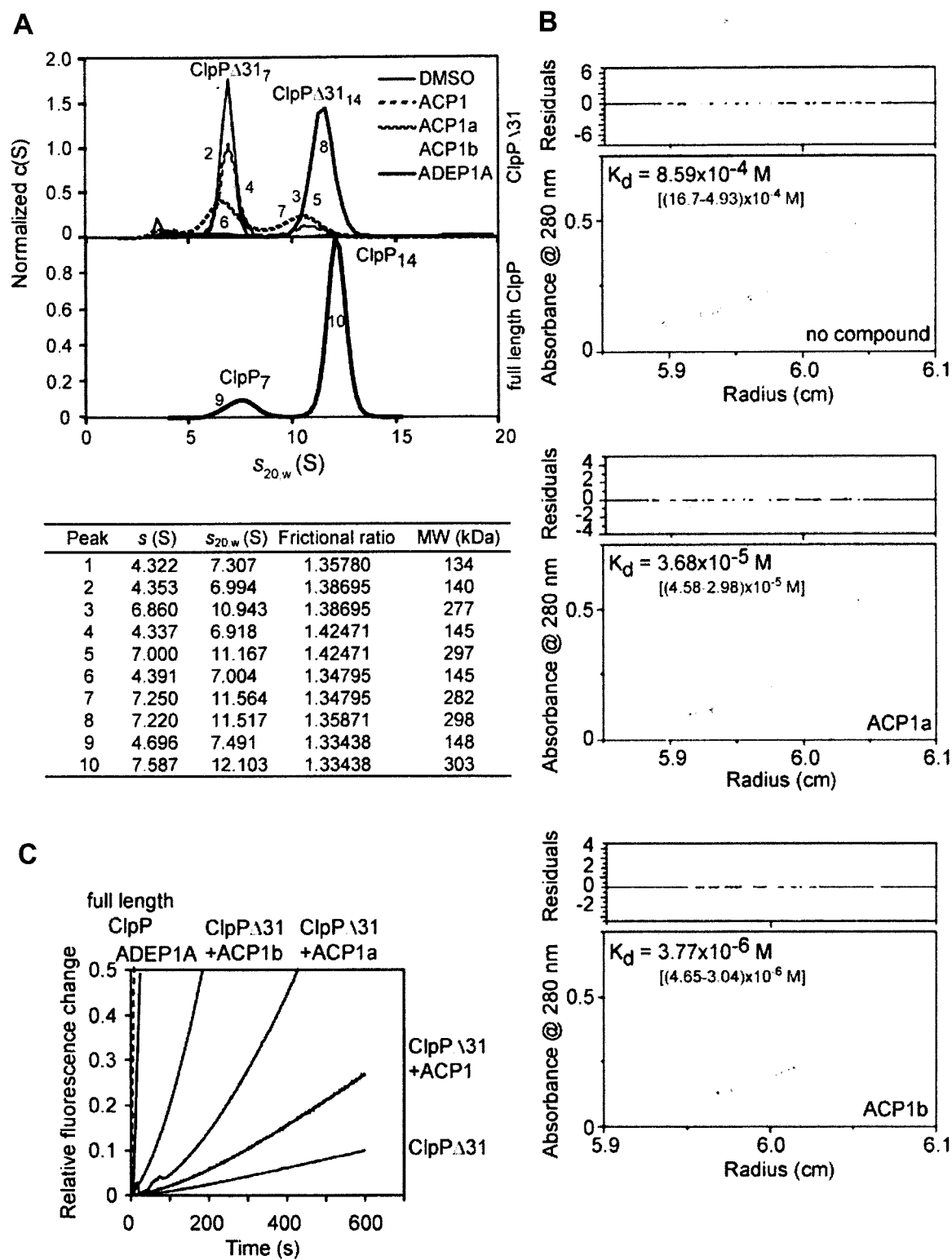


Figure 5

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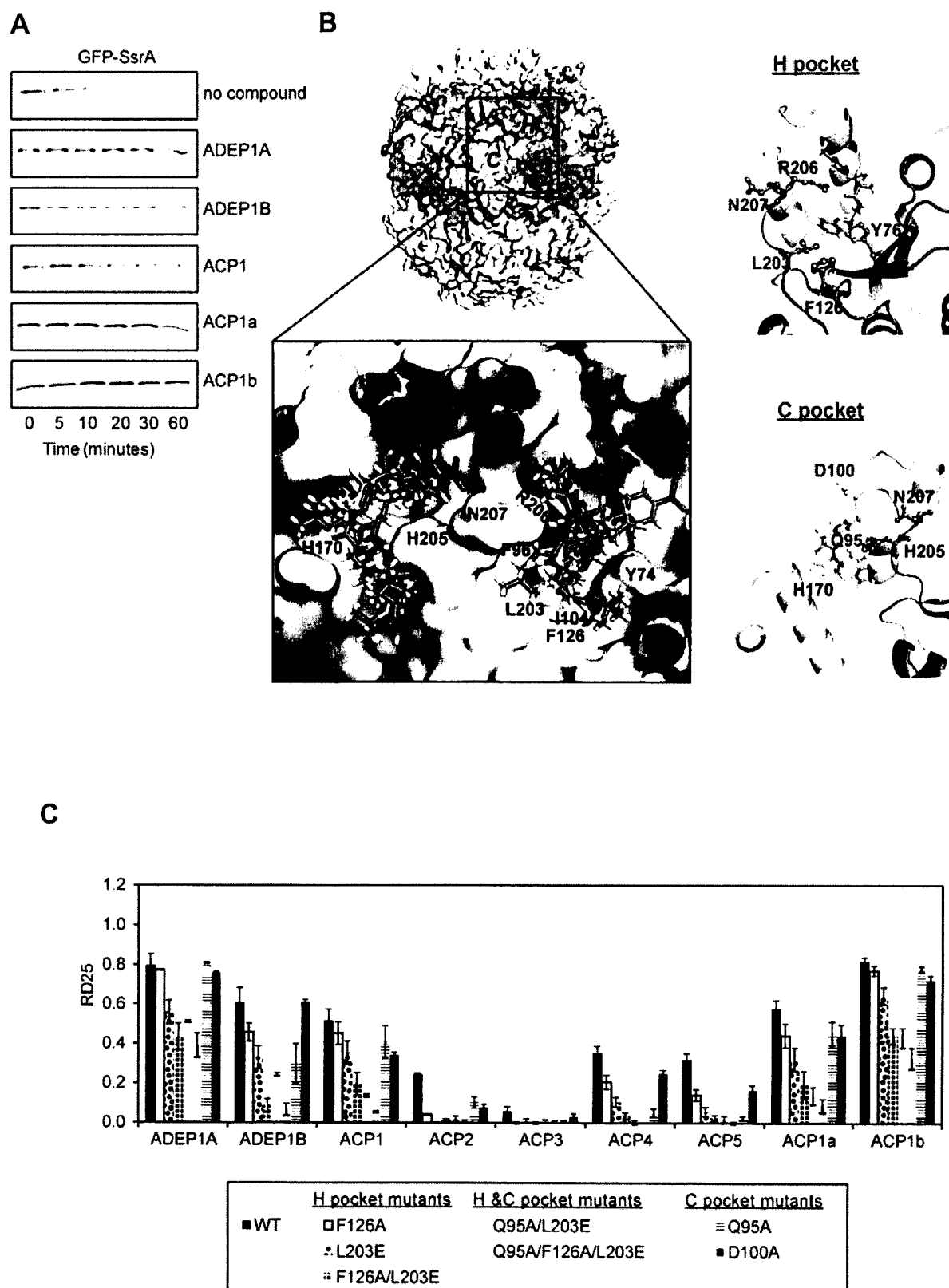
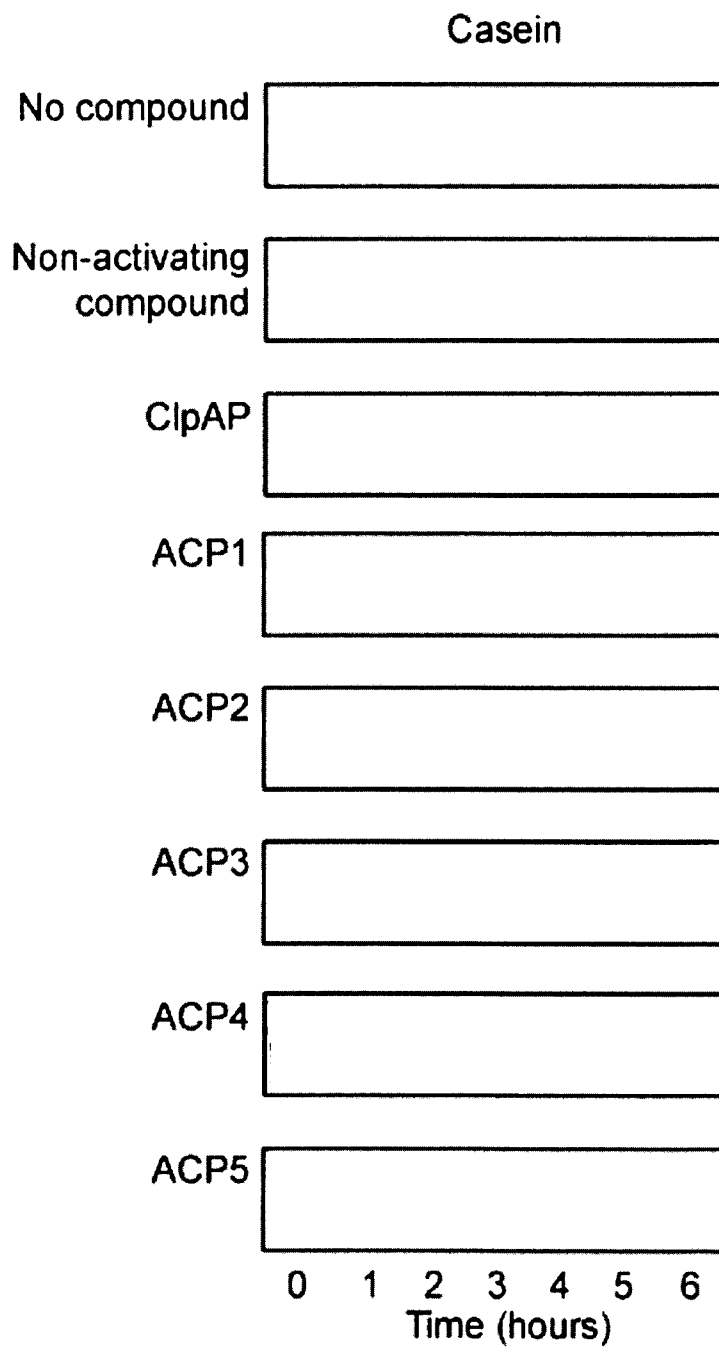


Figure 6

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	ADEP1A	ACP1	ACP1a	ACP1b
<i>N. meningitidis</i>	0.25	64	64	16
<i>H. influenzae</i>	8	16	32	8

Table 1

**Figure 7**

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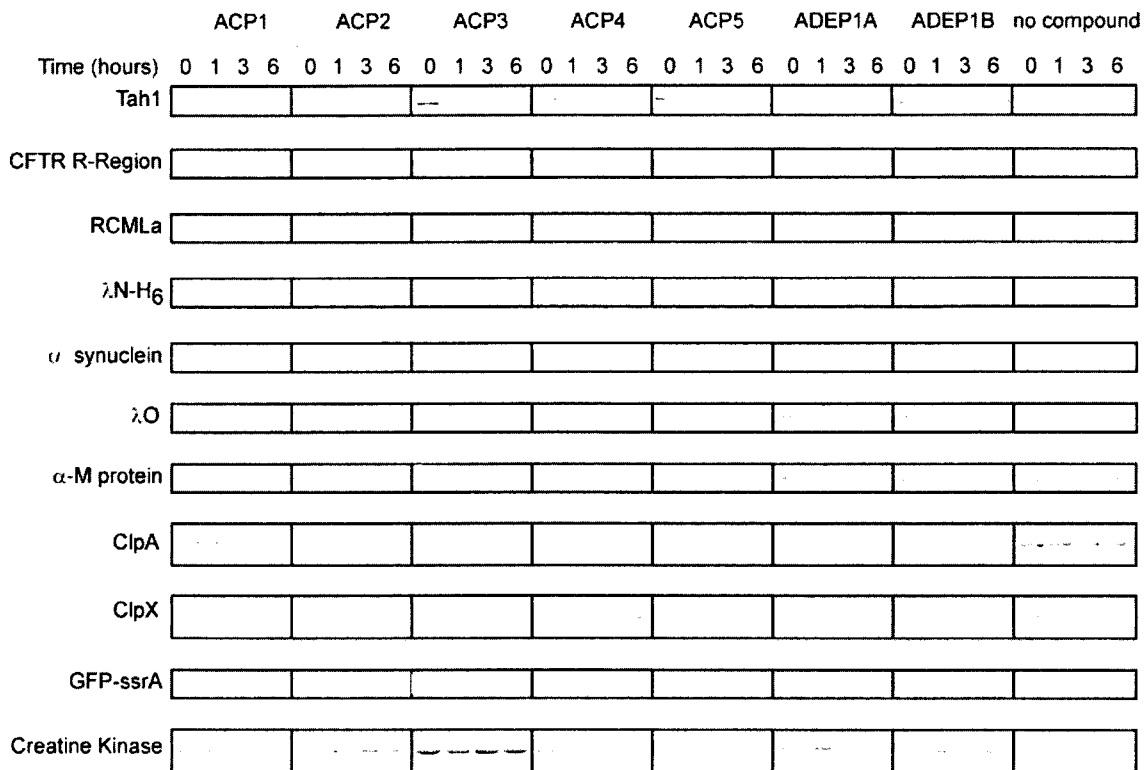
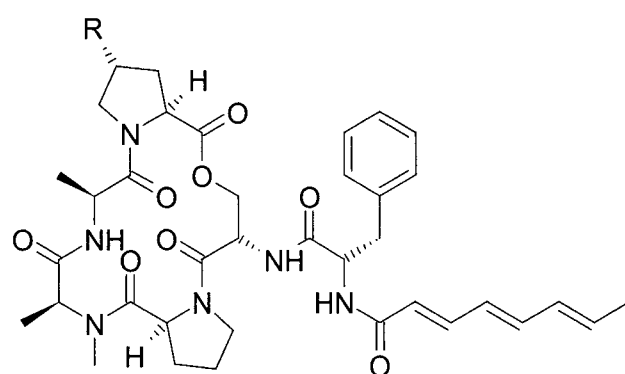


Figure 8

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ADEP1A (R = CH₃)
ADEP1B (R = H)

Figure 9

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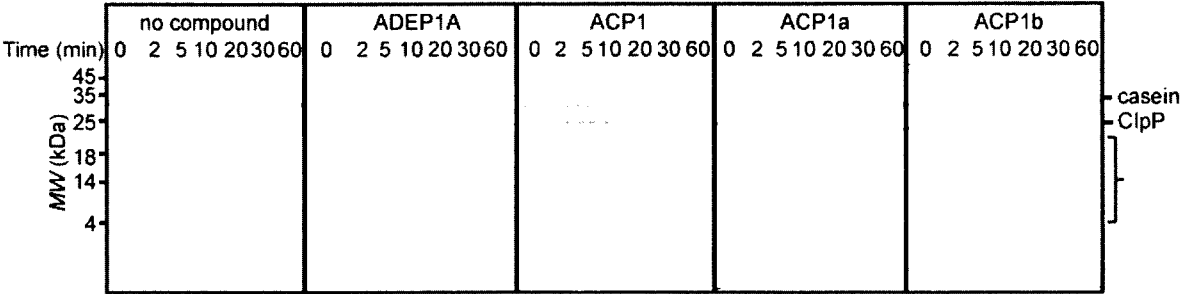
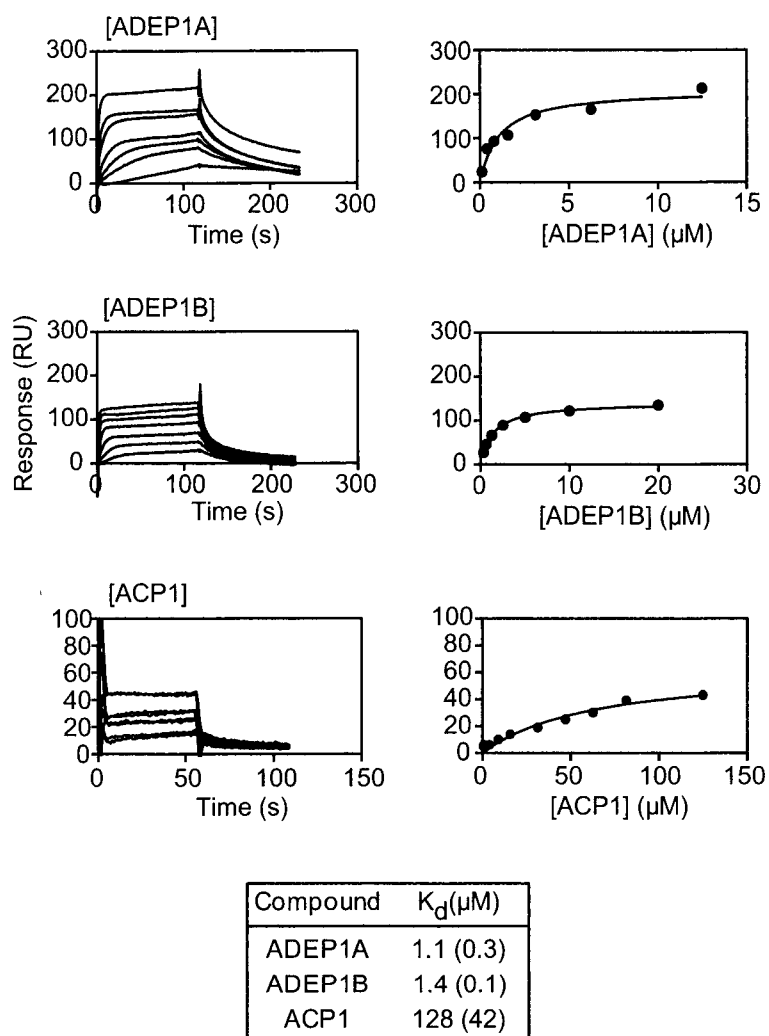
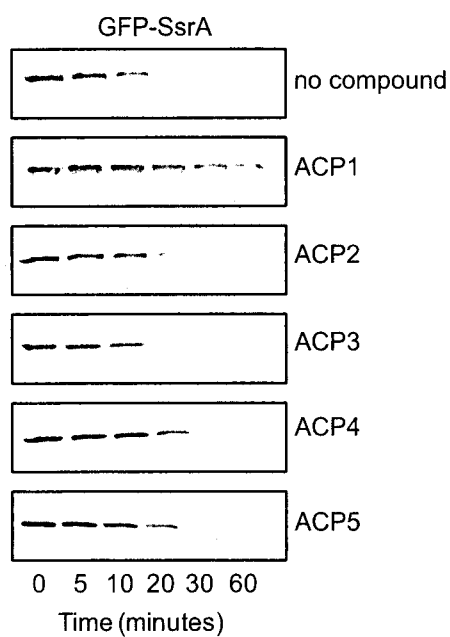


Figure 10

**Figure 11**

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**Figure 12**

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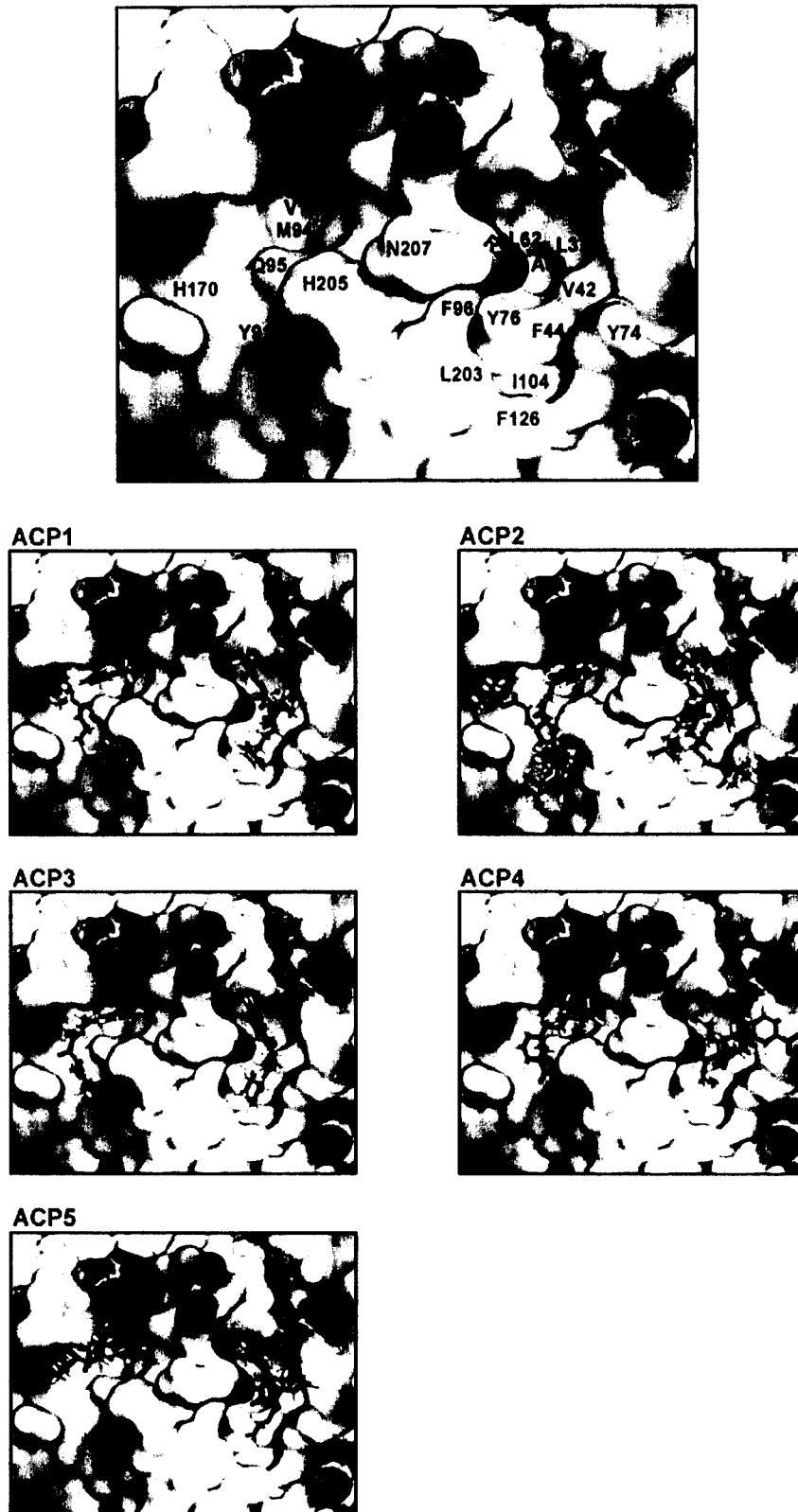
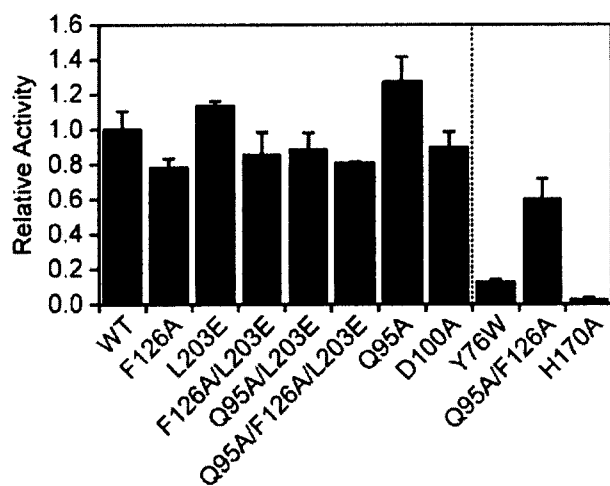


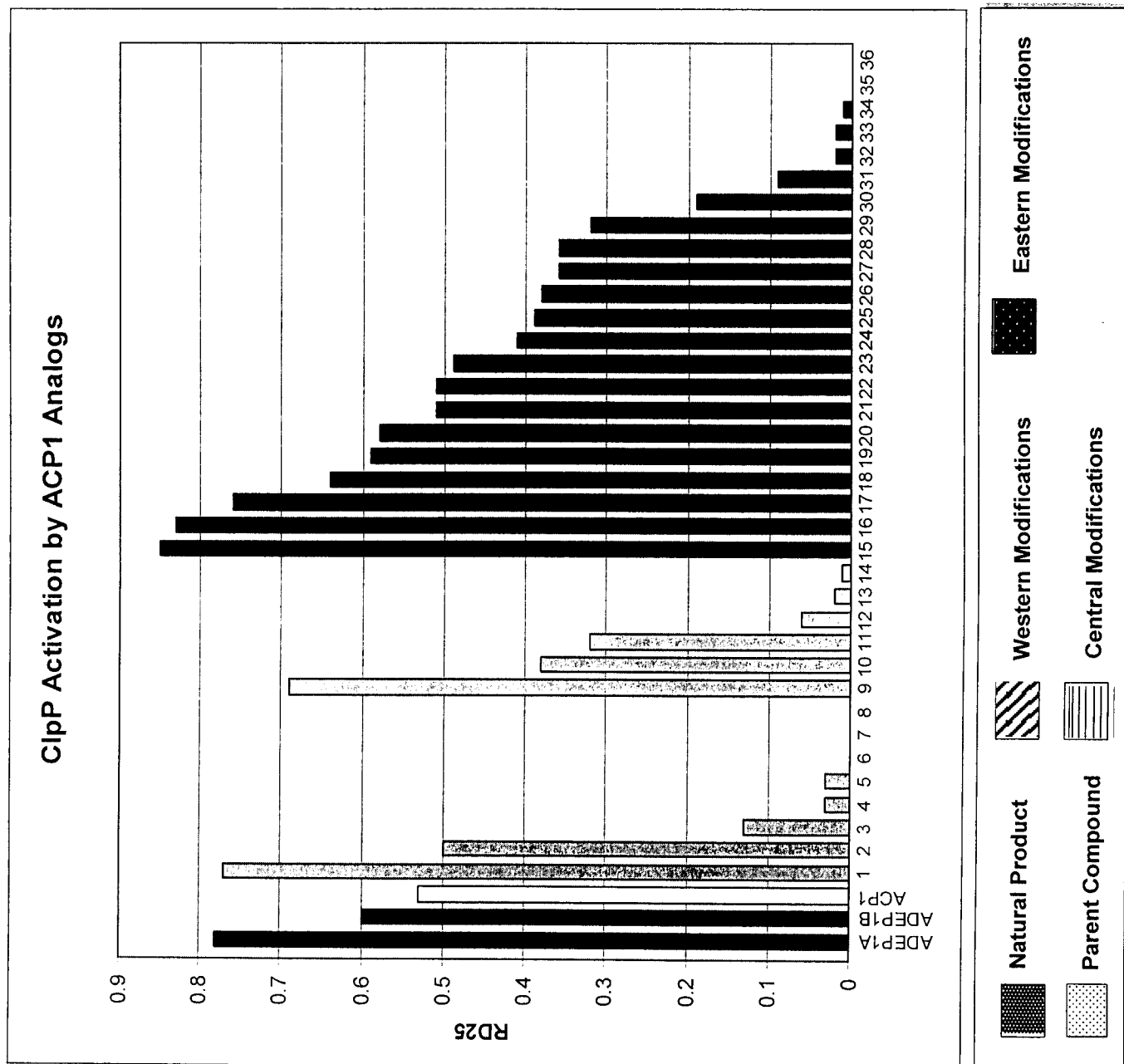
Figure 13



	H pocket mutants	H & C pocket mutants	C pocket mutants
■ WT	F126A	Q95A/L203E	Q95A
	L203E	Q95A/F126A/L203E	D100A
	F126A/L203E	Q95A/F126A	H170A
	Y76W		

Figure 14

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Substrate	MW (kDa)	Activator						
		ADEP1A	ADEP1B	ACP1	ACP2	ACP3	ACP4	ACP5
Bovine α -casein	25	++	++	++	++	++	++	++
Yeast Tah1	13	++	++	++	++	++	++	++
Human CFTR R-Domain	33	++	++	++	++	++	++	++
Bovine reduced carboxymethylated α -lactalbumin	14	++	++	++	++	++	++	++
Bacteriophage λ N-H ₆	15	++	++	++	++	-	++	++
Human α -synuclein	18	++	++	++	-	-	++	++
Bacteriophage λ O	33	+	+	+	-	-	+	+
<i>Neisseria</i> α -M protein	44	+	+	+	-	-	-	-
<i>E. coli</i> ClpA	84	+	+	+	-	-	-	-
<i>E. coli</i> ClpX	50	+	+	+	-	-	-	-
Jellyfish green fluorescent protein-ssrA	27	-	-	-	-	-	-	-
Rabbit creatine kinase	43	-	-	-	-	-	-	-

The double plus (++) denotes complete substrate degradation, the single plus (+) denotes partial degradation or clipping of the substrate, and the hyphen (-) denotes no visible degradation. The original SDS-PAGE gels are available in Fig. S2.

Table S1

PMBN	ACP1		ACP2		ACP3		ACP4		ACP5	
	+	-	+	-	+	-	+	-	+	-
<i>N. gonorrhoeae</i>	>256	>256	32	32	>256	>256	4	4	>256	>256
<i>N. meningitidis</i>	64	64	>256	64	>256	>256	32	32	>256	>256
<i>H. influenzae</i>	64	>256	8	>256	>256	>256	4	128	32	>256
<i>E. coli</i>	>256	>256	8	>256	>256	>256	16	>256	16	>256
<i>S. typhimurium</i>	>256	>256	>256	>256	>256	>256	>256	>256	>256	>256
<i>P. aeruginosa</i>	>256	>256	8	>256	>256	>256	>256	>256	>256	>256
<i>S. aureus</i>	N/A	>256	N/A	>256	N/A	>256	N/A	>256	N/A	>256
<i>S. pneumoniae</i>	N/A	>256	N/A	16	N/A	>256	N/A	8	N/A	>256
<i>L. monocytogenes</i>	N/A	>256	N/A	32	N/A	>256	N/A	>256	N/A	>256
<i>M. smegmatis</i>	>256	>256	>256	>256	>256	>256	128	128	256	256

4-8
16-64
128 or more

Table S2

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ZINC ID ^a	X ₁	X ₂	X ₃
ZINC15636003	S	S	S
ZINC04011799	S	S	R
ZINC01234770	S	R	S
ZINC04011798	S	R	R
ZINC15636001	R	S	S
ZINC00753769	R	S	R
ZINC15636005	R	R	S
ZINC04011797	R	R	R

^aACP4 is ethyl (1X₁,2X₂,4X₃)-2-(2,2-dichloroethenyl)-4-hydroxy-4-(3-nitrophenyl)-6-oxocyclohexane-1-carboxylate in IUPAC nomenclature, where X₁, X₂, and X₃ can adopt either S or R chirality.

Table S3

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ZINC ID ^a	X ₁	X ₂	X ₃
ZINC15635989	S	S	S
ZINC35327726	S	S	R
ZINC15635993	S	R	S
ZINC35327717	S	R	R
ZINC01234768	R	S	S
ZINC00752024	R	S	R
ZINC15635991	R	R	S
ZINC35327713	R	R	R

^a ACP5 is ethyl (1X₁,2X₂,4X₃)-4-(4-bromophenyl)-2-(2,2-dichloroethenyl)-4-hydroxy-6-oxocyclohexane-1-carboxylate in IUPAC nomenclature, where X₁, X₂, and X₃ can adopt either S or R chirality.

Table S4

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Name of Compound	Dock6 Score	
	H pocket	C pocket
ACP1	-43.36	-42.05
ACP2	-74.50	-71.10
ACP3	-37.91	-39.72
ACP4 ^a	-40.75	-42.14
ACP5 ^b	-38.78	-40.02

^aZINC15636003 and ZINC04011797 showed the lowest Dock6 score values on H and C pockets, respectively.

^bZINC00752024 and ZINC35327717 showed the lowest Dock6 score values on H and C pockets, respectively.

Table S5

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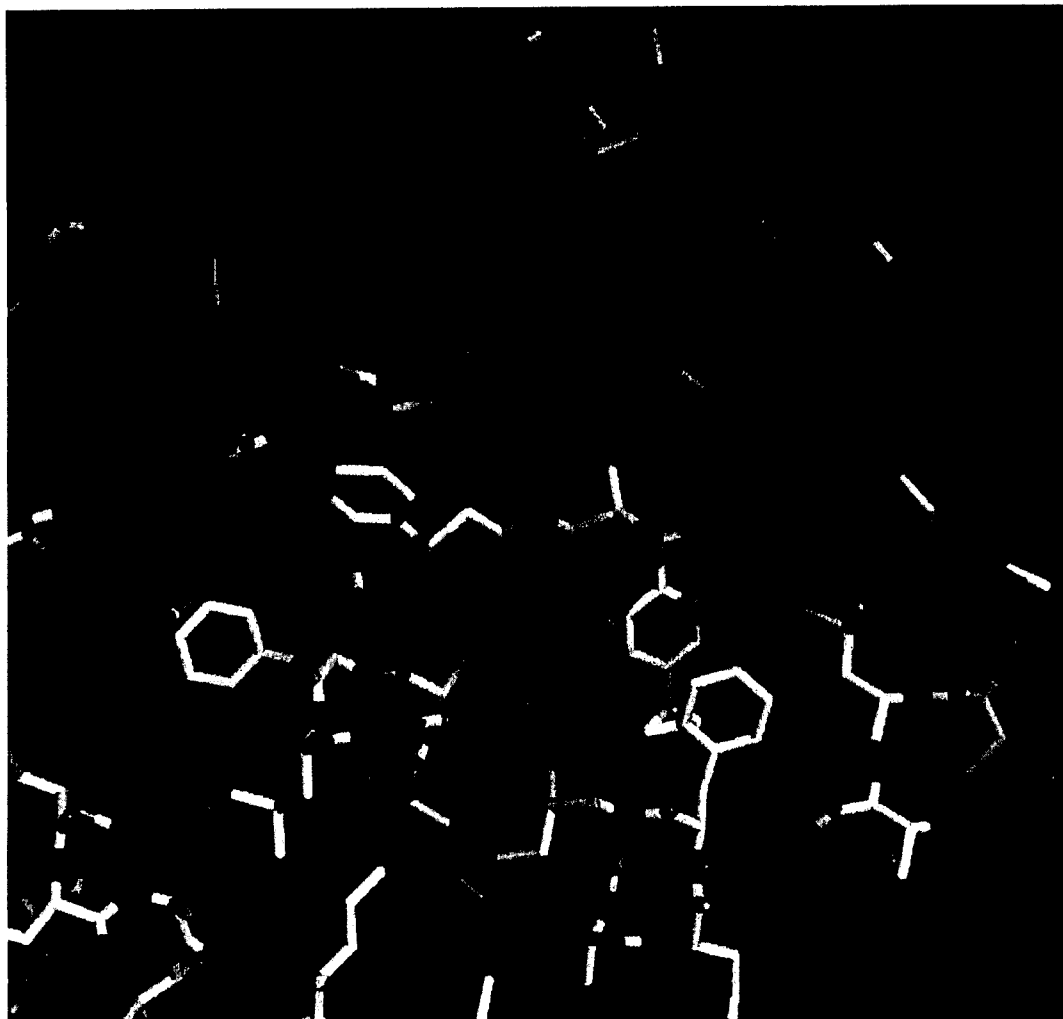


Figure 16

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TABLE 3

TABLE 3																											
Disk Assay - N.meningitis (diameter in cm)										Disk Assay - S.aureus (diameter in cm)						Disk Assay - E.coli (diameter in cm)											
compo und #	RD25				RD5		Test 1			Test 2			Test 3			Test 1		Test 2		Test 3		Test 1		Test 2		Test 3	
	Avera ge	Stde v	Avera ge	Stde v			wild type	CipP knocko ut	wildty pe	CipP knock out	wildty pe	CipP knock out	wildtyp e	wildtyp e	wildtyp e	wildty pe	CipP knock out	wildty pe	CipP knock out	wildty pe	CipP knock out	wildty pe	CipP knock out	wildty pe	CipP knock out	wildty pe	CipP knock out
1	0.13	0.03	N/A	N/A			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	0.59	0.03	N/A	N/A			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	0.09	0.03	N/A	N/A			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4	0.19	0.05	N/A	N/A			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	0.82	0.02	0.64	0.03			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	0.51	0.01	N/A	N/A			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	0.38	0.00	N/A	N/A			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	0.58	0.04	0.03	0.02			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	0.06	0.00	0.07	0.03			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10	0.50	0.13	N/A	N/A			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	0.05	0.03	N/A	N/A			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
12	0.19	0.02	N/A	N/A			0.7*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13	0.31	0.01	N/A	N/A			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
14	0.68	0.04	N/A	N/A			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	0.52	0.02	N/A	N/A			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	0.54	0.02	N/A	N/A			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	0.11	0.02	N/A	N/A			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	0.80	0.03	0.64	0.03			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	0.15	0.01	N/A	N/A			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	0.40	0.01	0.06	0.01			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	0.11	0.02	N/A	N/A			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	0.93	0.02	0.42	0.01			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	0.00	0.01	0.00	0.00			1.0*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	0.94	0.14	0.57	0.01			-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE 3

TABLE 3																											
Disk Assay - N.meningitis (diameter in cm)						Disk Assay - S.aureus (diameter in cm)						Disk Assay - E.coli (diameter in cm)															
compo und #	RD25		RD5	Test 1			Test 2			Test 3			Test 1	Test 2	Test 3	Test 1		Test 2		Test 3		wildty pe	CipP knock out	wildty pe	CipP knock out	Test 3	
	Avera ge	Side v		wild type	CipP knocko ut	wildty pe	CipP knock out	wildty pe	CipP knock out	wildtyp e	wildtyp e	wildtyp e				wildty pe	CipP knock out	wildty pe	CipP knock out	CipP knock out	wildty pe						CipP knock out
25	0.95	0.01	0.50	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28	0.49	0.03	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	0.64	0.03	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30	0.03	0.00	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
31	0.02	0.00	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32	0.01	0.01	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
33	0.02	0.00	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
34	0.02	0.00	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35	0.00	0.00	N/A	N/A	2.5	2.3	2.6	2.4	2.6	2.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
36	0.00	0.01	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
37	0.39	0.06	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
38	0.01	0.00	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
39	0.76	0.02	0.07	0.02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
40	0.36	0.01	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
41	0.77	0.01	0.07	0.03	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
42	0.03	0.01	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
43	0.69	0.01	0.04	0.01	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
44	0.19	0.02	0.00	0.00	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
45	0.32	0.01	0.01	0.02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
46	0.38	0.03	0.05	0.02	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
47	0.36	0.02	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
48	0.00	0.00	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
49	0.41	0.01	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
50	0.00	0.01	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE 3																																																																																																																																																																																																																																																																																																																																																																																																																														
compo und #	RD25				RD5				Disk Assay - N.meningitis (diameter in cm)						Disk Assay - S.aureus (diameter in cm)				Disk Assay - E.coli (diameter in cm)																																																																																																																																																																																																																																																																																																																																																																																																											
	Avera ge	Stde v	Test 1		Test 2		Test 3		wild type	ClpP knocko ut	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildtyp e	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3	wildty pe	ClpP knock out	Test 1	Test 2	Test 3</

TABLE 3																
compo und #	Disk Assay - N meningitis (diameter in cm)				Disk Assay - S aureus (diameter in cm)				Disk Assay - E.coli (diameter in cm)							
	RD25		RD5		Test 1		Test 2		Test 3		Test 1		Test 2		Test 3	
	Avera ge	Stde v	Avera ge	Stde v	wild type	ClpP knocko ut	wildty pe	ClpP knock out	wildty pe	wildtyp e	wildtyp e	ClpP knock out	wildty pe	ClpP knock out	wildty pe	ClpP knock out
75	0.14	0.04	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-
76	0.09	0.08	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-
77	0.00	0.00	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-
78	0.04	0.01	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-
79	0.00	0.00	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-
80	0.00	0.00	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-
81	0.16	0.01	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-
82	0.05	0.01	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-
83	0.05	0.03	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-
84	0.02	0.01	N/A	N/A	-	-	0.65*	-	-	-	-	-	-	-	-	-
85	0.01	0.01	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-
86	0.05	0.01	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-
87	0.00	0.00	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-
88	0.00	0.00	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-
89	0.00	0.00	N/A	N/A	1.2*	0.7*	1.2*	0.8*	1.4*	1.0*	-	-	-	-	-	-
90	0.11	0.03	N/A	N/A	1.0*	-	1.0*	-	1.0*	-	-	-	-	-	-	-
91	0.41	0.07	N/A	N/A	1.2*	0.65*	1.2*	0.9*	1.2*	0.9*	0.8*	0.65*	-	-	-	-
92	0.33	0.06	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-
93	0.00	0.04	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-
94	0.05	0.04	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-
95	0.00	0.00	N/A	N/A	-	-	-	-	-	-	0.8*	0.8*	-	-	-	-
96	0.23	0.02	N/A	N/A	1.2*	1.1*	1.3*	0.8*	1.2*	1.0*	-	0.7*	-	-	-	-
97	0.00	0.00	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-
98	0.55	0.02	N/A	N/A	1.0*	-	0.9*	-	1.0*	-	-	-	-	-	-	-

TABLE 3

compo und #	Disk Assay - N.meningitis (diameter in cm)						Disk Assay - S.aureus (diameter in cm)						Disk Assay - E.coli (diameter in cm)					
	RD25		RD5		Test 1		Test 2		Test 3		Test 1		Test 2		Test 3		Test 1	
	Avera ge	Stde v	Avera ge	Stde v	wild type	ClipP knocko ut	wildty pe	ClipP knock out	wildty pe	ClipP knock out	wildtyp e	ClipP knock out	wildty pe	ClipP knock out	wildtyp e	ClipP knock out	wildty pe	ClipP knock out
99	0.05	0.01	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
100	0.00	0.00	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
101	0.06	0.01	N/A	N/A	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26	0.82	0.04	0.23	0.02	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
27	1.11	0.03	0.69	0.04	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
102	0.06	0.01	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
103	0.02	0.01	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
104	0.00	0.00	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
104	0.00	0.00	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
105	0.04	0.02	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
106	0.04	0.02	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
107	0.00	0.00	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
108	0.26	0.03	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
109	0.02	0.01	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
110	0.52	0.01	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
111	0.15	0.01	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
112	0.45	0.03	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
113	0.06	0.00	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
114	0.22	0.02	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
115	0.00	0.00	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
116	0.00	0.00	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
117	0.03	0.01	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

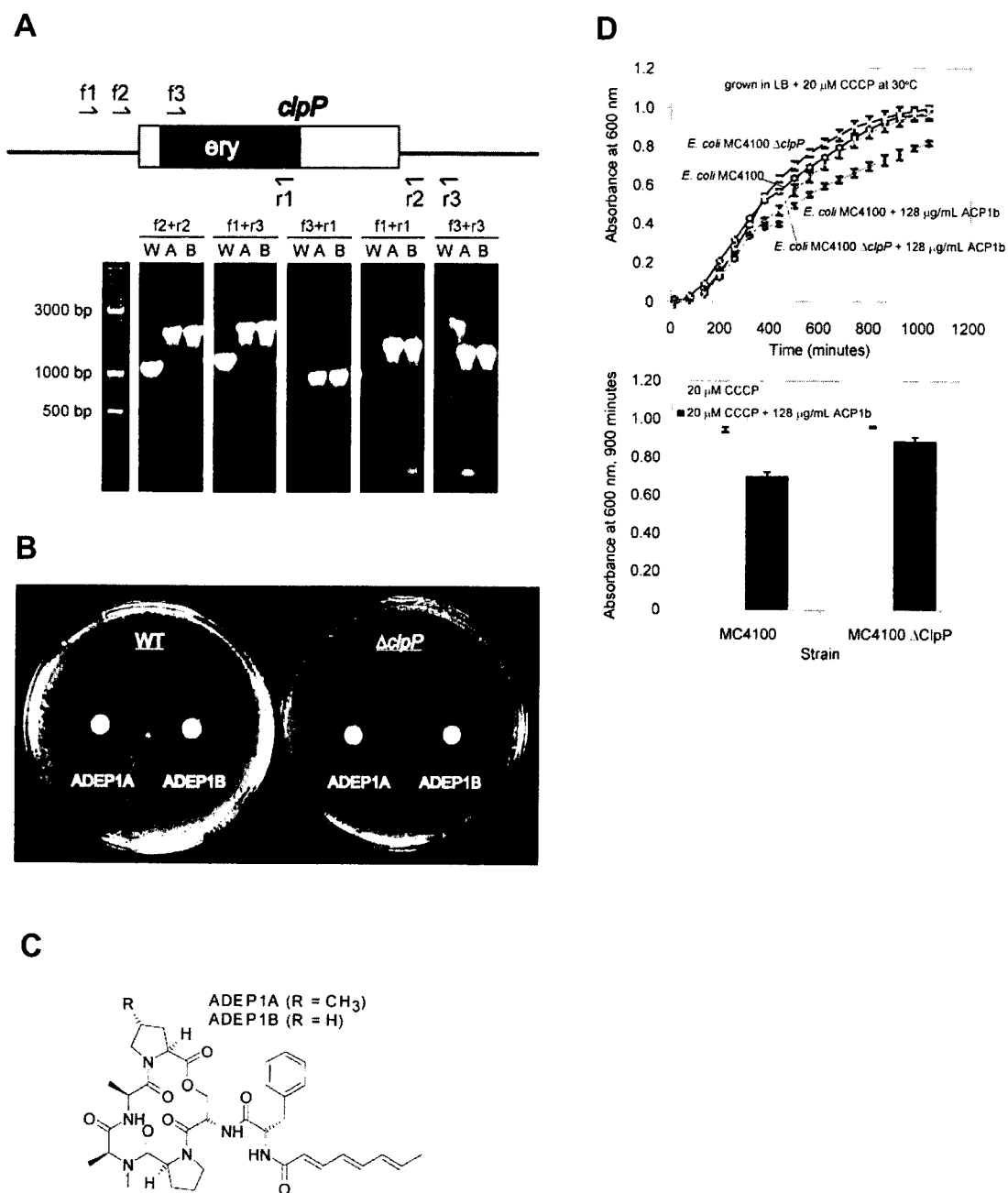


Figure 17

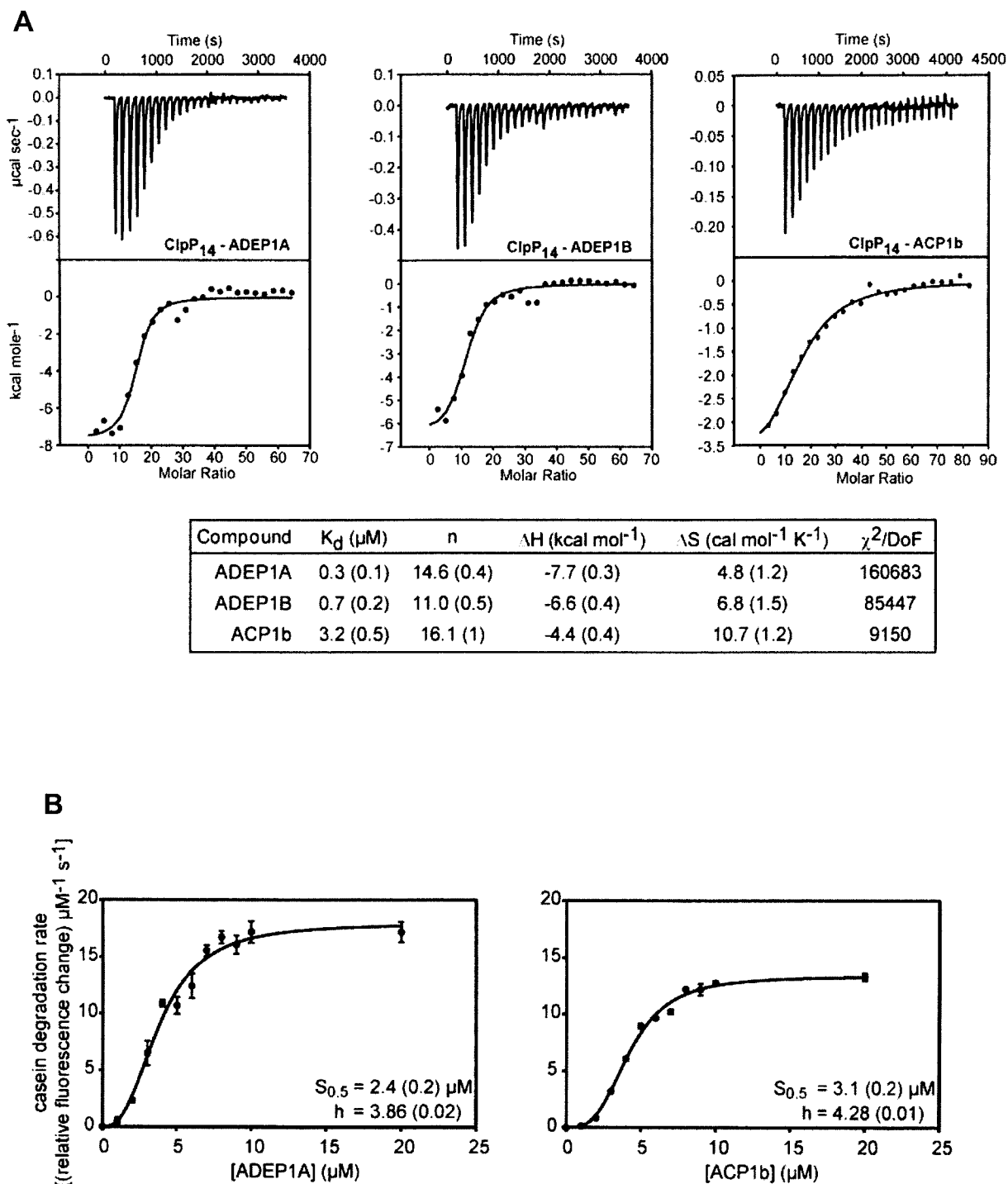


Figure 18

30/30

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2011/001406

A. CLASSIFICATION OF SUBJECT MATTER

IPC: *C07D 213/71* (2006.01) , *A61K 31/165* (2006.01) , *A61K 31/216* (2006.01) , *A61K 31/4164* (2006.01) , *A61K 31/428* (2006.01) , *A61K 31/433* (2006.01) (more IPCs on the last page)
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 (2006.01): *C07D 213/71*, *A61K 31/165*, *A61K 31/4164*, *A61K 31/44*, *A61K 31/4402*, *A61K 31/4409*, *A61K 31/496*, *A61K 31/505*, *A61P 31/04*, *C07C323/41*, *C07C213/70*, *C07C216/36*, *C07C239/38*

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

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
STN (Registry, Caplus, ChemCat), Canadian Patent Database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97/36584 A1 (HUTCHINSON), 09 October 1997 (09-10-1997) *see page 12, third compound and page 14, third compound*	1-9, 36
X	WO 83/00939 A1 (BAILEY et al.), 17 March 1983 (17-03-1983) *see page 57*	1-9
X	US 3,507,961 (WEBER et al.), 21 April 1970 (21-04-1970) *see the examples and the claims*	1-9, 36
X	GB 1,107,411 (CLINCH et al.), 27 March 1968 (27-03-1968) *see the table 2 wherein R is isopropoxyphenyl and R' is benzenesulphonylacetyl and the claims *	1-9
X	YOON et al., "γ-Lactam Synthesis via C-H Insertion: Elaboration of N-Benzyl Protecting Groups for High Regioselectivity toward the Total Synthesis of Rolipram", <i>Org. Lett.</i> , 2003, Vol. 5(13), pp. 2259-2262 *see table 1, scheme 5 and the supporting information*	1-9

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :	"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
"A" document defining the general state of the art which is not considered to be of particular relevance	"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
"E" earlier application or patent but published on or after the international filing date	"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
"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)	"&" document member of the same patent family
"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	

Date of the actual completion of the international search

12 April 2012 (12-04-2012)

Date of mailing of the international search report

24 April 2012 (24-04-2012)

Name and mailing address of the ISA/CA
Canadian Intellectual Property Office
Place du Portage I, C114 - 1st Floor, Box PCT
50 Victoria Street
Gatineau, Quebec K1A 0C9
Facsimile No.: 001-819-953-2476

Authorized officer

Guillaume Tessier (819) 934-0420

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. ☐ Claim Nos. :
because they relate to subject matter not required to be searched by this Authority, namely :

2. ☐ Claim Nos. :
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. ☐ Claim Nos. :
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows :

SEE EXTRA SHEET

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. : 1-9, 10 (in part), 21-30, 31-36 (in part)
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☒ No protest accompanied the payment of additional search fees.

Continuation of Box No. III:

The claims are directed to a plurality of inventive concepts as follows:

Group A - Claims 1-9, 10 (in part), 31-36 (in part) are directed to compounds of formulas (I) and (II), uses thereof and compositions thereof.

Group B - Claims 10 and 34-36 (all in part) are directed to compounds 4, 7 and 8, which comprise a 5-membered heteroring and are not of formula (I) or (II), uses thereof and compositions thereof.

Group C - Claims 11-18, 19-20 (in part) and 31-36 (in part) are directed to compounds of formula (III), uses thereof and compositions thereof.

Group D - Claims 19-20 and 34-36 (all in part) are directed to compounds not of formula (III), uses thereof and compositions thereof.

Group E - Claim 21-30 and 34-36 (in part) are directed to compounds of formula (IV), uses thereof and compositions thereof.

Group F - Claim 31, 32 and 34-36 (all in part) is directed to ACP2, uses thereof and compositions thereof.

Group G - Claim 31, 32 and 34-36 (all in part) is directed to ACP4, uses thereof and compositions thereof.

Group H - Claim 31, 32 and 34-36 (all in part) is directed to ACP5, uses thereof and compositions thereof.

Group I - Claims 34-35 (in part) is directed to the use of other ACP as antibacterial compounds.

An international application should relate to only one invention or, if there is more than one invention, the inclusion of those inventions in one international application is only permitted if all inventions are so linked as to form a single general inventive concept (Rule 13.1). With respect to a group of inventions claimed in an international application, unity of invention exists only when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. The expression "special technical features" is defined in Rule 13.2 as meaning those technical features that define a contribution which each of the inventions, considered as a whole, makes over the prior art.

The present claims are directed to allegedly novel compounds which are activators of self-compartmentalizing protease (ACP), particularly the caseinolytic protease P (ClpP). However, it is known that ClpP can be activated by other compounds such as acyldepsipeptides. Therefore, the fact that the present compounds are ACP cannot be considered a technical relationship among the different groups of alleged invention.

Continuation of Box. No. V:

A61K 31/44 (2006.01) , *A61K 31/4402* (2006.01) , *A61K 31/4409* (2006.01) , *A61K 31/47* (2006.01) ,
A61K 31/496 (2006.01) , *A61K 31/505* (2006.01) , *A61K 38/06* (2006.01) , *A61P 31/04* (2006.01) ,
C07C 323/41 (2006.01) , *C07C 69/757* (2006.01) , *C07D 213/70* (2006.01) , *C07D 215/18* (2006.01) ,
C07D 215/36 (2006.01) , *C07D 233/84* (2006.01) , *C07D 239/38* (2006.01) , *C07D 277/76* (2006.01) ,
C07D 285/125 (2006.01)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2011/001406

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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